

Ollscoil na hÉireann, Corcaigh  
**National University of Ireland, Cork**



**TAPHONOMY OF PIGMENTARY  
COLOURS IN FOSSIL INSECTS**

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for the degree of  
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Nidia Álvarez Armada

17<sup>th</sup> September 2020

**ABSTRACT**

Extant insects are renowned for the striking colouration displayed in their cuticles. Although fossil insects are abundant and the patternings in their cuticles highly resemble those found in extant insects, these are commonly monotonal, especially in those insects preserved across deep geological time. Colouration mechanisms in extant insects are majorly a combination of colour producing molecules called pigments and occasionally colour producing structures, which can be present in combination, but colouration mechanisms are largely unknown in their counterpart fossil taxa. Here, an experimental approach is utilized to elucidate the taphonomy of pigmentary colours in fossil insects. Decay and tumbling experiments are combined to explore environmental, biological and compositional biases constraining the potential for fossilisation of cuticles in various extant taxa. In addition, the trace elemental signatures embedded in patterned cuticles of extant taxa are characterized and compared with trace elemental signatures in fossil taxa. These analyses indicate that the fossilisation potential for insect cuticle is greatly diminished for those regions of the cuticle where the sclerotisation molecule, melanin, is not present. Environmental conditions also play an important role in the potential for fossilisation, where the increase of alkalinity in the media accelerates the process of internal decay and aids in the proliferation of decomposing bacteria, especially on those regions of the cuticle where melanin is absent. Physical decay of cuticles appears to be affected by the distribution of the pigment components throughout the cuticle; the rate of physical decay differs in cuticles with variables degrees of patterning, and those cuticles without heterogeneous distribution of pigments resist decay related to transport for longer periods of time. Trace elemental chemistry is taxonomically constrained in extant taxa at order and family levels. At genera level,

these broad taxonomical trends are overprinted by a strong pigment-specific signal. The trace element chemistry in fossil insects is controlled by both taxonomy and depositional context, indicating some diagenetic overprinting. Regions of the fossilized cuticles displaying different tones contain distinct suites of elements, which suggests that there is a preservation of a pigmentary component in the chemical signal of fossil insects. Revealing biases in the fossil record of insects assist to the interpretation of fossil insect assemblages, the conditions of the environment of deposition and any biological constraints in the preservation potential of fossil insects. Trace elemental chemistry of fossil insects aids in the interpretation of original colours of fossil insects, and consequently informs on the models of evolution of colour and its ecological functions in insects through deep time.



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Chapter 3: M.E.M. and F.L.G. conceived the research; M.E.M., F.L.G. and N.A.A. designed the research; N.A.A., S.I., K.W., K.J.M. and F.K. performed the GC- and HPLC-MS analyses. N.A.A. performed all other analyses; N.A.A. wrote the paper with contributions from all other authors.

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**R Script**

**Full Resolution Figures and Digital Files of Tables Included in the Main Text  
and Appendices of this Thesis**

[https://www.dropbox.com/sh/iddgmrhsonmnlcy/AAAqqmARCjrBDnqMwj3H  
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### **In text abbreviations**

4-AHP – 4-amino-3-hydroxyphenylalanine

AHPO – Alkaline Hydrogen Peroxide Oxidation

ANOVA – Analysis of Variance

BSTFA - Bis(trimethylsilyl)trifluoroacetamide

EDS – Energy Dispersive X-ray spectroscopy

EM - Eumelanin

EPMA – Electron Probe Microanalysis

GC-MS – Gas Chromatography-Mass Spectrometry

HI – Hydroiodic Acid Hydrolysis

HMDS – Hexamethyldisilamine or Bis(trimethylsilyl)amine

HPLC-MS – High Performance Liquid Chromatography-Mass Spectrometry

LDA – Linear Discrimination Analysis

MANOVA – Multivariate Analysis of Variance

MCA – Multi Channel Analysis

NHMO – Natural History Museum of Oslo

PCA – Principal Component Analysis

PM - Pheomelanin

PTCA – pyrrole-2,3,5-tricarboxylic acid

OUMNH – Oxford University Museum of Natural History

ROI – Region of Interest

SEM – Scanning Electron Microscopy

SRS-XRF – Synchrotron X-ray Fluorescence

SSRL – Stanford Synchrotron Radiation Lightsource

TM – Total melanin

TMS – Trimethylsilyl Functional Groups

YPMNH – Yale Peabody Museum of Natural History



# CHAPTER 1

## Colouration mechanisms in insects present and past

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### INTRODUCTION

#### 1. Taphonomy

Fossil remains have been objects of interest to naturalists for centuries, but it was not until the 18<sup>th</sup> century that palaeontology was established as a stand-alone branch of science (McGowan 2001). Palaeontology can be thought of as a conjunction of diverse sciences with a common objective to search and understand ancient life. Today, palaeontology agglomerates specialists from many backgrounds in science including, among others, geology, biology, chemistry and physics. Taphonomy is one important aspect of palaeontology. From the Greek “*taphos*” (burial) and “*nomos*” (law), taphonomy is the science that studies anything happening to an organism or any of its products from moments before its death until it is recovered as a fossil. It was first introduced in 1940 by Ivan Efremov (1940) to describe the transition from organisms or any of their products from the biosphere to the lithosphere.

**1.1. Susceptibility of becoming a fossil.** Fossil remains of uncountable organisms can be found in numerous varieties of sedimentary conditions; nevertheless, some organisms (or their tissues) are more prone for fossilisation than others (Briggs *et al.* 1993; Sansom *et al.* 2010; Parry *et al.* 2018). Vertebrate animals are composed of two main types of tissues regarding their propensity for fossilisation: tissues that are mineralized to some extent (“hard” tissues), such as bones or teeth, are more resistant to decay or recalcitrant, and tissues that are not mineralized (“soft” tissues), such as muscles and internal organs, are less resistant to decay or labile. Invertebrate animals are mostly composed of labile tissues, with the exception of marine invertebrates of which a great variety possess an external protective barrier composed of calcium carbonate. This is unfortunate, as labile tissues are usually the bearers of the most information in terms of physical appearance and environmental relationships (Briggs and Kear 1993c; Briggs 2003). Often, when organisms with non-biomineralized tissues are described, the morphological and chemical changes that take place during the process of decay are disregarded, although these changes are capable of producing profound taxonomical misplacement (Sansom *et al.* 2010). Consequently, taphonomical studies could not be complete without an experimental trial that investigates the potential of fossilisation of certain tissues and connected taxa (Purnell *et al.* 2018).

In recent years, experimental taphonomy has grown to become an important conduit attempting to clarify processes involved in decay and mineralisation of diverse tissues (Briggs 1995; Briggs and McMahon 2016; Purnell *et al.* 2018). Long discussed topics such as early evolution of vertebrates (Briggs 1995; Sansom 2010, 2011), mineralisation of soft tissues from arthropods and plants (Briggs 1999), and taxonomical affinities of incertae sedis (Donoghue and Purnell 2009;

Cunningham *et al.* 2012) have greatly benefited from taphonomical experimentation in recent years.

## **2. Experimental taphonomy.**

The use of controlled laboratory conditions for the interpretation of past environmental physical and chemical characteristics is essential to elucidate the modes of decay and subsequent fossilisation of exceptionally preserved organisms. Taphonomic experiments rely on the assumption that chemical and physical laws have remained the same throughout the geological history. These experiments are always based on two main premises: i) the natural process under study can be reproduced under a feasible timescale (or can be accelerated by substitution processes), and ii) the processes driving fossilisation can be simplified sufficiently for the role of individual factors to be identified (Briggs and McMahon 2016). Processes involved in the fossilisation of animals reproduced in laboratory conditions by previous studies include decay, mineralisation and maturation.

**2.1. Decay.** When trying to decipher new fossil material, decay is probably the most vital process to consider before interpretation of the original morphology (Briggs and Kear 1993b; Sansom *et al.* 2010), and phylogenetic positioning of the preserved animal (Briggs 2010; Sansom 2015). Rate of decay and favourable environments of decay for the preservation of certain tissues (Briggs and Kear 1993a; Sansom 2014) play an important role in the preservation of a carcass. Besides from chemical decay, an important factor to take into account is physical decay produced by transport of bodies prior burial (Allison 1986; Briggs 1995). One of the most physically damaging factors after death of an organism is transport

before deposition (Briggs 1995). Well-preserved soft-bodied fossils are considered to have undergone little transport (Whittington 1971; Stürmer and Bergström 1973; Allison 1986); however, pre-transport processes, particularly decay, have a greater impact in the potential for preservation of unmineralized organisms (Allison 1986; Duncan *et al.* 2003; Enright *et al.* 2021). Tumbling experiments in a rota barrel are regularly used as controlled homologues when reconstructing transport environments in laboratory conditions (Chave 1964; Behrensmeyer 1982; Allison 1986). Whilst tumbling experiments are well established as homologous of transport, some authors have designed alternative experimental conditions that they consider closely represent the physical processes acting in natural fluvial systems (Kuenen 1956; Duncan 1997).

**2.2. Mineralisation.** Generally, soft tissues decay so rapidly that any evidence of their presence is obliterated from the fossil record. The presence or absence of these tissues in the fossil record is dependent on their replication via the mineralisation of authigenic minerals in early stages of fossilisation and/or organic preservation (Briggs and McMahon 2016). Bacterial activity is a significant incentive for preservation of soft tissue either from direct mineralisation (Hippler *et al.* 2012) or bacterial pseudomorphing of internal soft tissues (Raff *et al.* 2008; Butler *et al.* 2015). Unfortunately, extensive mineralisation has been challenging to achieve in laboratory conditions, particularly when the concentration of constituent mineral ions is low, nevertheless mineralisation of tissues has been achieved in previous laboratory experiments (Briggs and Kear 1993c; Briggs *et al.* 1993; Hof and Briggs 1997, Grimes *et al.* 2001).

**2.3. Maturation.** After decay and burial, organic tissues still have to undergo maturation, which includes processes both of loss of biological information and stabilisation of the organic compounds that will result in their survival over geological times (Gupta *et al.* 2006; Gupta *et al.* 2009; McNamara *et al.* 2016b).

### 3. Colour in nature

*Colour*, in common terms, refers to the aspect of a visual experience generated by the sight of an object. In reality colour is not far from this description, it is the way an intensity of light absorbed, reflected, refracted, scattered and/or diffracted by a matter is perceived by the brain via a visual organ (Nassau 2003; Shevell 2003). Visible light can be described as part of the electromagnetic spectrum from 380 to 740 nm; the shortest wavelengths appear violet, while the longest appear red. Contained in this range, the colours change from one to the next continuously following this sequence from shorter to longer wavelengths: violet–indigo–blue–green–yellow–orange–red (Fig. 1.1).

In view of the fact that no wavelength of light is endowed with a colour, it is clear that colour appearance is a mental not a physical phenomenon. This becomes more apparent when analysing the evolution of colour in visually displayed regions of animals or plants and their correlations with advances in sensory organs destined for vision in their counterpart animals, as though it were a weaponry race. For example, the early evolution of UV-blue-green perception by the insect compound eye (Briscoe and Chittka 2001) clearly coincides with the evolution of pigmentation characteristic of these colours in patterned wings of insects (North and French 1994; Parchem *et al.* 2007; Werner *et al.* 2012). Some

butterfly species have been confirmed to use colouration as a communication method for conspecific recognition (Bernard and Remington 1991; Kronforst *et al.* 2006; Stavenga and Arikawa 2006; Piszter *et al.* 2011). But perhaps the most significant colour/sight co-evolutions are those concerning plant genitalia and their potential pollinators (Menzel and Shmida 1993; Weiss 2001). It is important to note that not all sensory systems are prepared to perceive the same range of wavelengths from the electromagnetic spectrum, for instance, insects perceive additional wavelengths that the human eye is incapable to perceive; from 300 to 700 nanometres, including the ultraviolet range (Morgan 2004b).

Colour in animals is achieved through two separate pathways; chemical and structural colour, notwithstanding these frequently act in combination to create further colourations and other light-interaction properties such as iridescences (Vukusic *et al.* 2004; Shawkey and Hill 2005; Shawkey *et al.* 2009).

**3.1. Chemical colour.** This type of colouration is formed by selective absorption of light by organic compounds stored in animals or plants called pigments (Shawkey *et al.* 2009). This type of colouration derives from the absorption and emission of photons from unpaired electrons in transition metal compounds within organic molecules (Nassau 2003). These pigments can be specific to a group, such as psittacofulvins, exclusive to parrots (McGraw and Nogare 2004), or to an entire phylum such as ommochromes present in arthropods (Nijhout 1997; Insausti and Casas 2008; Riou and Christidès 2010; Figon and Casas 2019) and also in the chromatophores of some cephalopods (Williams *et al.* 2016; Figon and Casas 2019) or widespread throughout the animal kingdom such as melanin (Hadley and Oldman 1969; Fitzstephens and Getty 2000; Bezzerides

and Loofbourrow 2014; Edwards *et al.* 2016; Galvan and Solano 2016).

Descriptions of pigments are far more numerous in extant taxa than in fossil taxa for vertebrate and invertebrate animals (Fig. 1.4), this is most likely the result of a preservational bias rather than an exponential increase of pigmentary compounds late in evolution. Described colours for specific pigments have to be considered individually since analysis in birds indicate that some pigments can produce different colours than those commonly described (McGraw *et al.* 2004).

Bioluminescence present in numerous vertebrate and invertebrate marine animals (Widder 2010), fungi (Purtov *et al.* 2015), various microorganisms such as bacteria (Meighen 1988; Widder 2010) and some terrestrial arthropods such as fireflies (Wood 1995) is an additional type of chemical colouration that it is not within the scope of this thesis.

**3.2. Structural colour.** This type of colouration is formed by coherent or incoherent scattering of light from highly structured tissues (Shawkey *et al.* 2009). All substances, without exception, scatter light due to small fluctuations in their refractive indexes (Nassau 2003). There is a great variety of structured tissues described in both fossil and extant specimens of vertebrate (Prum 1999; Prum and Torres 2003; Bagnara *et al.* 2007) and invertebrate (Shawkey *et al.* 2009; Vigneron and Simonis 2010; McNamara *et al.* 2011; Shevtsova *et al.* 2011; Balint *et al.* 2012; Barrows and Bart 2014) animals (Fig. 1.4). Structural colour in vertebrate tissues is most commonly produced by pigment filled cells, such as leucophores (e.g. DeMartini *et al.* 2013), xanthophores (e.g. Mahalwar *et al.* 2014) or melanophores (e.g. Logan *et al.* 2006). In addition, melanosomes have been extensively reported from tissue of extant and fossil vertebrates (e.g. Zhang *et al.*

2010; Rossi *et al.* 2020). Common structural colouration in extant and fossil invertebrate taxa are produced by diffraction gratings (e.g. Vigneron and Simonis 2010) and other biophotonic nanostructures (e.g. Vukusic *et al.* 2004; Vigneron and Simonis 2010), other colour producing structures such as disordered structures, remain unidentified in fossil findings (Fig. 1.4).

#### **4. Importance of insects in past and present ecosystems**

Insects are the most diverse and ecologically- and numerically dominant group of animals on Earth (Sabrosky 1953; Mayhew 2007). Three-quarters of all animal species named to this date are insects; they are a hyper-diverse group with obvious evolutionary benefits across-the-board in terrestrial niches (Wheeler 1990; Grimaldi and Engel 2005; Clapham *et al.* 2016). The group is incredibly speciose everywhere in Earth, especially the tropics, where the environmental conditions are optimal. Several authors have approached the mission of assessing the total number of insect species living today (Erwin 1991; Mayhew 2007); whether trying an extrapolation of extant tropical communities (Erwin 1983), census of colonies of known insect species (Sabrosky 1953) or extrapolation from known fossil data (Clapham *et al.* 2016) this has proven an arduous task. Regardless of the uncertainty of the exact number of living insect species, the fact that insects have been important components of terrestrial ecosystems for the last 400 million years stands undeniably truthful (Jarzembowski 1989; Labandeira and Sepkoski 1993; Mayhew 2007). Insect diversity has increased throughout their evolution, with five orders wielding dominance in current days: Coleoptera, Diptera, Hemiptera, Hymenoptera, and Lepidoptera. From the oldest fossil insect, only known from fragmentary body parts (396–407 Myr), preserved in cherts from the Old Red



Sandstones of Rhyne in Scotland (Engel and Grimaldi 2004), there was a long history of novel anatomical, physiological and behavioural evolutionary characters, which without a doubt, were responsible for the class' evolutionary success (Mayhew 2007; Nicholson *et al.* 2014). Probably the most striking of these innovations, including a winged bauplan, reproductive traits or limited size, are the highly variable colour patterning present in cuticles of extant insects, e.g. spots, bands and eyespots, that function primarily in intra- and interspecific communication. These patternings are achieved through the spatial distribution of various pigments (e.g. Pfeiler 1968; Wilson 1985; Bezzerides and Loofbourrow 2014; Stavenga *et al.* 2014) and/or structural colours (Rutowski *et al.* 2005; Yoshioka and Kinoshita 2006; Seago *et al.* 2009).

## 5. Structure of insect cuticle

The cuticular structure of insects consists of a series of overlying layers of chitin, protein and various lipids that aid in body support and protection from the environment (Locke 1965, 2001; Andersen 2009a, b). The thickness and sturdiness of arthropod cuticles vary among species, sex, ontogeny and even laterally from location to location in the cuticle (Neville 2012b; Andersen 2009a, b; Moussian 2013) but fortunately, all cuticles can be defined in reference to a basic plan (Fig. 1.2). Insect cuticle is formed by three layers, from external to internal: epicuticle, exocuticle and endocuticle.

**5.1. Epicuticle.** The epicuticle is a thin (1–4  $\mu\text{m}$  thick) layer composed of proteins and lipids, probably covalently cross-linked (Andersen 2009a; Moussian 2013). This layer is responsible for waterproofing (Locke 1965), mechanical

protection (Hopkins and Kramer 1992), structural colouration via multilayer reflectors (Schultz and Rankin 1985; Cai *et al.* 2020) and contains various glands and sensory structures and cuticle-producing epidermal cells (Andersen 2009a; Ghiradella 2010).

**5.2. Exocuticle.** The exocuticle lies immediately beneath the epicuticle and is highly sclerotized. It is composed of chitin fibrils in parallel organisation into sub-micron layers whereby successive layers show helical displacement of chitin fibrils, the organisations of these fibrils and chitin/protein ratio have important input in the mechanical structure of the cuticle (Andersen 2009a; Ghiradella 2010) as well as taking part in structural colouration, such as originating polarized colours (Blahó *et al.* 2012).

**5.3. Endocuticle.** The endocuticle, in conjunction with the exocuticle, makes the bulk of the insect cuticular thickness. It also contains chitin and proteins but it is less sclerotized than the overlying exocuticle. Endocuticle is composed of a cross-ply arrangement of chitin bundles in successive layers surrounded by protein matrix and chitin (Wigglesworth 1948, 1957; Raabe *et al.* 2005; Moussian 2013).

## **6. Chemistry of insect cuticle**

The cuticle of insects is formed by chitin, lipids and proteins in various ratios conditional to the elasticity of the cuticle (Andersen 1979; Hopkins and Kramer 1992; Andersen 2009a) and ecdysis state (Schaefer *et al.* 1987).

**6.1. Chitin in the cuticle.** Chitin, as a major component of the insect

cuticle (Kramer *et al.* 1995; Locke 2001; Merzendorfer and Zimoch 2003; Neville 2012a), is an acylated polysaccharide with the monosaccharide units joined by  $\beta$ -(1,4) links which produce straight, ribbon-like molecules (Cohen 1987; Merzendorfer and Zimoch 2003; Vincent and Wegst 2004). Chitin is found in the exo- and endocuticle (Andersen 1979) and functions as a light but strong building material in the exoskeleton of insects (Merzendorfer and Zimoch 2003).

**6.2. Proteins in the cuticle.** Proteins seem to be synthesized within the epidermis and secreted into the underlying layers of the cuticle (Locke *et al.* 1994). All cuticles contain a wide mixture of proteins, while there is evidence that some proteins are synthesized by epidermal cells, it is possible that different proteins are formed elsewhere, transported and stored in the cuticle at a later stage (Andersen 1979). Proteins interact, and stabilize the chitin fibrils in exo- and endocuticle layers in narrow, hyper water-saturated zones called Schmidt layers aiding in elasticity and hydrophobicity to the cuticle (Vincent and Wegst 2004). Some proteins have highly specialized roles, such as resilin, present in regions of the cuticle with rubber-like properties (Weis-Fogh 1960).

**6.3. Lipids in the cuticle.** Cuticular lipids in insects are present in the outer layer of the integument and consist of a complex mixture of apolar compounds including straight-chain and branched, saturated and unsaturated hydrocarbons, waxes, sterols, alcohols and acids (Jackson and Baker 1970; Andersen 1979; Blomquist *et al.* 1987). The composition of cuticular lipids depends to some extent of dietary habits (Andersen 1979; Blomquist and Jackson 1979; Liang and Silverman 2000), seasonality (Hackman 1984; Savarit and Ferveur 2002; Rouault

*et al.* 2004), ontogenic stage (Monnin 2006) and sex of a species (Hackman 1984).

In some instances, the interspecific variation of cuticular lipids in insects is sufficiently large as to use them for species characterisation (Hadley 1984; Lockey 1988).

**6.3.1. Surface waxes.** The cuticle of insects is covered by thin layer of epicuticular wax, majorly formed by long-chain hydrocarbons but also including a variety of other lipids such as esters, alcohols, sterols and carboxylic acids, all with saturated or unsaturated chains (Andersen 1979; Blomquist and Jackson 1979; Blomquist *et al.* 1987). It mainly functions as a water repellent barrier against the environment (Beament 1964; Hadley 1984).

**6.3.2. Hydrocarbons.** Long-chain hydrocarbons are common lipid components of many insect cuticles, yet proportions are highly variable across species (Blomquist and Jackson 1979; Lockey 1988; Howard and Blomquist 2005; Cvačka *et al.* 2006; Blomquist and Bagnères 2010). These are usually a mixture of alkanes and alkenes of variable carbon number, ranging between C<sub>11</sub>-C<sub>36</sub>, however those with odd carbon numbers are predominating (Blomquist and Jackson 1979). Often aid in protection against various conditions (e.g. water-loss and pathogens), recognition and communication between insects (Drijfhout *et al.* 2009).

**6.3.3. Fatty acids.** Free fatty acids are common components of the lipid content in the cuticle of insects (Thompson 1973; Blomquist and Jackson 1979; Blomquist *et al.* 1991). These may be saturated or unsaturated chains of even carbon numbers and among those, C<sub>14</sub>-C<sub>18</sub> are vastly predominant (Gilbert 1967; Thompson 1973; Blomquist and Jackson

1979; Jackson 1981; Stanley-Samuelson and Dadd 1983; Hackman 1984).

The fatty acid component seems to be similar across orders, commonly composed by myristic (14:0), myristoleic (14:1), palmitic (16:0), palmitoleic (16:1), stearic (18:0), oleic (18:1), linoleic (18:2) and linolenic (18:3) acids (Thompson 1973). Fatty acids in the cuticle of insects are utilized as structural support (Stanley-Samuelson *et al.* 1988) and components of the hydrophobic barrier (Evans *et al.* 1985). Likewise, fatty acids are stored for other functions such as energy production (Downer and Matthews 1976; Beenakkers *et al.* 1985; Athenstaedt and Daum 2006; Arrese and Soulages 2010), synthesis of different organic compounds such as pheromones (Lockey 1988; Prestwich and Blomquist 2014) or phospholipids and waxes (Stanley-Samuelson 2006).

**6.3.4. Sterols.** Sterols and sterol esters are usually minor components of the total cuticular lipids in insects (Gilbert 1967; Beenakkers *et al.* 1985; Lockey 1988). Sterols are formed by three cyclohexane and one cyclopentane rings fused together with a hydroxyl functional group at C<sub>3</sub> (Morgan 2004c). Cholesterol and  $\beta$ -sitosterol usually compose the majority of the sterols found in the cuticle, with cholesterol commonly comprising at least 80% of the total percentage (Arnold *et al.* 1969; Hutchins and Kaplanis 1969; Beenakkers *et al.* 1985). Sterols are stored in free form in the insect's body and are not self-biosynthesized; they can either be directly absorbed through diet with minor alterations or derived from dietary phytosterols (Clayton 1964; Gilbert 1967; Beenakkers *et al.* 1985; Morgan 2004c). Nevertheless, some insects have been found to utilise the sterol biosynthetic abilities of their symbionts (Clayton 1964). Other common

sterols in the lipid content of insect cuticles, but not necessarily biosynthesized by insects, are phytosterols such as campesterol and stigmasterol (Hutchins and Kaplanis 1969; Beenakkers *et al.* 1985). Sterols mainly function as structural components of biological membranes. In addition, they are important precursor in other metabolic roles such as the biosynthesis of ecdysteroids, the most important being ecdysone, moulting hormone, which plays a major role in moulting and development (Beenakkers *et al.* 1985; Svoboda and Lusby 1994). The prospect of cholesterol or a metabolite of cholesterol having a control on reproductive functions has been explored by previous studies with limited data (Clayton 1964; Morgan 2004d).

**6.3.5. Alcohols and phenols in the cuticle.** Free alcohols are minor components of the total lipid content, generally saturated and of even carbon number. Alcohols with C<sub>22</sub>–C<sub>34</sub> chains are the most commonly found (Blomquist and Jackson 1979; Hackman 1984). Phenols are found in the cuticle as tyrosine and others, these react with phenoloxidases to create a chemical defense against microorganisms in the form of quinones (Andersen 2009b).

**6.3.6. Other lipids.** Triglycerides, stored in its anhydrous form, have a high caloric content, consequently are the most important component of energy reserves in insects (Gilby and McKellar 1970; Arrese and Soulages 2010). Phospholipids can also be present in the total count of lipids in the cuticle (Gilby and McKellar 1970). Other lipids are more obscure in content and function, for example cuticulin which combined with proteins, serves to stabilize the cuticle before sclerotisation occurs

(Wigglesworth 1970).

**6.4. Sugars in the cuticle.** Sugars are important components of the nutrient reserve of all insects, which is composed in its majority by glycogen and triglycerides (Arrese and Soulages 2010). Glycogen is a multibranched polysaccharide of glucose which is stored in its hydrated form forming bulky supplies, which are then broken down to produce energy (Thompson 2003; Arrese and Soulages 2010). Mono- and polysaccharides such as glucose and glycogen are also stored for chitin production and for synthesis of sugar alcohols (Lockey 1988), which are needed in periods of drought (Watanabe *et al.* 2002) or stressing conditions of temperature (Storey 1997).

**6.5. Pigments in the cuticle.** Pigments such as melanin, pterins and carotenoids are stored in the cuticle and epidermis of insects (Andersen 2009c). These offer protection against external factors, particularly light (Hackman 1984; Hu *et al.* 2013), parasitic resistance (Charnley 2003) and even act as resistance to mechanical penetration of the cuticle via sclerotisation (Mills and Fox 1972; Andersen 2010). Most pigments are biosynthesized by the organism storing them for display; however, some pigments such as flavonoids or carotenoids are biosynthesized by plants and stored in the animal body through ingestion (Goodwin 1986). For a review of biosynthesising pathways of pigments in insects see Shamim *et al.* (2014).

**6.5.1. Melanins.** Melanins in insects, are polymers of quinonoid substances with a benzene nucleus, two internal double bonds and two external double bonds at ortho and para positions (Fig. 1.3a) (Britton 1983b). They are insoluble and unreactive organic compounds, which are

deposited and stored in the exocuticle as irregular polymers or small granules synthesized from the amino acid tyrosine (Hackman 1984; Morgan 2004b; Ghiradella 2009; Hu *et al.* 2013) and produce black, brown, tan or reddish visual colours (Ghiradella 2009). Melanins may be synthesized in insects as eumelanins or phaeomelanins.

**6.5.1.1. Eumelanin.** Eumelanin is composed by a polymer of dihydroxyindole carboxylic acid and its reduced forms (Shamim *et al.* 2014). This pigment appears black in insect cuticle and requires dopamine and tyrosine as precursors (Nakagoshi *et al.* 1984; Nijhout 1985; Morgan 2004b; Ghiradella 2009).

**6.5.1.2. Phaeomelanin.** Phaeomelanin is a polymer of benzothiazine units (Shamim *et al.* 2014). This pigment appears yellow to red in insect cuticle but its biosynthesis is still not completely understood (Morgan 2004b; Ghiradella 2009).

**6.5.2. Pterins.** Pterins belong to a class of nitrogen heterocycles called pteridines with a keto and an amino group in their structure (Fig. 1.3b) (Morgan 2004b). The so called “pigments of butterflies” were first discovered in Lepidoptera wings but have since been described as body pigments in some Hymenoptera (Leclercq 1950) and Hemiptera (Merlini and Nasini 1966; Bel *et al.* 1997), crustaceans (Palmer *et al.* 2018), amphibians (Hama and Obika 1960), reptiles (Steffen and McGraw 2007) and plants (Galland and Senger 1988). The number of known animal pigments with the basic skeleton of pterins is now extensive; the most common in insects are xanthopterin, leucopterin, chrysopterin and erythropterin.



**6.5.2.1. Xanthopterin.** Xanthopterin absorbs in the violet range of wavelengths (Wijnen *et al.* 2007) and thus this pigment appears yellow when stored in insect cuticle (Fuzeau-Braesch 1972). When in its form isoxanthopterin, is common on arthropod eyes and appears bright fluorescing blue-violet (Rawson 1968; Palmer *et al.* 2018).

**6.5.2.2. Leucopterin.** Leucopterin absorbs exclusively in the ultraviolet (Wijnen *et al.* 2007), consequently this pigment appears colourless in insect cuticle (Fuzeau-Braesch 1972).

**6.5.2.3. Chrysopterin.** This pigment appears yellow when stored in insect cuticle (Fuzeau-Braesch 1972).

**6.5.2.4. Erythropterin.** Erythropterin is a blue-absorbing pigment (Wijnen *et al.* 2007), consequently appears orange-red when stored in insect cuticle (Fuzeau-Braesch 1972).

**6.5.3. Ommochromes.** The basic structure of ommochromes is a phenoxazine ring (Fig. 1.3c), which gives rise to two subgroups: ommatins and ommins (Figon and Casas 2019; Britton 1983d). These pigments are restricted to protostomes and are mainly components of insect ommatidia, but are also found in cephalopod eyes and in most protostomian integuments; however, they are common as colour producing pigments in other tissues (Cromartie 1959; Nijhout 1991; Nijhout 1997; Figon and Casas 2019). Ommochromes show strong absorption of light in the ultraviolet as well as the range of 440–500 nm in the visible spectrum, they can produce a range of colours from pale yellow to dull brown and can also achieve bright red to deep purple (Figon and Casas 2019; Britton 1983d).

Most commonly found ommochromes in insect cuticles are xanthommatin and rhodommatin. These organic compounds also have important roles in other functions in the insect body, such as transporting electrons, reacting with oxidants, as well as functioning in vision (Stavenga 2002; Insausti *et al.* 2013; Romero and Martínez 2015).

**6.5.4. Flavonoids.** Flavonoids are *O*-heterocyclic compounds with two benzene rings, joined by a C<sub>3</sub> unit (Fig. 1.3d). The C<sub>3</sub> unit can exist in several oxidation states, each giving rise to a different flavonoid class (Britton 1983c). These pigments are exclusively plant synthesized and stored in the cuticle of insects via ingestion (Ford 1944; Nijhout 1985; Shamim *et al.* 2014). Flavonoids produce cream or yellow colours in insect cuticle, especially in Lepidoptera (Morris and Thomson 1964; Wilson 1985; Wilson 1987; Hopkins and Ahmad 1991; Ghiradella 2009).

**6.5.5. Carotenoids.** Carotenoids are derived from tetraterpenes, composed of eight C<sub>5</sub> isoprene units, totalling a C<sub>40</sub> molecule, and in occasions completed with a six or five membered ring at each end of the molecule (Britton 1983a). Insects acquire carotenoids via ingestion and are then deposited in the cuticle either unaltered or metabolically modified as free molecules or water-soluble protein complexes (Cromartie 1959; Goodwin 1986); however, previous studies indicate animal biosynthesis and/or metabolic conversions of carotenoids are achievable (Britton *et al.* 1977; McGraw *et al.* 2002). Carotenoids are important in a great variety of animals responsible for photoprotection, photoreception, contribution in coloured tissues (Britton 1983a; Bezzerides *et al.* 2007) and in antioxidant and/or immune defences (Blount 2004; Heath *et al.* 2013). Carotenoids are

present in the majority of insect groups (e.g. Czeuczuga 1980; Czeuczuga 1981; Czeuczuga 1986). These pigments display yellow, orange, red and blue visual colours when oxidized to their derivatives; xanthophylls (Ghiradella 2009). The colour displayed in the cuticle is highly dependant of the content of carotenoids (Bezzarides *et al.* 2007), changes in colour related to sexual maturity have been described by previous authors in a variety of animal orders (e.g. Loher 1961; McGraw *et al.* 2003).

**6.5.5.1. Xanthophylls.** Carotenoids with molecules containing oxygen such as lutein and canthaxanthine (Britton 1983a). Xanthophylls produce blue colouration in insect cuticle (Ghiradella 2009).

**6.5.5.2. Carotenes.** Oxygen-free carotenoids, typically only contain carbon and hydrogen groups, such as  $\beta$ -carotene, and lycopene (Britton 1983a). Carotenes produce yellow to red colours in insect cuticle, but in many instances they are grouped with a blue pigment (often bilins see below) to produce green (Ghiradella 2009).

**6.5.6. Bile pigments.** Tetrapyrroles, or commonly known as “bile pigments”, are produced in animals when the haem of haemoglobin and other haemoproteins are degraded (Britton 1983e). These pigments are found in several insect orders, including Lepidoptera, Mantodea and Orthoptera; for instance, the green colour of the cuticle of numerous grasshoppers and many larvae of Lepidoptera is due to bilins of the biliverdin type (Morgan 2004b).

**6.6. Other organic compounds in the cuticle.** Alkaloids can be stored in the cuticle of certain insects repurposed as venom acquired via ingestion (Morgan 2004a). Pteridines are stored as pigments in the cuticle as a form of permanent excretion from the insect body (Harmsen 1966).

## 7. Fossil biomolecules

When any biomolecule from the distant past is found preserved, it offers a unique window into the history of life; unfortunately, this does not occur frequently because the processes of diagenesis usually destroy or alter organic molecules beyond recognition; nevertheless, certain molecules, such as alkanes, are chemically stable and generally inherently unchanged during diagenesis (e.g. Tu *et al.* 2004). Not all molecules in the same organism have the same preservation potential; for example, DNA carries the most phylogenetic and taxonomical information of all molecular remains, yet it only survives a few tens of thousands of years under optimal conditions of preservation (Barnes *et al.* 2007; Haile *et al.* 2009), however, recently the first DNA sample one-million-year-old was sequenced (Roca 2021). On the other hand, more robust molecules, such as chitin in cuticles of insects (Stankiewicz *et al.* 1997b; Flannery *et al.* 2001) or lignin in fossil plants (Gupta *et al.* 2007), are capable of surviving for millions of years. Through the loss of the hydrophilic functional groups, organic molecules become a solvent-insoluble macromolecule of fossil carbon (kerogen) (Eigenbrode 2008; Stankiewicz *et al.* 2000). Molecular fossils are particularly important when they are taxonomically specific; in this case, they are referred to as biomarkers (Brocks and Pearson 2005; Briggs and Summons 2014). Nucleic acids are the most definite biomarkers in all kingdoms, but unfortunately, can only be used in very recent

fossils or sequences of permafrost (e.g. Willerslev *et al.* 2004; Lewis *et al.* 2008; Schwarz *et al.* 2009). Other useful biomarkers include lipids, such as hydrocarbons, which have a great preservation potential, fatty acids or sterols, proteins and aromatic carotenoids (Brocks and Pearson 2005; Briggs and Summons 2014; Buckley 2015).

**7.1. Fossil chitin.** The oldest chitin molecules preserved are from the Late Cambrian (Ehrlich *et al.* 2013); however, chitin molecules are usually altered to more stable biopolymers in the fossil record (Stankiewicz *et al.* 1997a). Biopolymers of chitin are formed via polycondensation (Briggs 1999) or *in situ* polymerisation (Stankiewicz *et al.* 1997a), which involves the incorporation of lipids into the aliphatic macromolecule of chitin through cross-linking reactions (Gupta *et al.* 2006; Gupta *et al.* 2009). Although, chitin is a labile molecule it is moderately resistant to decay when forming proteinic complexes in invertebrate cuticles (Bass *et al.* 1995), consequently, cross-linked protein-chitin systems (Kramer *et al.* 1995; Sugumaran 1998) have been observed in the recent fossil record (Flannery *et al.* 2001; Briggs and Summons 2014).

**7.2. Fossil lipids.** Lipids are indubitably the most common organic molecules found in fossilized sediments (e.g. Schouten *et al.* 2010). All living organisms on Earth have lipid membranes that protect the cellular contents from the ambient environment and act as a dynamic medium affecting cellular processes (Eigenbrode 2008). The bulk of hydrocarbons on Earth are from fossil fuels (or bitumen); however, they are originally from biological origin; consequently, compound-specific isotopic compositions of hydrocarbons may be essential in

identifying earliest life on Earth (Brocks *et al.* 2005).

**7.3. Fossil pigments.** In recent years, research on fossil pigments has acquired great importance since the discovery of fossil melanosomes in isolated feathers (Vinther 2016) and the potential for their utilisation as colour indicators of ancient animals (Li *et al.* 2010; Zhang *et al.* 2010; Rossi *et al.* 2020). Melanin is a well known pigment in fossil animals; it has been described in fossils of all major animal groups including cephalopods (Glass *et al.* 2012), reptiles (Lindgren *et al.* 2014, Rossi *et al.* 2019; Rossi *et al.* 2020), fish (Lindgren *et al.* 2012), arthropods (Lindgren *et al.* 2019; McNamara *et al.* In prep.), birds (Barden *et al.* 2011; Rossi *et al.* 2019; Rossi *et al.* 2020) and mammals (Colleary *et al.* 2015; Rossi *et al.* 2019; Rossi *et al.* 2020). Other pigments are considerably more obscure in the fossil record, for instance, carotenoid granules were found in the xanthophores of fossil snakes (McNamara *et al.* 2016a), quinone pigments in fossil echinoderms (Wolkenstein *et al.* 2006) and biliverdin and protoporphyrin in dinosaur eggshells (Wiemann *et al.* 2018). As a side note, carotenoids are rare in the fossil record; however, the fossil derivatives from the aromatic pigments isorenieratene and chlorobactene are common biomarkers for fossil carotenoids (Lee and Brocks 2011). An account of colour producing structures and pigments described in the literature are represented on Figure 1.4.

Considerable efforts have been made determining viable biomarkers of fossil melanins. When biomolecules are no longer preserved *in vivo* form, other evidences can be used for its characterisation, such as elemental concentrations of various metals (Wogelius *et al.* 2011; Rogers *et al.* 2019; Rogers *et al.* 2021; Rossi *et al.* 2020; Rossi *et al.* 2021). Non-melanin pigments, e.g. chlorophylls in

cyanobacteria (Gupta 1986) and plants (Droppa and Horváth 1990; Fernandes and Henriques 1991), can also associate with distinct suites of elements, but similar signatures for non-melanin pigments in extant and fossil insects have not been reported.

## 8. Aims

This thesis intends to address our limited understanding on diverse aspects of the taphonomy of pigmentary colours in fossil insects by: i) investigating the environmental biases in the fossil record of insects related to pigment content in the cuticle; ii) exploring the characteristics of decay of several pigments in the cuticle of insects; iii) determining the possible biases that pigmentary patterning have on the preservation potential of insects; iv) assessing the effectiveness of diverse chemical methods for characterising changes of the organic and inorganic chemistry of insect cuticles during decay and their possible uses in recognising pigments in the fossil record; v) compiling information on trace elemental chemistry and its associations with cuticular pigments in extant insects; vi) investigating the efficacy of various trace metals in the cuticle of insects as viable biomarkers for original pigmentation in fossil insects.

Specific hypotheses to be tested include the following:

*Hypothesis 1:* Contrasting environments of decay, particularly that of pH conditions, affect the rate of physical and chemical decay of insect cuticles differently.

*Hypothesis 2:* Regions of varying pigment content in the cuticle of insects (i.e. patterned cuticles) are affected distinctively through chemical decay when the conditions of decay are identical.

*Hypothesis 3:* Differently coloured regions of the cuticle of insects (i.e. containing distinct pigment molecules) behave disparately under the same transport conditions in a fluid medium.

*Hypothesis 4:* The pigmentary patterning and spatial distribution of the associated pigment molecules on the cuticle of extant insects plays a strong role on their preservation potential and suggests a biological bias in the fossil record of insects.

*Hypothesis 5:* Various elements or sets of elements are characteristic of distinct pigment molecules in the cuticle of modern insects prior to decay.

*Hypothesis 6:* The association between certain trace elements and pigment molecules (i.e. regions of the cuticle with specific visual colouration) persists during decay.

*Hypothesis 7:* Distinctive trace elements or sets of elements can be used as biomarkers for the original pigmentary content in the cuticle of fossil insects.

Chapter two details the reasoning behind the methodology used in the various parts of this thesis, outlining pros and cons of the methodology used and presenting solutions to concerns raised during practice.

Chapter three investigates the effects of environmental conditions on the rate of decay of patterned insect cuticles and the chemical and physical changes experienced by regions of the cuticle characterized by individual pigment content. Investigations on the viability of liquid and gas chromatography analyses for the characterisation of pigments or their degradation products after decay are also explored in this chapter.



Chapter four explores the effect of pigment type and amount of patterning on the consequences of transport, resultant damage produced in the cuticle and possible biases generated in the fossilisation of insects through tumbling experiments of cuticles of *Harmonia axyridis*.

Chapter five investigates the trace element inventory of cuticles and potential controls of pigmentary colours in the cuticle of patterned insects from diverse extant insects through synchrotron rapid-scanning X-ray fluorescence analysis (SRS-XRF) and assesses the potential of this method for characterising preserved pigments in the fossil record of insects.

Chapter six synthesises all previous chapters, and explores broader conclusions with effects at high taxonomical ranges affecting all environmental conditions and possible media of insect fossilisation in deep time. Critical analysis of methodology used and proposal of future work concludes the chapter.

Chapter three to five of this thesis will be submitted for publication, and they have been formatted with this in mind. For this reason, the main topics of this thesis are included within the introduction to the relevant chapters.

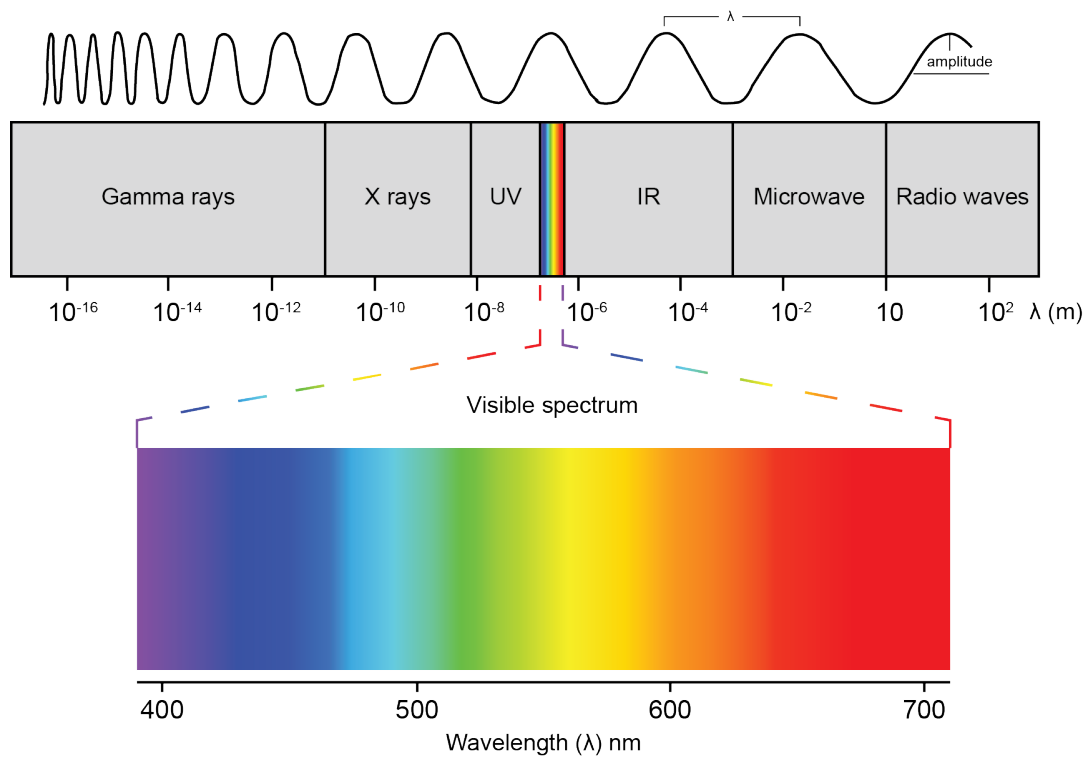
**LIST OF FIGURES AND TABLES:**

**Fig. 1.1:** Electromagnetic spectrum comprising visible light wavelengths from 380 to 740 nm.

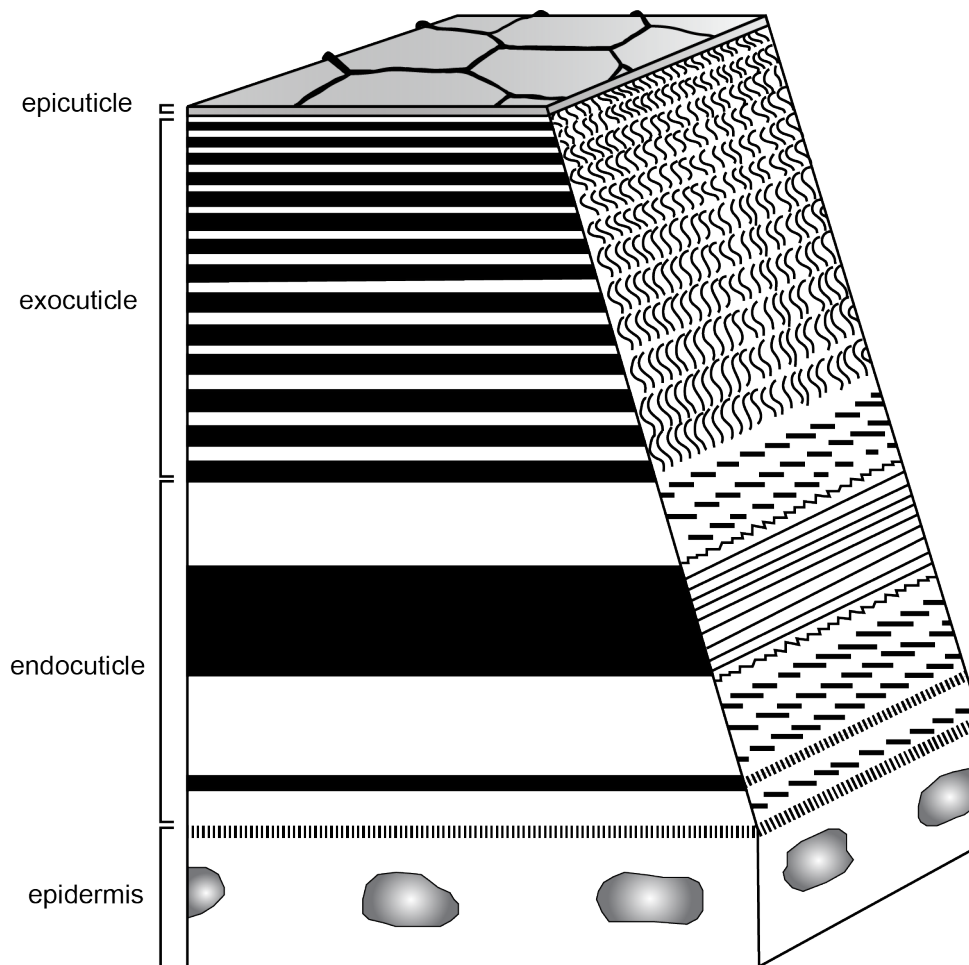
**Fig. 1.2:** Diagram of a transverse section of insect cuticle.

**Fig. 1.3:** Pigment molecules commonly found in the cuticle of insects and visual colours capable of producing.

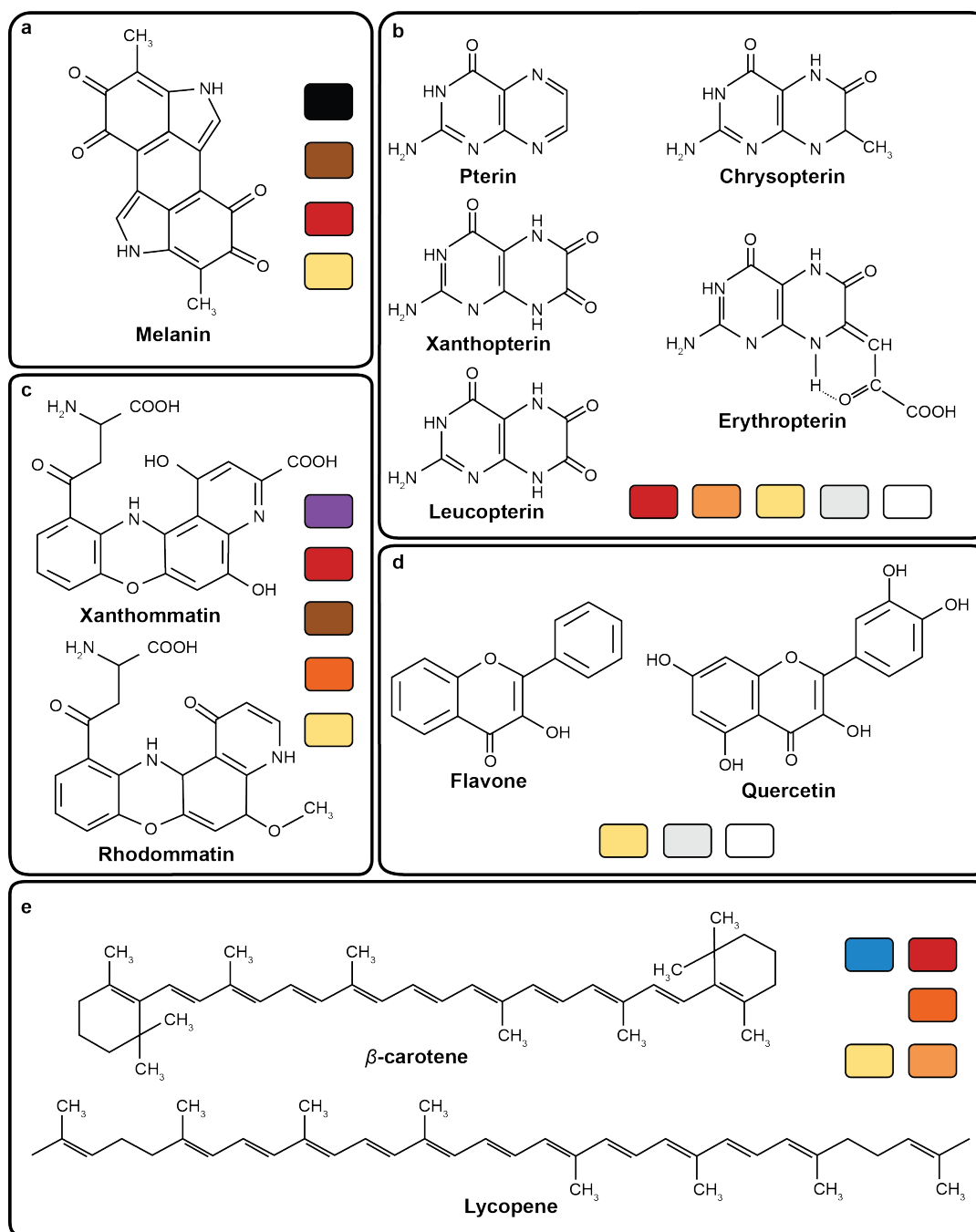
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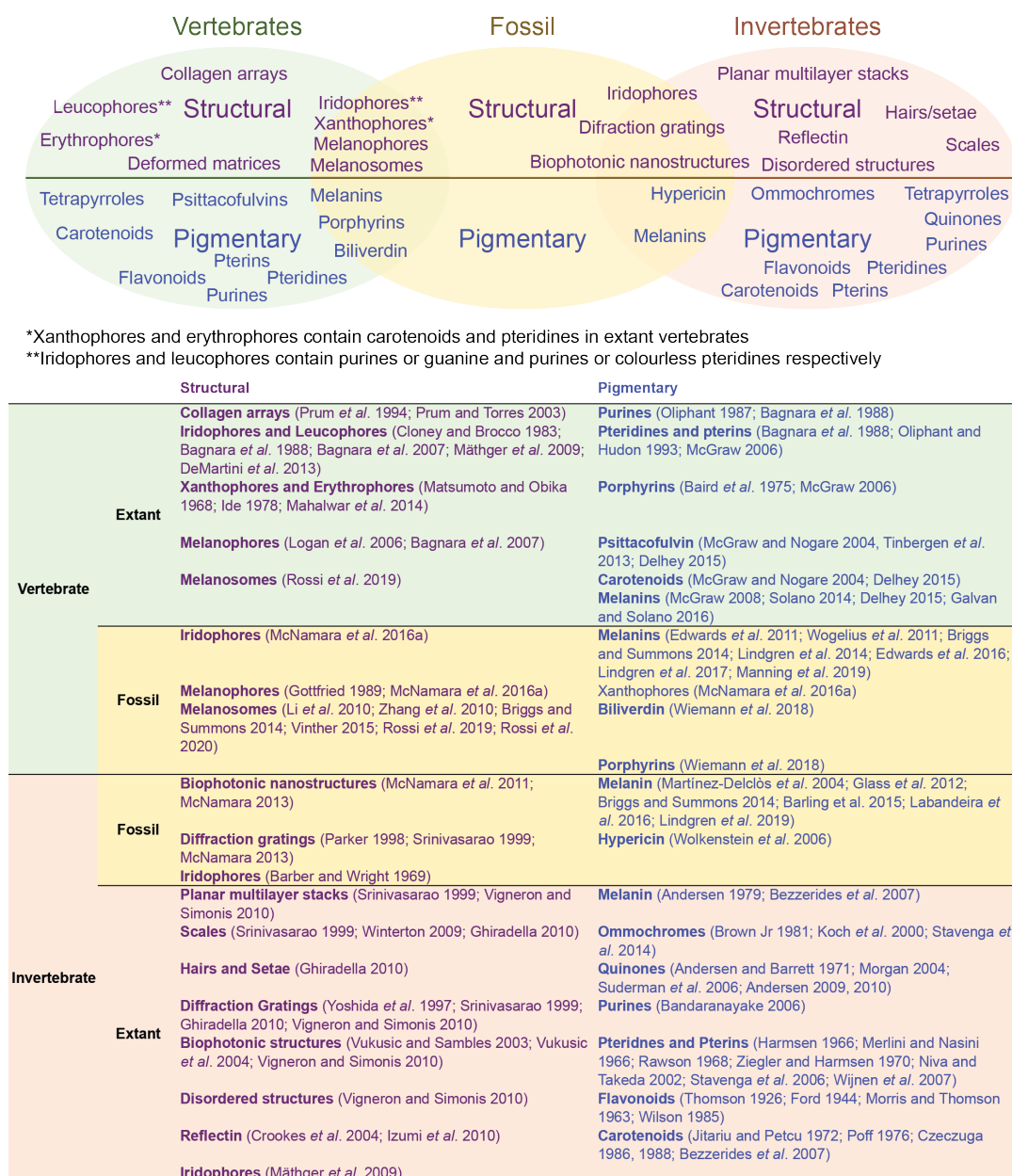
**Fig. 1.1. Electromagnetic spectrum comprising visible light wavelengths from 380 to 740 nm.**



**Fig. 1.2. Diagram of a transverse section of insect cuticle.** The exocuticle and endocuticle are modelled as they would appear in the polarising microscope (left) and in the electron microscope (right). The changing orientation of chitin fibrils in successive layers of the exocuticle creates a cross-linked pattern shown on the right of the diagram. Chitin fibrils are embedded on a protein matrix which gives rise to birefringence when seen under polarized light (white regions on the left) and when sectioned perpendicularly are isotropic (black regions). The endocuticle consists of an alternation of wide layers of chitin helicoidally twisted between them. (Adapted from Caveney 1971).



**Fig. 1.3. Pigment molecules commonly found in the cuticle of insects and visual colours capable of producing.** **a** Simplified melanin molecule; **b** Pterin molecules; **c** Ommochrome molecules; **d** Flavonoid molecules; **e** Carotenoid molecules.



**Fig. 1.4. Known colour producing structures and pigments in extant and fossil animals.**

## CHAPTER 2

# Methods rationale

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### 1. EXPERIMENTAL TAPHONOMY

#### 1.1. Taxa selection and euthanizing process

Specimens for decay were selected based on cuticle patterning and pigmentation, availability and external properties of the cuticle. i) All specimens displayed a minimum of one type of patterning in the cuticle controlled by at least two visual colours and clearly defined boundaries. Taxa with characterized pigment content in previous studies, whether at species- or family-level, were selected. ii) Taxa had smooth cuticle surfaces lacking protuberances, setae or other irregularities, and cuticles with slight curvature. iii) Taxa with large numbers of individuals in museum collections and/or in the wild were preferred.

Specimens were entirely retrieved from museum collections dead and desiccated excluding the Coleoptera *Goliathus goliathus* and *Harmonia axyridis*, which were acquired dead and alive respectively from private sellers, and the Blattodea *Therea petiveriana*, which were acquired from the live collection of OUMNH. Live specimens were euthanized upon arrival via freezing at -40°C in the dark, and desiccated at 20°C overnight.

## 1.2. Decay experiments

Experiments involving various processes of decay of extant organisms are crucial to interpret the history of fossilisation of taxonomically close ancient organisms, especially those composed of labile tissues. Numerous researchers have applied experimental decay practices as a controlled approach of elucidating the preservation conditions of ancient soft-tissue organisms in various environmental settings, and the importance of these methods has been increasingly addressed in recent years (Purnell *et al.* 2018).

### 1.2.1. Chemical decay

**1.2.1.1. Decay protocols.** Protocols were concocted to recreate insect decay in natural settings constrained by laboratory conditions. Individual elytra from each specimen were treated in separate vials due to number requirements, and to avoid cross-contamination. Particular taxa (e.g. *Goliathus goliathus*) possessed large elytra, which were dissected to reduced fragments for treatment. Samples were treated in aqueous media in the dark recreating natural conditions of insect decay, and to prevent extensive degradation of photosensitive pigments (i.e. carotenes). The temperature of decay was set at  $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$  consistent with previous analogous published taphonomic experiments (Sansom *et al.* 2010, 2011) whilst maintaining constant and time efficient rates of decay (Briggs and Kear 1994; Sansom 2014).

**1.2.1.2. Sampling.** Sampling points were increasingly more spaced as the experiment progressed. The spacing of the sampling was designed to capture rapid changes during early stages of decay decreasingly thereafter as the rate of decay slows (Sansom, *et al.* 2010; Sansom 2014). Each



sample point terminated the experiment to avoid cross- and external-contamination (Briggs and Kear 1993b; Sansom, *et al.* 2010; Sansom 2014).

### **1.2.2. Physical decay**

**1.2.2.1. Selection and previous treatment of samples.** Specimens for tumbling were selected based on cuticle patterning and pigmentation, media of decay and extent of decay. i) Specimens of the same taxon displayed high phenotypic plasticity in pigment concentration of two characterized pigment molecules in the cuticle resulting in various groupable degrees of patterning. ii) Specimens treated in basic media for 180 days were selected due to the accelerated rate of decay shown in experiments conducted in chapter 3.

**1.2.2.2. Decay protocols.** Protocols were devised to recreate transport and breakage of insect cuticles in natural settings constrained by laboratory conditions at the same time as enabling for collection of data in a reasonable amount of time. Individual elytra from each specimen were treated in separate vials to avoid cross-contamination. Samples were treated in aqueous media recreating natural conditions of insect decay. Siliceous sediment 1-2 mm sand sized was introduced to achieve abrasion of cuticles in a manageable experimental time. In trial experiments, any grain size smaller than 1 mm did not produce any effect in the cuticle after 168 hours of tumbling.

**1.2.2.3. Sampling.** Sampling points were designed to capture any changes displayed by individual cuticles during the duration of the

experimental conditions, and allow for periods of laboratory closure, these were set to approximately 48 hour intervals.

## **2. PHYSICAL AND CHEMICAL PROPERTIES OF INSECT CUTICLE**

### **2.1. Microspectrophotometry analysis**

Since colour is the personal visual experience generated by the sight of an object (Chapter 1), individual perceptions of colours are qualitative and nontransferable. In order to quantitatively analyse the variation of colour in insect cuticles during decay, it is essential to generate countable analysis of variations of intensity within the light absorbed, reflected, refracted, scattered and/or diffracted by these cuticles before and during progression of decay. An extensively used method to quantify visual colour produced by various matters is microspectrophotometry.

Microspectrophotometry permits to measure absorption or transmission of light in either transmitted or reflected light. In addition, other characteristics of the emitted light such as luminescence, common occurrence in animal taxa, are measurable with this method. Spectrophotometer systems in research institutions provide a variety of configurations, such as filters and shutters, which can be easily reconfigured and adapted to the characteristics of individual experiments.

**2.1.1. Data collection.** Topographic variations on the surface of materials analyzed must usually be kept to a minimum. In the case of insects, elytra are commonly convexly curved, which can generate shifts in the readings. To solve this issue, the field of view was adjusted to occupy regions of the cuticle with minimal changes in topography and displaying a single visual colour as region of interest for the analysis. In all cases, regions of interest (ROIs) were analyzed three

consecutive times and an average of the three scans were used in the analysis to minimise error.

**2.1.2. Data processing.** Data was normalized following common standards (Johnsen 2016).

## **2.2. Electrobeam analysis**

Analysis of materials using electrobeam methods has advanced uninterruptedly since the 60s, and there are now numerous techniques for extracting detailed chemical and structural information of any materials exploiting electrobeams. Scanning electron microscopy (SEM) uses a beam of electrons to recreate high magnification images of the surface of a sample. Energy-dispersive X-ray spectroscopy (EDS) permits the quantitative acquisition of elemental composition. Many research centres possess SEM-EDS systems that can be easily switched and combined to produce a highly informative analysis of numerous types of samples. As a disadvantage of this method samples are required to be good conductors of electricity; however, samples with low conductivity, such as many organic tissues, can be sputter coated with a metal alloy of high conductivity (e.g. Cu or Au).

**2.2.1. Data collection.** Samples can be damaged due to the beam action at high voltages or beam focus in a single spot over a long period of time. Organic samples analyzed showed beam damage; for this reason, the maximum voltage used was 20kV, and for taxa with thinner cuticles, such as *Pyrrhocoris apterus*, the maximum voltage used was 5kV. SEM and EDS analysis are highly affected by topographic changes in the surface of samples, those samples with topographic differences in the surfaces can produce regions of “shadows” or false readings in EDS analysis.

**2.2.2. Data processing.** Images acquired were altered for fine tuning brightness and contrast parameters.

### **2.3. Synchrotron X-ray fluorescence (SRS-XRF)**

SRS-XRF is widely used in analyses at atomic and molecular level of various natural and synthetic materials. Usually, synchrotron facilities are equipped with a variety of beam lines, which are capable of achieving a range of emittances with specific characteristics for a wide assortment of analyses, including tomography, fluorescence and spectroscopy amongst others. XRF analyses are probably the most informative non-destructive analysis, but they are time consuming depending on sample size as well as expensive. Shutters are frequently small and sample holders usually require some planning allowing for specimen placement without disturbing data collection. These analyses can explore the atomic chemistry of various elements at the same time, are highly sensitive to minute variation in absorption energy, and can simultaneously collect a variety of structural and chemical data.

**2.3.1. Data collection.** Calibration of the beam is dependant on the stability of the same over a period of usage, in the best-case scenario a beam line should be calibrated once at the start of the experiment. The beam penetrates the specimen to certain depth, which depends on the nature of the material; in the case of extant insects, the beam usually penetrates completely throughout the width of the body. For this reason, just tissue of interest was positioned under the beam, prompting, in some cases, dissection of the sample with consequent destruction. This is not an issue with fossil samples, where the beam penetrates microns into the surface.

**2.3.2. Data processing.** Software for data calibration and processing is usually specific for each beam line. Standards readings should be collected at the start of the experiment to be calibrated against once the sample data is collected.

## **2.4. Chromatography – mass spectrometry**

Chromatography is widely used in analytical chemistry as a powerful tool to analyse and quantify chemical compounds separated from a mixture. Complex laboratory protocols must be followed to extract the targeted suit of chemical compounds from each sample, these are usually at risk of contamination of the sample and time consuming. In addition, chemical reactants used for molecular targeting methods, such as pyridine in derivatisation of lipids, carry high health risk for the investigators, as they are usually well-documented human carcinogens.

**2.4.1. Gas Chromatography – mass spectrometry (GC-MS).** Gas chromatography separates target chemical compounds that can be vaporized from a mixture to then be analyzed in a mass spectrometer. In the case of lipid analysis, the extraction through solvents of the lipid content from a sample is at risk of being contaminated at every step of the process by human or any by-product of crude oil located in the laboratory. Laboratories destined for lipid extractions should be free of any plastic products, all glassware furnaceed before usage and all other materials should be rinsed with solvents at least three times before any contact with the sample. Lipid extractions are intricate, and easily miscarried, they require skill, and absolute concentration for a successful result. For derivatized samples, the analysis must be completed within a time window of 24-36 hours before molecules lose the trimethylsilyl (TMS) functional groups.

**2.4.1.1. Data collection.** Many institutions are capable of performing GC-MS analysis, systems in place cover an extensive range of possible analytics from diverse groups of molecules. The nature and length of the column attached to the mass spectrometer is of great importance and usually specific for the target molecule or molecules in the analysis. Some columns are capable of analysing a broader suit of molecules, however, precautionary measures should be taken regarding the nature and especially the size of the molecules to be analyzed. Systems in place for analysis usually include a loading tray, which aids in an autonomy but extends the period of preparation and the risk for accidental delays.

**2.4.1.2. Data processing.** Software for data processing is usually specific for each provisioner of scientific instrumentation; files created during the analysis are non-transferable, and built-in libraries are commonly basic and incomplete.

**2.4.2. High performance liquid chromatography – mass spectrometry (HPLC-MS).** Liquid chromatography separates target chemical compounds that can be diluted in a specific solvent to then be analyzed in a mass spectrometer. In the case of carotenoids, extraction of targeted molecules is complex and a time-consuming process, but the risk of contamination is considerably inferior than that of lipid extractions.

**2.4.2.1. Data collection.** Various institutions are capable of performing HPLC-MS analysis, systems in place cover an extensive range of possible analytics from diverse groups of molecules. The solvent nature is specific for the target molecule or molecules in the analysis and usually a combination of solvents in determined proportions are used. Systems in

place for analysis usually include a loading tray, which aids in an autonomy but extends the period of preparation and the risk for accidental delays.

**2.4.2.2. Data processing.** Software for data processing is usually specific for each provisioner of scientific instrumentation; files created during the analysis are non-transferable, and built-in libraries are commonly basic and incomplete.

## **2.5. Alkaline hydrogen peroxide oxidation, hydroiodic acid hydrolysis and high performance liquid chromatography**

Quantification of melanins in living tissues generally requires of isolation of the melanin molecules, which disallows for the characterisation of eumelanin against pheomelanin. Permanganate oxidation of eumelanin allows the formation of pyrrole-2,3,5-tricarboxylic acid (PTCA), and hydriodic acid (HI) reductive hydrolysis of pheomelanin permits the formation of 4-aminohydroxyphenylalanine (4-AHP) isomers, these degradation products can be considered as markers for eumelanin and pheomelanin respectively. PTCA and AHP can be determined using HPLC via UV detection and electrochemical detection respectively (Wakamatsu and Ito 2002a). This method for characterisation of melanins is quick, simple and highly sensitive, and requires a small sample size.

Data collection and processing present the same limitations as those introduced in HPLC-MS.

## **2.6. Statistical analyses**

In all cases the datasets were explored for normality, and in those cases where the nature of the data required it, standard procedures for normalising were applied. In

datasets expressing normal distributions, multivariate analyses were used to assess trends within the data. Principal component analysis (PCA) and linear components analysis (LDA) were conjunctively used in an effort to assess the viability and independency of these methods. Both, PCA and LDA were found to yield uniform results in all analyses, regardless the grouping assumptions imposed by LDA, however, the percentage of variability of the data explained by the first two components is always slightly larger in LDA. When the datasets resolved as non-normal distributed, non-parametric analyses were used to analyse trends in the data.



## CHAPTER 3

# Environmental bias in the fossil record of insects

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### ABSTRACT

Insect species exhibit an abundant variety of patternings on their cuticles. These are formed by the interplay of pigments and/or structural colours. Previous studies on the colouration of fossil insects have focused on structural colours and melanin taphonomy whilst other common pigments have been omitted. Here, an experimental approach is used to understand the taphonomy of pigmentary colours in insects. Beetle specimens of *Harmonia axyridis* (n = 150), *Leptinotarsa decemlineata* (n = 15), *Goliathus goliathus* (n = 4) and *Typocerus velutinus* (n = 12) and the true bug *Pyrrhocoris apterus* (n = 16) were decayed in acidic and basic pH conditions at 25°C for 2 and 6 months. These results show that the preservation potential of pigmented cuticles is controlled by the pH of decay medium and by the original pigment content of the cuticle. Energy-dispersive X-ray spectroscopy (EDS) and electron microprobe microanalysis (EPMA) show that the spatial partitioning of trace elements associated with pigmented cuticle regions changes during decay, and occasionally is even reversed. Gas and high performance liquid chromatography–mass spectrometry (GC-MS and HPLC-MS) reveal that the suite of organic compounds associated with the specimens varies over time due to decay,

and pictures decay by-products after two months decay. The pH of the media of decay and the pigment composition of the insect cuticle, particularly when discrete pigments are spatially distributed, are important factors controlling the rate of decay, where acidic conditions and melanin-rich regions in the cuticle delay the rate of decay. This study provides insights into biases that constrain the fossilisation potential of pigments within cuticles of insects and, by extension, major biases within the fossil record of insects in deep time. Future studies will elucidate the impact of these profound biological and environmental biases on the fossil record of different insect groups.

## **1. PREVIOUS WORK**

### **1.1. Introduction**

Insects have been important components of terrestrial ecosystems for the last 400 Myr (Jarzembowski 1989; Labandeira and Sepkoski 1993; Duncan 1997; Mayhew 2007). Fossil insects are known from a large variety of depositional sediments, from the oldest known in chert (Engel and Grimaldi 2004) to astoundingly high fidelity preservation in amber (Henwood 1992). The fossil record of insects is biased towards small, wingless forms, with highly sclerotized cuticle preserved as carbonaceous impressions in lacustrine shales (Labandeira and Sepkoski 1993; Duncan 1997; Smith 2012; Karr and Clapham 2015). Insects have been highly adaptable to a great variety of niches since their appearance, yet one of the most striking adaptations acquired are diverse cuticle patterning which primarily functions in intra- and interspecific communication. Patterning can consist of spots, eyespots and bands, these are formed by the uneven nature of the spatial distribution of pigments such as carotenoids (Jitariu and Petcu 1974; Czeuczuga

1981; Bezzerides *et al.* 2007), melanin (Koch *et al.* 2000a; Bezzerides *et al.* 2007; Bezzerides and Loofbourrow 2014), pterins (Pfeiler 1968; Stavenga *et al.* 2006; Wijnen *et al.* 2007), flavonoids (Morris and Thomson 1963; Wilson 1985), ommochromes (Koch *et al.* 2000b; Stavenga *et al.* 2014) among others and/or structural colours (Rutowski *et al.* 2005; Yoshioka and Kinoshita 2006; Seago *et al.* 2009). Some fossil insects exhibit similar patterning defined by monotonal colours; usually varying tones of brown and black but the colouration mechanisms responsible are not completely understood.

## **1.2. Recognisable biases in the fossil record of insects**

Fossil insects are found in diverse biotas with various characteristics of host sediment (Duncan 1997; McCobb *et al.* 1998; Engel and Grimaldi 2004; Smith 2012; Barling *et al.* 2015; Anderson and Smith 2017), the majority occur in restricted lacustrine Konservat-Lagerstätten with preservation of soft tissues (Smith 2012; Karr and Clapham 2015; Anderson and Smith 2017). Fossil insects are usually disarticulated and frequently incomplete (Duncan 1997; Barling *et al.* 2015; Anderson and Smith 2017), particularly in those fossils older than 160 Myr (Karr and Clapham 2015). The incompleteness of the fossil record of insects is the result of three main biases: i) nature of tissues and pre-burial processes; ii) lithology and environment of deposition; iii) position of the body respective to the sediment at the time of burial.

**1.2.1. Nature of tissues and pre-burial processes.** The potential for fossilisation of different tissues from animals is dependant on physical, and chemical factors such as composition, structure and nature of autolysis (Briggs 1995; Orr *et al.* 1998; Smith 2012; Butler *et al.* 2015; Karr and Clapham 2015).

Recalcitrant tissues such as legs or thorax are positively biased for fossilisation against more labile tissues such as antenna or wings (personal observation from Labandeira and Sepkoski 1993). Insects with thicker exoskeleton seem to preserve more commonly than those with a lesser degree of sclerotisation and thinner cuticle; for example, beetles (Coleoptera) are the most copious fossil finds while butterflies (Lepidoptera) are rare in the fossil record (Labandeira and Sepkoski 1993). In addition, the conditions of decay, the extent of time of the process of decay (Allison 1986; Gupta *et al.* 2009; Sansom 2014) and early mineralisation of tissues (Briggs and Kear 1994; Orr *et al.* 1998; Briggs 2003; Anderson and Smith 2017) are major drivers for the potential of preservation of any labile tissue. Exogenous microbial activity and autolysis are the important agents promoting decay of tissues; however, endogenous microbial activity is also a driver of decay, but while consuming internal tissues can form biofilms, which can prompt early mineralisation and subsequent preservation of the internal anatomy (Butler *et al.* 2015). Additionally, pH is an important factor that shapes the structure of bacterial activity in modern lacustrine environments, for instance, acidic media has been observed to decrease the richness and diversity of the bacterioplankton in the community and greatly reduce the bacterial activity (Ren *et al.* 2015).

**1.2.2. Lithology and environment of deposition.** The taphonomic processes involved in fossilisation vary depending on the lithology and the environment of deposition (Butterfield 1995; Smith 2012). Whilst fossil insects are found from numerous depositional environments such as deltaic (Karr and Clapham 2015), fluvial (White *et al.* 2017), lagoonal (Barling *et al.* 2015), marine settings (Beaury *et al.* 2017) and as amber inclusions (Martínez-Delclòs *et al.* 2004), the majority of the fossil insects are preserved in lacustrine sediments

(Labandeira and Sepkoski 1993; Smith 2012). In general, fossil insects are preserved as carbonaceous impressions in lacustrine shales (Duncan 1997; Smith 2012; Karr and Clapham 2015; Anderson and Smith 2017), although amber enclosed and fine-grained carbonate sediments are important components of the Cretaceous and Tertiary insect fossil records (Labandeira and Sepkoski 1993; Martínez-Delclòs and Martinell 1993; Martínez-Delclòs *et al.* 2004). Lacustrine insect assemblages are usually overabundant in allochthonous taxa from smaller size classes due to transportation and deposition processes (Smith 2012), additionally, the quality of the preservation is largely dependant on the type of lake, associated sediments, water chemistry and the sampling region within the lake (Park 1995; Smith and Moe-Hoffman 2007; Henning *et al.* 2012; Smith 2012). Fossil insects preserved in amber display high fidelity preservation of minute morphological details that in other environments of deposition are lost during fossilisation (Henwood 1992; Martínez-Delclòs *et al.* 2004). Nevertheless, some mineralising pathways, such phosphatisation (Schwermann *et al.* 2016) can prompt rapid fossilisation resulting in morphological preservation of insects comparable to that preserved in amber, but mineralisation results in loss of important chemical information.

Lacustrine settings can be closed or open environments; the former records an autochthonous sedimentation with unchanging composition whereas the latter, is highly influenced by allochthonous outputs (Smith 2012). Pelitic and carbonate rocks can be produced in lacustrine settings, for instance, saline-alkaline lakes tend to release CO<sub>2</sub> and NH<sub>3</sub> from decomposition of organic matter, which increases the alkalinity of the pore water and the carbonate ion concentration and prompts carbonate precipitation (Park 1995). In pelitic rocks, such as mudstones or shales,

fossilisation of insects depends on the microenvironment (Park 1995), particularly the pH conditions of the media of decay and/or the presence of organics in the sediment (Briggs and Kear 1993c; Sagemann *et al.* 1999), which can decrease pH and prompt the precipitation of  $\text{CaCO}_3$  or  $\text{CaPO}_4$  creating a protective layer enclosing the carcass. The taphonomic processes undertaken by fossil insects preserved in carbonate rocks are majorly biologically controlled (Martínez-Delclòs *et al.* 2004), such as the formation of microbial mats, which protect the carcass from bio- and environmental erosion (Gall 1995) and induce authigenic mineralisation (Butler *et al.* 2015).

**1.2.3. Position of the body respective to the sediment at the time of burial.** Insect specimens in the same biota may be preserved differently controlled by the position of the body relative to the sediment at the time of burial. Insects found in lacustrine environments would have broken the air-water interface, and fallen through the water column before reaching the lake bottom (Wang *et al.* 2013; Karr and Clapham 2015); this process is controlled by biological factors explained by the wing surface/area-to-mass ratio (Wagner *et al.* 1996) and environmental factors such as sediment load (Tian *et al.* 2020). Experiments on extant specimens and inspection of the fossil record of insects shows that larger wings would increase the flotation time (Wagner *et al.* 1996), and therefore result in decomposition on the water surface, dorsoventral settlement in the sediment and lower fidelity preservation (Wang *et al.* 2013).

### 1.3. Insect cuticle

Insect cuticle, as in all arthropods, is formed by three layers, from external to internal: epicuticle, exocuticle and endocuticle. Each of these layers is highly

variable in thickness, and structural content depending on species, ontogeny, sex and laterally from location to location in the cuticle (Neville 2012b; Andersen 2009a, b; Moussian 2013) but fortunately all cuticles can be defined in reference to a basic plan (Fig. 1.2).

Major organic compounds of insect cuticle include chitin, lipids and proteins (Andersen 1979). Chitin is a polysaccharide that functions as the basic building material of all insect cuticles (Merzendorfer and Zimoch 2003). Cuticular lipids in insects are present in the outer layer of the integument and consist of a complex mixture of apolar compounds including straight-chain and branched, saturated and unsaturated hydrocarbons, waxes, sterols, alcohols and acids (Jackson and Baker 1970; Andersen 1979; Blomquist *et al.* 1987). Pigments are another important component of the cuticle, these not only produce striking colouring and patterning but also offer protection against external factors such as ultraviolet damage (Hackman 1984; Hu *et al.* 2013) and mechanical penetration (Mills and Fox 1972; Andersen 2010).

An extensive account of insect cuticle in both physical and chemical components has been made in Chapter 1 of this thesis.

#### **1.4. Pigments in fossil insects.**

Fossil biomolecules present a valuable opportunity to have a peek at long extinct organisms and their ecological relationships. Most fossilized biomolecules endured great chemical changes that permitted them to be more stable over long periods of time (Blumer 1965; Stankiewicz *et al.* 1997a), however, the original structure is often preserved enabling recognition after fossilisation. Whereas the chemical changes undergone by arthropod chitin through decay and preservation processes

have been extensively investigated in insects and arthropods in general (e.g. Baas *et al.* 1995; Flannery *et al.* 2001; Gupta *et al.* 2007; Cody *et al.* 2011), there has been little attempt to characterise other possible molecular remains in insects.

Pigments hold important information about fossil organisms, they inform us not only about original colour but also its intra- and interspecific relations and interactions in the ecosystem. Melanin is probably the most recognized pigment in both extant and fossil insects (e.g. Hackman 1967; Hu *et al.* 2013; Bezzerides and Loofbourrow 2014; Barling *et al.* 2015). It is a particularly recalcitrant molecule (Fig. 1.3), which is insoluble and unreactive with other organic compounds (Morgan 2004a) and seems to easily bind to a series of trace elements that have been investigated as possible trace elemental biomarkers of melanin in the fossil record (Wogelius *et al.* 2011; Edwards *et al.* 2016; Rogers *et al.* 2021; Rossi *et al.* 2021). While the taphonomy of melanin has been investigated (Rogers *et al.* 2021; Rossi *et al.* 2021), and the trace element chemistry of melanin and other pigments has been characterized (Chapter 5), there has been no attempt to compare the taphonomy of insect cuticle containing different pigments.

Taphonomic experiments under controlled laboratory conditions are crucial to test and understand the fossilisation processes involved in the preservation of soft tissue (Purnell *et al.* 2018). Environmental conditions such as temperature (Sansom *et al.* 2010; Sansom 2014) and pH of the media of decay (Briggs *et al.* 1993) are important direct and indirect controls on the rate of decay of soft tissue of vertebrates but whether this applies to insect cuticle is unknown. In addition, the impact of cuticle chemistry, in particular presence / absence of certain pigments on preservation remain unidentified. This is the first experimental study of alteration of pigments during decay of insect cuticles.



## 2. AIM, SCOPE AND JUSTIFICATION OF EXPERIMENTS

Laboratory controlled experiments were conducted to investigate the effect of pH of decay medium and specific pigment content and distribution within the cuticle on the chemical and physical properties of cuticles of *H. axyridis*, *L. decemlineata*, *G. goliathus*, *T. velutinus* and *P. apterus*. This investigation informs controls on the potential for fossilisation of patterned insect cuticles and possible preservational biases generated by the conditions of decay and the spatial distribution of certain pigments in the cuticle of insects.

These key issues are explored for the first time by assessing the extent of chemical and physical changes occurring during distinctive conditions of decay and associating them with pigment content in the insect cuticle. This analysis provides insights into environmental and biological biases that constrain the fossilisation potential of pigments within cuticles of insects and, by extension, major biases within the fossil record of insects in deep time.

This chapter addresses the following hypotheses as formulated in Chapter 1.

*Hypothesis 1:* Contrasting environments of decay, particularly that of differing pH conditions, affect the rate of physical and chemical decay of insect cuticles differently.

*Hypothesis 2:* Regions of varying pigment content in the cuticle of insects (i.e. patterned cuticles) are affected distinctively through chemical decay when the conditions of decay are identical.

*Hypothesis 6:* The association between certain trace elements and pigment molecules (i.e. regions of the cuticle with specific visual colouration) persists during decay.

*Hypothesis 7:* Distinctive trace elements or sets of elements can be used as biomarkers for the original pigmentary content in the cuticle of fossil insects.

### 3. MATERIAL AND METHODS

#### 3.1. Samples

**3.1.1. *Harmonia axyridis*.** The elytra of *H. axyridis* show black spots of variable number and size on a dark yellow to bright red background. Elytra are smooth and lack setae or other irregularities. Carotenoids have been reported from non-black regions of the cuticle (Britton *et al.* 1977; Bezzerides, *et al.* 2007); the concentration of carotenoids in the cuticle is positively correlated with the hue and intensity of the displayed visual colour (Bezzerides, *et al.* 2007). Black regions of the cuticle contain quantified amounts of pheomelanin and eumelanin (this study). All specimens used in this study (n = 94) were collected alive, are from a single commercial supplier (Blades Biological, UK) with similar ontogenic stage and a common diet.

**3.1.2. *Leptinotarsa decemlineata*.** The elytra of *L. decemlineata* show four to five black stripes with a bright yellow background. Elytra are smooth and lack setae or other irregularities. Melanins (Gortner 1911; this study) and carotenoids including Xanthophylls (Gortner 1911; Cze Czuga 1971; Jitariu and Petcu 1972; Jitariu *et al.* 1973; Jitariu and Petcu 1974; Cze Czuga 1980; Cze Czuga 1981) and keto-carotenes (Leuenberger and Thommen 1970; Poff 1976) have been reported from black and yellow regions of the cuticle respectively. All specimens (n = 14) were loaned for destructive analysis by Yale Peabody Museum of Natural History.

**3.1.3. *Goliathus goliathus*.** The elytra of *G. goliathus* show white to light grey amorphous patches on a dark brown background. The colouration in the

lighter regions is achieved via reflection of the light in short setae (Jiang *et al.* 2012) clustered in the surface of the cuticle containing an ultraviolet reflective pigment (Pope and Hinton 1977). These lighter surfaces in the cuticle are covered by a thin layer of wax-producing colouration (Hagen 1872). Melanins have been reported in dark regions of the cuticle at family level (Hackman 1953; Kuusijärvi *et al.* 2015). All specimens (specimens  $n = 4$ ; dissected samples  $n = 42$ ) were females and were purchased from a private collector. The cuticles were dissected into smaller pieces, each containing two distinct visual colours, and treated as individual samples.

**3.1.4. *Typocerus velutinus*.** The elytra of *T. velutinus* show two to four bright yellow elongated spots in a dark brown background. Elytra are smooth and contain long, thick, black setae. Colouration in the yellow regions is produced by carotenoids in other members of the family Cerambycidae (Czeczuga 1988). Melanins (eumelanin and pheomelanin) have been reported in brown regions of the cuticle (this study). All specimens ( $n = 14$ ) were loaned for destructive analysis by Oxford University Museum of Natural History.

**3.1.5. *Pyrrhocoris apterus*.** The elytra of *P. apterus* show two circular and one triangular black spot on a dark orange to dark red background. Elytra are smooth and lack setae or other irregularities. Red colouration in the cuticle is produced by pterins (Smith and Forrest 1969; Porcar *et al.* 1996; Socha and Nemec 1996; Bel *et al.* 1997) and carotenoids (Lederer 1938). Melanins have been reported in dark regions of the cuticle (Slama 1998). All specimens ( $n = 14$ ) were loaned for destructive analysis by Oxford University Museum of Natural History.

### 3.2. Decay protocol.

Specimens were degraded in a series of concurrent experiments in controlled laboratory conditions designed to investigate the impact of environmental pH on the taphonomy of pigmented insect cuticle. Live specimens were euthanized via freezing and stored at -40°C or -80°C prior to the start of the experiments. All other specimens had been dried prior to storage via standard procedure by the hosting institutions and frozen on arrival prior to experimentation. Frozen specimens (n = 197) were dissected and individual elytra were transferred to glass vials with a solution of ca. 5 ml double distilled water, potassium hydroxide (KOH) and hydrochloric acid (HCl) adjusted to one of the conditions illustrated in Table 3.1. Control specimens (n = 52) were placed in double distilled water adjusted to pH 7. Vials were placed in a constant temperature incubator at 25°C ± 1°C in the dark.

### 3.3. Sampling.

In all cases, four replicates, or 12 in the case of *G. goliathus*, for each combination of conditions of decay were destructively sampled at every sampling interval. Two replicates of control samples for *H. axyridis*, *L. decemlineata*, *T. velutinus* and *P. apterus* and 6 replicates of *G. goliathus* were destructively sampled at 60 days of decay, additionally a further two replicates of *H. axyridis* treated in neutral conditions were destructively sampled each at 120 and 180 days. Four specimens of *H. axyridis* (two replicates for acidic and another two for basic conditions) were sampled at pH 6 and pH 8 at 1, 3, 7, 14, 30, 60, 120 and 180 days; each sampling point terminated the experiment. Four specimens of *H. axyridis* at pH 5 and pH 10 *L. decemlineata*, *T. velutinus* and *P. apterus* (two replicates for acidic and another two for basic conditions) were sampled at 14, 30 and 60 days when experiment was

terminated; each sampling point terminated the experiment. Twelve samples of *G. goliathus* (six replicates for acidic and another six for basic conditions) were sampled at 14, 30 and 60 days when experiment was terminated; each sampling point terminated the experiment. In all taxa, except *G. goliathus*, each replicate was dissected into three smaller pieces immediately after retrieval, producing 12 samples per interval and taxon, and processed separately following described procedures below for each analytical method. Table 3.1 summarises the number of replicates treated and relevant experimental conditions.

### **3.4. Fixing and dehydration of cuticles.**

Samples of elytra were fixed in a solution of 2.5% glutaraldehyde in 0.1M phosphate buffer (PB) at 4°C overnight. For specimens analyzed using scanning electron microscopy (SEM), EDS and EPMA fixed cuticles were dehydrated in ethanol following McNamara and colleagues protocol (2006) with various adjustments enumerated here. Fixed cuticles were dehydrated via immersion in the following solutions: 0.1M PB (60 min, three times), distilled water (20 min, three times), 10% ethanol (10 min, twice), 25% ethanol (10 min, twice), 50% ethanol (10 min, twice), 75% ethanol (10 min, twice), 90% ethanol (10 min, twice), 100% ethanol (20 min, twice), hexamethyldisilamine (HMDS) (20 min).

### **3.5. Microspectrophotometry.**

Untreated and fixed elytra were imaged using a Leica MSV266 stereomicroscope and a Leica DFC 290 HD camera with a polarizing filter. The brightness and contrast of images were adjusted using GNU Image Manipulation Program (GIMP 1995-2017). Colour spectra were measured from the dorsal surface of untreated

and treated samples in individually coloured regions of the cuticle using a Zeiss Axioskop 2 Epifluorescence Microscope, an Ocean Optics 2000+ microspectrophotometer and Oceanview software. Three measurements (each representing an average of three scans using an integration time of 1 s) were taken for each region of interest (ROIs). Resultant data was normalized to the highest absorption peak for individual colour readings in each sample operating the formula below in Excel.

$$(C - C_{x\min}) / (C_{x\max} - C_{x\min})$$

C – Cell

C<sub>x</sub> – Column

For all colours displayed on the cuticle of each specimen, hue ( $\lambda_{\max}$ ), intensity ( $R_{\max}$ ) and purity (S) were calculated using standard protocols (Srinivasarao 1999; Briscoe *et al.* 2010) executed as follows.

Hue is the similarity of a colour to a wavelength on the red, blue, green or yellow spectrum (Hill 2002). It was determined calculating the position of the peak at its maximum nanometres (maximum lambda:  $\lambda_{\max}$ ) in the colour spectrum (Hill 2002; Piszter *et al.* 2011). Intensity refers to the brightness of the material producing the colour, and it deals with its black/white components (Hill 2002; Srinivasarao 1999). It was calculated by finding the equivalent amplitude (maximum height of the peak) at the maximum lambda in the colour spectrum (Srinivasarao 1999). Purity or saturation is the range of intensity of colour from white to maximally saturated (Hill 2002). A very saturated colour has most of the intensity close to the dominant wavelength, while an unsaturated colour would have contributions from many other wavelengths of light (Srinivasarao 1999). It

was calculated by estimating the total area occupied by the peak in the colour spectrum (Srinivasarao 1999).

Mean, variance and standard deviation values were calculated for each ROI for each sampling point. Variation of the data among experimental conditions was assessed using two-sample F-test for variances (*H. axyridis*, n = 13; *L. decemlineata*, n = 5; *G. goliathus*, n = 5; *T. velutinus*, n = 5; *P. apterus* n = 5) with a 95% confidence interval in StatPlus 7.3.3.0. Individual samples from all experimental conditions were compared to untreated samples.

### **3.6. Electrobeam analysis.**

Dehydrated and untreated samples were sputter coated with an Au-Pd mixture, and analyzed with a FEI Quanta 650 field emission scanning electron microscope at an accelerating voltage of 10–20 kV. EDS maps of C, O, F, Na, Mg, Al, Si, P, S, Cl, K, Ca, Ti, Cr, Mn, Fe, Co, Cu, Zn, Pd and Au of coated specimens were obtained using a Hitachi S-3500N variable pressure SEM equipped with a Thermo Noran energy dispersive X-ray spectrometer (University of Bristol, UK) and a FEI Quanta 650 FEG SEM equipped with an Oxford Inca EDS at an accelerating voltage of 10–20 kV. EPMA maps (Na, Mg, Al, Si, P, S, K, Ca, Ti, Mn, Fe, Cu and Zn) of coated specimens were obtained using a JEOL JXA8530F Hyperprobe at an accelerating voltage of 20 kV.

### **3.7. Gas chromatography-mass spectrometry.**

Untreated (n = 8) specimens, specimens treated for 60 days (n = 36) and 180 days (n = 8) were analyzed by GC-MS, as well as decay media for specimens degraded for 60 (n = 16) and 180 (n = 8) days. All samples were freeze-dried prior to lipid

extraction. All glassware was furnace at 450°C for 4 hours and metal tools were rinsed three times with methanol (MeOH) prior to, and in between, uses to avoid contamination. Natural melanin (*Sepia officinalis*), synthetic melanin and natural  $\beta$ -carotene were used as standards (Sigma-Aldrich). Total lipid extracts (TLE) were acquired as follows. Freeze-dried samples were crushed in 10 ml glass boiling tubes using a thick glass stirring rod. Crushed samples were dissolved in a solution of ca. 2 ml of a 2:1 dichloromethane (DCM):methanol (MeOH) mixture. The mixture was ultrasonicated for 15 mins and centrifuged for 5 mins at 3000 rpm and the supernatant pipetted to a clean tube. This procedure was repeated twice. All extracts for each sample were combined before the solvent was removed by drying under a gentle stream of nitrogen. Blank samples were run concurrently with the tissue extractions to test for contamination. All extractions were derivatized immediately before analysis as follows: i) 50  $\mu$ l of N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 50  $\mu$ l of pyridine were added to each vial; ii) vials were sealed and placed on the heating block at 70°C for 1 h; iii) the vial and heating block were allowed to cool to 40°C and derivatisation agents dried using a gentle stream of nitrogen; and iv) 500  $\mu$ l of ethyl acetate were added to resolubilise the samples. All derivatized samples were analyzed within 24–36 h. Samples were analyzed using a Trace 1300 gas chromatograph with flame ionisation detector (Thermo Scientific), equipped with a non-polar-fused silica capillary column (CPSil-5CB, 50m x 0.32mm x 0.12 mm, Agilent Technologies - Flame Ionisation Detector (GC-FID)). The temperature program was as follows: initial temperature 40°C, rising to 130°C at 20°C min<sup>-1</sup>, then rising to 300°C at 4°C min<sup>-1</sup>, holding at 300°C for 25 min. Concentrations were adjusted to optimal values via addition or evaporation of the solvent. Samples were then analyzed



using a Trace 1300 gas chromatograph and column previously described, with identical temperature program, coupled to an ISQ mass spectrometer (Thermo Scientific, Hemel Hempstead, UK). The ion source and transfer line were maintained at 300°C. The emission current was set to 50  $\mu$ A and the electron energy to 70 eV. The analyser was set to scan at  $m/z$  50–650 with a scan cycle time of 0.6 s. Data were analyzed using Xcalibur software (Thermo Fisher Scientific, UK). Organic compounds in the spectra were identified by reference to the NIST Spectral Library, AOCS Lipid Library (AOCS 2019), Lipid Web (Christie 2019; Foster *et al.* 2013) and published literature. Between 10 and 20 peaks of interest (excluding phthalate due to possible contamination), i.e. those representing the highest percentage area, were selected from each sample for statistical analysis. Combining these data for all specimens produced a data matrix with a total of 66 compounds of interest (combined to yield 23 generalized groups of compounds; Supplementary Table 3.2; NOTE: Supplementary files mentioned in this chapter are located in Appendix 3). For each sample, peaks of interest were assigned their percentage area value determined by manually delimiting the base of the peak in Xcalibur or a value of 0 if absent in the sample. Trends in the data were assessed using paired-correlation analyses and ANOVA at 95% confidence intervals in Microsoft Excel and linear discrimination analysis (LDA), principal component analysis (PCA) in the packages "stats" and "MASS in R (R CORE TEAM 2013).

### **3.8. High Performance Liquid chromatography-mass spectrometry of carotenoids.**

The carotenoid-rich regions (red-orange) of untreated ( $n = 2$ ) and treated cuticles (decayed for 60 ( $n = 4$ ), 120 ( $n = 6$ ) and 180 ( $n = 6$ ) days) of *H. axyridis* were

dissected with a sterile scalpel, crushed in a porcelain mortar, transferred to individual vials using ethanol and dried under a gentle stream of nitrogen. Decay media for 60 (n = 4), 120 (n = 6) and 180 (n = 6) days were freeze-dried in order to separate suspended particulates from the liquid and analyzed concurrently with degraded insects. Extractions of all samples proceeded as follows: i) powders from insect cuticles and particulates from liquid samples were dissolved in 1 ml of a 1:1 solution of hexane: *tert*-butyl methyl ether (MTBE) and shaken vigorously for 10 s. The resulting supernatant was transferred to a 1.5 ml plastic vial and the solvent evaporated using a gentle stream of nitrogen. The pigment residue was resuspended in 200 µl of a (42:42:16) solution of methanol:dimethyl:acetone and shaken vigorously for 10 s. Solutions were centrifuged for 3–4 s at 100 rpm prior to analysis. A three-step gradient solvent system was used to analyse both xanthophylls and carotenes in a single run at a constant flow rate of 1.2 mL/min: first, isocratic elution with 42:42:16 methanol:acetonitrile:dichloromethane for 11 min, followed by a linear gradient up to 42:23:35 methanol:acetonitrile:dichloromethane for 21 min, held isocratically at this condition until 25 min, and finishing with a return to the initial isocratic condition from 25–30 min. Organic compounds in the spectra were identified by reference to published literature (Mercadante *et al.* 1997; Bezzerides *et al.* 2007; Van Breemen *et al.* 2012).

### **3.9. Alkaline hydrogen peroxide oxidation (AHPO), hydroiodic acid hydrolysis (HI) and high performance liquid chromatography (HPLC) of melanins.**

Dark regions of the cuticle of specimens treated for 180 days (n = 9), in acidic (pH 6; n = 3), neutral (pH 7; n = 3) and basic (pH 8; n = 3) conditions, *H. axyridis*, *L.*

*decemlineata* and *T. velutinus*, were dissected with a sterile scalpel, and transferred to individual foil containers, which were shipped for analysis. Freeze dried tissue samples (9-17 mg) were homogenized in water with Ten-Broeck homogenizner at a concentration of 10 mg/mL and and 100  $\mu$ L aliquots were subjected to alkaline hydrogen peroxide oxidation (Ito *et al.* 2011; Barek *et al.* 2018) and hydroiodic acid hydrolysis (Wakamatsu *et al.* 2002b). For PTCA samples were dissolved in 100  $\mu$ L of double distilled water and placed in 10 ml screwed-capped test tubes. Test tubes were filled with 375  $\mu$ L of 1M potassium carbonate ( $K_2CO_3$ ) and 35  $\mu$ L 30% of hydrogen peroxide ( $H_2O_2$ ). The tubes were vigorously mixed at  $25 \pm 1^\circ C$  for 1200 min. The residual hydrogen peroxide was decomposed by adding 50  $\mu$ L 10% sodium sulphite ( $Na_2SO_3$ ) and the mixture was acidified with 140  $\mu$ L 6M of hydrochloric acid (HCl). Reaction mixtures were centrifuged for 1 min at 10,000 rpm, and an aliquot (80  $\mu$ L) of the supernatant was directly injected into the HPLC system (Ito *et al.* 2011). HI to measure the degradation product of pheomelanin, 4-amino-3-hydroxyphenylalanine (4-AHP), was performed following previous published protocols (Wakamatsu *et al.* 2002b). Sample were crushed in a porcelain mortar and resultant powder was suspended in 100  $\mu$ L of double distilled water and placed in 10 ml screwed-capped test tubes. Test tubes were filled with 20  $\mu$ L of phosphinic acid ( $H_3PO_2$ ) and 500  $\mu$ L 57% of hydroiodic acid (HI). The tubes were heated at  $130^\circ C$  for 1200 min. Cooled aliquots (100  $\mu$ L) of the hydrolysates were evaporated to dryness by a vacuum pump and the residues were dissolved in 200  $\mu$ L 0.1M of hydrochloric acid (HCl). Reaction mixtures were centrifuged for 1 min at 10,000 rpm, and an aliquot (10  $\mu$ L) of the supernatant was directly injected into the HPLC system (Wakamatsu *et al.* 2002b; Barek *et al.* 2018). Determination of melanin markers was completed using HPLC as previously reported (Wakamatsu

*et al.* 2002b, 2012). EM and PM contents were calculated by multiplying by factors of 25 and 7, respectively. For PTCA in HCl-hydrolyzed tissue, we used 200 uL

### **3.10. Image processing and analysis.**

Digital images of all specimens were obtained using a Leica MSV266 stereomicroscope and a Leica DFC 290 HD camera with a polarising filter. Stacks of images were combined onto a single file using CombineZP.Ink for Windows (<https://combinezp.software.informer.com/>). SEM images were acquired with a FEI Quanta 650 field emission scanning electron microscope at an accelerating voltage of 10–20 kV. The author declares that disclosed images were not altered in any other mode furthermore of fine-tuning brightness and contrast parameters.

## **4. RESULTS**

### **4.1 Optical Microscopy.**

For all samples analyzed, the changes in regions of the cuticle displaying black visual colour were minimal regardless of the duration of the experiment. Variations of colour in regions of the cuticle displaying non-black colours were taxon dependant.

**4.1.1. *Harmonia axyridis*.** Cuticle colour did not change for the first 14 days. From 14 days red cuticle regions altered from red to orange and light yellow after 30 days regardless of the conditions of decay; colour did not change thereafter (Fig. 3.1i–p, y–af and Supplementary Fig. 3.1). At the end of the experiment, black cuticle regions showed minimal or no visible alteration in colour from the original state regardless of the conditions of decay (Fig. 3.1y–af). Red cuticle regions were

visibly less saturated changing to paler red to light yellow in colour, particularly in basic conditions of decay (Fig. 3.1y–af).

**4.1.2. *Leptinotarsa demlineata*.** Cuticle colour did not change for the first 14 days regardless of the conditions of decay. At the end of the experiment, black cuticle regions showed minimal or no visible alteration in colour from the original state regardless of the conditions of decay (Fig. 3.2e–h). Yellow cuticle regions were visibly less saturated, particularly in basic conditions of decay (Fig. 3.2e–h).

**4.1.3. *Goliathus goliathus*.** Cuticle colour did not change for the first 14 days regardless of the conditions of decay. At the end of the experiment, black and grey cuticle regions showed minimal or no visible alteration in colour from the original state regardless of the conditions of decay (Fig. 3.2m–p).

**4.1.4. *Typocerus velutinus*.** Cuticle colour did not change for the first 14 days regardless of the conditions of decay. At the end of the experiment, brown cuticle regions showed little alteration in colour from the original state regardless of the conditions of decay (Fig. 3.2u–z). Yellow cuticle regions were visibly less saturated, particularly under basic conditions of decay (Fig. 3.2u–z).

**4.1.5. *Pyrrhocoris apterus*.** Cuticle colour did not change for the first 14 days regardless of the conditions of decay. At the end of the experiment, black cuticle regions showed little alteration in colour from the original state regardless of the conditions of decay (Fig. 3.2ae–ah). Red cuticle regions were visibly much less saturated than the same regions at the start of the experiment, nearly approaching white, particularly in basic conditions of decay (Fig. 3.2ae–ah).

The results described above indicate in regions of the cuticle in *P. apterus* containing pterins and carotenoids show the largest quantifiable shift of visual colour; these dark red/orange regions have lost the red saturation and appear white

after 60 days of decay, black regions of the cuticle, melanin-rich, show little colour alteration (Fig. 3.2 ae–af). Specimens of *G. goliathus* show the least alteration of visual colour during decay; brown and grey regions contain melanin in the cuticle whilst the grey regions achieve their colouration through short setae containing an ultraviolet reflective pigment (Fig. 3.2m–p).

#### 4.2. Microspectrophotometry.

For all samples analyzed, changes of hue ( $\lambda_{\max}$ ) and purity (S) in regions of the cuticle displaying black visual colour were minimal regardless of the duration of the experiment. Changes of hue and purity in regions of the cuticle displaying lighter colours were taxon dependant. Intensity ( $R_{\max}$ ) is taxon dependant in all visual colour and experimental conditions. The duration of the experiment, pH conditions and biological constraints related to the pigment content in the cuticle affect the extent of decay. For instance, samples of *H. axyridis* decayed for 180 days show the greatest changes in hue, purity and intensity but the results are also more heterogeneous among experimental conditions (Fig. 3.3a, Supplementary Fig. 3.2a–d and Supplementary Table 3.1). This is confirmed through two-sample F-test, which shows that only in those suites of tests where experimental conditions had the most disparate pH values, the null hypothesis was rejected (in bold; Supplementary Table 3.1). Additionally, the visual colour of melanin-rich regions of the cuticle is largely unaffected by decay regardless of the conditions of decay or the duration of the experiment (Fig. 3.3, Supplementary Fig. 3.2a, b, e, f, i, j, m, n and Supplementary Table 3.1). The hue, purity and intensity of the visual colour from regions lacking melanin pigmentation are more affected with increasingly alkaline conditions (Fig. 3.3, Supplementary Fig. 3.2c, d, g, h, k, l, o, p and

Supplementary Table 3.1). For regions of the cuticle lacking melanin pigments, the F-values are larger than the critical values in *H. axyridis*, *L. decemlineata* and *P. apterus* regardless of the pH conditions of decay (Supplementary Table 3.1), which demonstrates that the variance within the data is not randomized at 95% confidence. Acidic conditions of decay in *G. goliathus* and *T. velutinus* create F-values are larger than the critical values regardless of the visual colour displayed by the cuticle (Supplementary Table 3.1).

Microspectrophotometry analyses of the variation of quantifiable visual colour in the cuticle of these 5 insect taxa confirm that samples decayed in alkaline media ( $\text{pH} < 7$ ) display a larger extent of decay after the experiment is terminated in all cases, thus supporting hypothesis 1. Additionally, the rate of decay occurs faster and decay is more extensive in regions of the cuticle where the main pigment molecule is not melanin, thus supporting hypotheses 1 and 2.

**Hypotheses 1 (*contrasting environments of decay, particularly that of pH conditions, affect the rate of decay*) and 2 (*regions of varying pigment are affected distinctively through chemical decay when the conditions of decay are identical*) are accepted.**

Supplementary Text in Appendix 3 contains a detailed account of the microspectrophotometry results for all taxa analyzed.

#### **4.3. Electrobeam analysis.**

SEM analysis reveals that there is morphological evidence for a more extensive decay in melanin free regions of the cuticle for all taxa. Bacteria are more abundant on the surface of the cuticle in non melanin-rich regions for all experimental conditions and taxa; furthermore, bacteria are qualitatively more abundant and

diverse in samples treated in basic media (Fig. 3.4g, h, k, l, o, p, s, t, w, x and Supplementary Figs. 3.3g, h, k, l, 3.4g, h, k, l, 3.6g, h, k, l, 3.7g, h, k, l). Cross sections of the cuticle show that decay occurs mainly within the chitin bundles located in the endocuticle for all taxa (Fig. 3.4e, f, i, j, m, n, q, r, u, v, y, z, aa, ab and Supplementary Figs. 3.3e, f, i, j, 3.4e, f, i, j, 3.6e, f, i, j, 3.7e, f, i, j.); proteinic matrix is virtually absent after 60 days of decay in all samples treated in basic media (Fig. 3.4e, f, m, n and Supplementary Figs. 3.3e, f, 3.4e, f, 3.6e, f, 3.7e, f). The exocuticle in all taxa is barely changed during decay; however, in basic samples it seems to be further altered (Fig. 3.4e, f, m, n and Supplementary Figs. 3.3e, f, 3.5, 3.4e, f, 3.6e, f, 3.7e, f). Decay is more evident in specimens treated in basic media; although, differences seem to be correlated with the extent of decay in each experimental condition and the colour associated on the insect cuticle, the exact structural nature of these differences cannot be confidently identified with the images available.

This analysis shows that regions of the cuticle containing carotenoids, in *H. axyridis* and *P. apterus*, and pterins, in *P. apterus*, show the largest alteration of the cuticular structure; endocuticular chitin bundles are more prominent in these non melanin-rich regions, which indicate the degradation of the surrounding proteinic matrix (Supplementary Figs. 3.3e, i, 3.7e, i). Additionally, these dark red/orange regions have distinctively more numerous and diverse bacterial communities in the surface after 60 days of decay, black regions of the cuticle, melanin-rich, seem to attract less bacteria and these communities are less diversified, particularly in acidic media (Supplementary Figs. 3.3g, h, k, l, 3.7g, h, k, l). Specimens of *G. goliathus* show the least alteration of cuticular structure; brown and grey regions contain melanin in the cuticle, in this taxon, the cuticular structure is affected



similarly regardless of the visual colour displayed in its surface (Supplementary Fig. 3.5).

Electrobeam analyses of the variation of the micro-structural properties of insect cuticle associated with visual colour in these 5 insect taxa indicate that samples decayed in alkaline media ( $\text{pH} < 7$ ) display a larger extent of decay after the experiment is terminated in all cases, thus supporting hypothesis 1. Additionally, the rate of decay occurs faster and decay is more extensive in regions of the cuticle where the main pigment molecule is not melanin, thus supporting hypothesis 2.

**Hypotheses 1 (*contrasting environments of decay, particularly that of pH conditions, affect the rate of decay*) and 2 (*regions of varying pigment are affected distinctively through chemical decay when the conditions of decay are identical*) are accepted.**

Supplementary Text in Appendix 3 contains a detailed account of the electrobeam results for all taxa analyzed.

#### **4.4. Electron microprobe microanalysis mapping.**

EPMA maps of *H. axyridis* reveal that Mg, K and Ca show strong spatial partitioning between red and black cuticle regions in untreated specimens; spatial partitioning is not apparent for other elements (Fig. 3.5 and Supplementary Fig. 3.8). Mg and Ca are enriched in black cuticle regions, whereas K is enriched in red-coloured regions (Fig. 3.5d, f and Supplementary Fig. 3.8). Spatial partitioning of Mg and Ca persist and are enhanced during decay (Fig. 3.5d, f and Supplementary Fig. 3.8). In addition, during decay Na is preferentially lost in red cuticle regions (Fig. 3.5c and Supplementary Fig. 3.8). Intriguingly, spatial partitioning of Na, Mg and Ca is more striking after decay (Fig. 3.5c, d, f and Supplementary Fig. 3.8c, d);

this feature is apparent in acidic media only for Na (Fig. 3.5c and Supplementary Fig. 3.8d), but in all media for Mg and Ca (Fig. 3.5d, f and Supplementary Fig. 3.8b–d). Spatial partitioning of K is reversed during decay, whereby it is enriched in dark regions post-decay, especially in basic media (Fig. 3.5e and Supplementary Fig. 3.8c). Although melanin seems to strongly bind to Mg and Ca *in vivo*, the original cuticular chemistry of insect cuticles appears to be overprinted by chemical signals acquired during decay, this is particularly evident when the analysis includes more than one taxon, see Chapter 5.

Electron microprobe analyses of the variation trace elemental signatures associated with visual colour in the cuticle of *H. axyridis* during decay confirm that the rate and conditions of decay exert a strong influence in the resultant spatial partitioning of trace elements in the cuticle of insects and the effects of chemical overprinting are not restricted to diagenetic processes, thus not supporting hypotheses 6 nor 7.

**Hypotheses 6 (*the association between certain trace elements and pigment molecules persists during decay*) and 7 (*distinctive trace elements or sets of elements can be used as biomarkers for the original pigmentary content in the cuticle of fossil insects*) are rejected.**

#### **4.5. Gas chromatography–mass spectrometry.**

In the majority of taxa (80%), the lipid with highest concentration in the cuticle was identified as C<sub>18</sub> monounsaturated free fatty acid (Fig. 3.6a–h, Supplementary Figs. 3.9–3.13 and Supplementary Tables 3.2–3.7). Straight-chained hydrocarbons, such as alkanes, are common in all cuticles, especially those with odd carbon numbers (Fig. 3.6a–h, Supplementary Figs. 3.9–3.13 and Supplementary Tables

3.2–3.7). Lower molecular weight compounds (e.g. C<sub>14</sub> and C<sub>16</sub> fatty acids) are relatively rare in all samples, but are more common in experimentally degraded cuticles, especially in basic (pH 8) and neutral (pH 7) media (Fig. 3.6c, d, g, h, Supplementary Figs. 3.9d, h, 3.10b, d–3.12 b, d, 3.13a, c and Supplementary Tables 3.2–3.7). Higher molecular weight compounds e.g. long chain alkenes (e.g. >C<sub>30</sub> straight-chained hydrocarbons) and sterols (e.g. cholesterol), are more abundant in specimens treated in neutral (pH 7) and basic (pH 8) media (Fig. 3.6b, d, f, h, Supplementary Figs. 3.9c, g, 3.10b, d–3.12b, d, 3.13a, c and Supplementary Tables 3.2–3.7). Said higher molecular weight compounds are less abundant in specimens degraded in acidic (pH 6) conditions (Fig. 3.6 c, g, Supplementary Figs. 3.9f, 3.11c, 3.13b and Supplementary Tables 3.2, 3.4, 3.6, 3.7), and are absent in untreated specimens (Fig. 3.6 a, e, Supplementary Figs. 3.9c, 3.10a–3.12a and Supplementary Tables 3.3–3.5, 3.7). Sugars are abundant in the cuticles of all taxa analyzed yet rare in cuticles degraded in neutral (pH 7) and acidic (pH 6) media (Fig. 3.6b, c, f, g, Supplementary Figs. 3.9b, e–f, 3.11a, c, 3.12a, c, 3.13a–b and Supplementary Tables 3.2–3.7). GC-MS analyses of standard melanin and  $\beta$ -carotene resulted in unresolved chromatograms, which are not included in this study. Liquid residue samples were excluded from the analyses due to high levels of external contamination after breakage of various vials during transport to the analysing institution.

GC-MS analyses of the variation of lipid molecules in the insect cuticle during decay indicate that cuticle chemistry will differ for the same taxa in different pH conditions of decay, thus supporting hypothesis 1.

**Hypothesis 1 (*contrasting environments of decay, particularly that of pH conditions, affect the rate of decay*) is accepted.**

Supplementary Text in Appendix 3 contains a detailed account of the gas chromatography-mass spectrometry results for all taxa analyzed.

#### 4.6. Multivariate analysis.

Trends in the GC-MS data were explored using PCA and LDA, which reduces the dimensions of multivariate data into linear combinations of variables in order to maximise separation among data classes. The resultant PCA plot indicates a clear taxonomical trend in terms of chemical signatures of the cuticle, where all taxonomical groupings analysed occupy distinct regions of the morphospace with minimal overlapping (Supplementary Fig. 3.14a). Additionally, there is a clear chemical trend associated with the pH conditions of decay (Supplementary Fig. 3.14b–f). LDA plots show similar trends; for instance, *H. axyridis* displays a clear differentiation between 60 and 180 days decayed samples (Fig. 3.6i and Supplementary Fig. 3.15b). Samples decayed for 60 days (filled dots on the chemospace) show a wider occupancy of the area in the chemospace and are fully separated by conditions of decay (Fig. 3.6i and Supplementary Fig. 3.15b). There is no overlap among samples decayed in neutral (grey), acidic (orange) and basic (blue) conditions (Fig. 3.6i and Supplementary Fig. 3.15b). These results indicate that as decay progresses in cuticles of *H. axyridis* the relative abundance in low C number fatty acids (i.e. C<sub>16</sub> and C<sub>18</sub> fatty acids) increases, whereas the relative abundance of odd number, straight chain hydrocarbons (i.e. C<sub>23</sub>–C<sub>31</sub> alkanes, alkenes and dienes) decreases (Supplementary text, Supplementary Figs. 3.14b, h, 3.15b, h and Supplementary Table 3.7). The increment of relative abundance of molecules of C<sub>18</sub> fatty acid remains constant for all taxa analysed regardless of the pH conditions of decay; however, for samples treated under basic conditions the

relative abundance is constantly higher than for those samples treated under acidic conditions (Supplementary Table 3.7). These taxonomical and chemical controls are driven by differences in variability of the lipid molecules found in the cuticles intra-specifically, particularly fatty acids (Supplementary Text, Supplementary Figs. 3.14b–l, 3.15b–l); however, there is no clear chemical trend associated with determined conditions of decay across all taxonomical groups (Supplementary text, Supplementary Figs. 3.14, 3.15 and Supplementary Table 3.7).

Multivariate analyses of the variation of lipid molecules in the insect cuticle during decay confirm that when the same taxon is decayed in disparate pH conditions the resultant cuticular chemistry is overprinted by the specific chemistry of each combination of pH conditions, thus supporting hypothesis 1 but contradicting hypothesis 7.

**Hypothesis 1 (*contrasting environments of decay, particularly that of pH conditions, affect the rate of decay*) is accepted and hypothesis 7 (*distinctive trace elements or sets of elements can be used as biomarkers for the original pigmentary content in the cuticle of fossil insects*) is rejected.**

Supplementary Text in Appendix 3 contains a detailed account of the multivariate results for all taxa analyzed.

#### **4.7. High performance liquid chromatography–mass spectrometry.**

In summary, as decay progresses, carotenoids with higher molecular weight gain relative abundance in the chromatograms of *H. axyridis* regardless of the conditions of decay (Supplementary Fig. 3.16g–i and Supplementary Table 3.9). In addition, carotenoids with lower molecular weight make their appearance in the chromatograms and occupy the first minutes of the analysis, especially in samples

decayed in neutral and basic conditions (Supplementary Fig. 3.16 and Supplementary Table 3.9). Analysis of the liquid residue from decay experiments yielded blank results in all HPLC-MS runs; thus, the concentration of the samples was found to be lower than the sensitivity of the MS detector.

HPLC-MS analyses of the variation of carotenoid molecules in the cuticle of *H. axyridis* during decay indicate that cuticle chemistry will differ for the same taxa in different pH conditions of decay.

**Hypotheses 1 (*contrasting environments of decay, particularly that of pH conditions, affect the rate of decay*) and 2 (*regions of varying pigment are affected distinctively through chemical decay when the conditions of decay are identical*) are accepted.**

#### **4.8. Alkaline hydrogen peroxide oxidation and hydroiodic acid hydrolysis.**

Quantification of melanin molecules via HPLC analysis of the degradation products of permanganate oxidation of eumelanin and reductive hydrolysis of pheomelanin (PTCA HPLC-MS) concludes that there is melanin presence (eumelanin and pheomelanin) in the dark cuticle regions of *H. axyridis*, *L. decemlineata* and *T. velutinus* after 60 days of decay (Supplementary Table 3.10). Absolute proportions of melanin content within samples were not calculated due to the lack of information on specific sample weight after freeze-drying, although it ranged between 9-17 mg/g. Samples of *H. axyridis* show similar concentration of eumelanin and pheomelanin regardless of the conditions of treatment; nevertheless, in all cases the concentration is lower in those samples decayed under basic pH conditions (Supplementary Table 3.10). Analysis of the dark regions of *L. decemlineata* indicates that the relative abundance of eumelanin and pheomelanin

is higher in abundance in those samples treated in neutral and acidic conditions (Supplementary Table 3.10). Samples of *T. velutinus* show similar concentration of eumelanin and pheomelanin regardless of the conditions of treatment; nevertheless, in all cases the concentration is lower in those samples decayed under basic pH conditions (Supplementary Table 3.10).

PTCA HPLC-MS analyses of the variation of melanin molecules in the cuticle of *H. axyridis*, *L. decemlineata* and *T. velutinus* during decay indicate that cuticle chemistry will differ for the same taxa in different pH conditions of decay.

**Hypotheses 1 (*contrasting environments of decay, particularly that of pH conditions, affect the rate of decay*) and 2 (*regions of varying pigment are affected distinctively through chemical decay when the conditions of decay are identical*) are accepted.**

## 5. DISCUSSION

Analysis of extant insects reveals that the preservation potential of pigmented cuticles is critically controlled by the pH of the media during decay and the chemical components of the pigment molecules in the cuticle. Albeit specimens were collected from diverse sources and in some instances, the time of death is not fully constrained; it does not seem to have an effect on the chemical signature of pigment molecules in the cuticle (Supplementary Fig. 5.4 in Appendix 5). Colour changes in regions of the cuticle containing melanin were unrecognisable with the naked eye after 60 days of decay regardless of the media of decay in *H. axyridis*, *L. decemlineata*, *G. goliathus* and *T. velutinus* (Figs. 3.1, 3.2a–z and Supplementary Fig. 3.1); melanin-rich regions in the cuticle of *P. apterus* was noticeably lighter after 60 days of decay (Fig. 3.2aa–ah). These results are substantiated by minimal

changes in quantitative hue, purity and intensity of melanin-rich regions in the cuticle (Fig. 3.3, Supplementary Fig. 3.2 and Supplementary Table 3.1). All specimens experienced great changes in hue, purity and intensity in regions of the cuticle where melanin is known to be absent but regardless of any other pigment content, particularly on specimens decayed under basic conditions (Figs. 3.1, 3.2 and Supplementary Fig. 3.1). These results are validated by extensive changes in quantitative hue, purity and intensity in these regions of the cuticle (Fig. 3.3, Supplementary Fig. 3.2, and Supplementary Table 3.1). In addition, analysis of the ultrastructural variations during decay of cuticles of *H. axyridis*, *L. decemlineata*, *T. velutinus* and *P. apterus* indicate that when the media of decay is acidic, the rate of decay is slower than in alkaline media for the first 14 to 30 days of decay, after this point decay slows down in all experimental conditions (Fig. 3.4 and Supplementary Figs. 3.3–3.7). Lower pH generally inhibits the precipitation of calcium carbonate and promotes the formation of apatite, and is regarded as the ideal condition to fossilise soft tissue (Martill 1988; Briggs and Kear 1994). With constant temperature, the pH of the media is the main driver for the growth rate and kinetics of various types of bacteria; generally, the fastest growth rates occur at pHs between 5.5 and 8.5 (Sreekrishnan *et al.* 1996; Min *et al.* 2014). Previous studies had already recognized substantial reduction of pH as direct result of decay of soft tissues (Allison 1988; Briggs and Kear 1993c, 1994; Wilby *et al.* 1996), additionally, acidic lakes have a low diversity of bacterial communities and the bacterial activity is reduced with reduction of alkalinity (Ren *et al.* 2015). Our experiments support an acceleration of the rate of decay in alkaline media, possibly prompted by the increasing numerically and diversified allochthonous bacterial



community, or inversely, a deceleration of the rate of decay in acidic media due to the lower bacterial activity (Supplementary Figs. 3.3–3.7).

Furthermore, melanin-rich regions of the cuticle retard the effects of decay while regions of the cuticle containing other pigments appear to have faster rates of decay (Supplementary Figs. 3.3b, f, j–3.4b, f, j, 3.6b, f, j–3.7b, f, j). Melanin is found widespread throughout the cuticle of *G. goliathus*, grey regions of the cuticle are achieved through a combination of structural and pigmentary colour overlaying the cuticle's surface (Supplementary Fig. 3.5a). Ultrastructural analysis of the cuticle of *G. goliathus* indicates that there is no difference in the decay rates among differently coloured regions of the cuticle. On the other hand, the ultrastructure of non melanin-rich regions in the cuticles of the other taxa indicates that the absence of melanin is associated with the acceleration of decay in these regions (Supplementary Figs. 3.3a, e, i–3.4a, e, i, 3.6a, e, i–3.7a, e, i). Cuticle loss and reduction in thickness had already been reported in other arthropods due to decay processes (Gupta *et al.* 2009), but this is the first time that cuticle loss has been correlated to spatial distribution of pigments in the cuticle. Melanin is considered to be a resistant molecule, it is insoluble and unreactive with other organic compounds (Morgan 2004a). Instead, carotenoids, pterins and some flavonoids are water-soluble molecules (Goodwin 1986; Matsukawa *et al.* 2009; Ragone *et al.* 2012) and are clearly photo-oxidated by light (Carmona *et al.* 2006; Lavelli *et al.* 2007), or degraded by temperature changes (Olsen *et al.* 2009). In addition, some ommochromes, such as ommatin D, spontaneously degrade into smaller molecules when stored at ambient temperature in direct contact with light (Nijhout 1997; Figon and Casas 2019). The potential for deterioration of cuticular ultrastructure in regions where these labile pigments are stored is the result of accelerated decay due

to their high water-solubility and susceptibility for degradation at average environmental conditions is unquestionable. In addition, the majority of bacterial growth observed on the surfaces of cuticles of *H. axyridis*, *L. decemlineata*, *T. velutinus* and *P. apterus* coincides with regions of the cuticle where melanin is absent (Supplementary Figs. 3.3c, g, k–3.4c, g, k, 3.6c, g, k–3.7c, g, k). Melanin-rich regions of the cuticle contain fewer bacteria and are usually less diversified (Supplementary Figs. 3.3d, h, l–3.4d, h, l, 3.6d, h, l–3.7d, h, l). These regions do not present any modifications of morphological features neither at the surface nor in the internal ultrastructure of the cuticle. Previous studies have correlated the rate of bacterial decay to pigment-specific content, such as accelerating decay in melanin-rich in feathers of various bird taxa (Grande *et al.* 2004) or increasing resistance to decay in psittacofulvins-rich feathers specific to parrots (Burt Jr *et al.* 2011). The accumulation of bacteria in non melanin-rich regions of the cuticle may be representative of the ideal chemical conditions for the proliferation of these types of communities. The accentuated decay in these regions in conjunction with the high content of molecules with weaker C–C bonds (e.g. carotenoids), in contrast to the high number of double bond C=C and C=O bonds in melanin molecules, ought to create a more favourable environment for the multiplication of bacteria.

Chemical fluctuations during decay, affect both the inorganic trace element signatures and the organic molecules contained in the cuticles analyzed. Spatial partitioning of trace elements in the cuticle of insects, although strongly taxonomically controlled (see Chapter 5), acquires new signatures in *H. axyridis* during decay, particularly in acidic media (Fig. 3.5 and Supplementary Fig. 3.8). However, applicable to only one taxon, the results indicate that Mg, K and Ca have

a large input in the variability of trace elemental chemistry of the cuticle during decay (Fig. 3.5 and Supplementary Fig. 3.8); those elements with a relatively enrichment in melanin-rich regions of the cuticle in untreated specimens (e.g. Mg and Ca), increase in proportion as decay progresses, particularly in basic and neutral decayed samples (Fig. 3.5). Elements with a relative depletion in melanin-rich regions prior to decay (e.g. K) overturn during decay and result enriched in these regions regardless of the media of decay after two months of decay (Fig. 3.5). In addition, lipid content in the cuticle of all taxa analyzed is affected during decay; however, quantification of these fluctuations was unviable due to absence of information on sample concentrations, the following statements are based on a qualitative analysis of the results. Lower molecular weight compounds are rare in untreated cuticles, although, their concentration increases during decay and are more common in experimentally degraded cuticles, especially in basic and in neutral media of decay (Fig. 3.6b, d, f, h, Supplementary Figs. 3.9d, h, 3.10b, d–3.12 b, d, 3.13a, c and Supplementary Tables 3.2–3.6). In addition, higher molecular weight compounds, such as sterols (e.g. cholesterol) and long chained alkanes are relatively more abundant in those samples decayed in basic and neutral media (Fig. 3.6b, d, f, h, Supplementary Figs. 3.9c, g, 3.10b, d–3.12b, d, 3.13a, c and Supplementary Tables 3.2–3.6). Sugars are rare in untreated samples, yet they become increasingly more common as decay occurs in basic media (Fig. 3.6d, h, Supplementary Figs. 3.11b, g–h, 3.13a, d–3.15a, d and Tables 3.2–3.6). Differences in variability of the of lipid content suggest that there is a chemical control in the cuticles, at least at order-level, and this is related to the pH conditions of decay. Variations in the lipid content linked to the concentration of chitin macromolecules have already been reported during decay (Gupta *et al.* 2009;

Gupta 2014) and fossilisation processes (Stankiewicz *et al.* 2000; Gupta *et al.* 2006) of other arthropods. In addition, the increment of simple polysaccharides (i.e. sugars) in samples decayed in basic and neutral conditions ought to be another by-product of chitin degradation (Briggs and Kear 1994; Briggs 1999; Gupta *et al.* 2009). A molecule with similar spectrum to that of vitamin A was encountered in various samples of *L. decemlineata* (present in 100% neutral, 100% basic and 50% acidic decayed samples) and *P. apterus* (present in 100% basic and 50% acidic decayed samples) (Supplementary Text and Supplementary Table 3.7). The appearance of this unidentified molecule is curious since the carbon rings at either side of most common carotenoid molecules in insects, including lycopene,  $\beta$ - and  $\alpha$ -carotenes, lutein and zeaxanthin, are of similar chemical structure (Fig. 1.3 in chapter 1) and resultant spectral analysis of some carotenoid molecules can be analogues to vitamin A (Reid *et al.* 1973). In fact, carotenoids are well-studied precursors of retinoid molecules (chemically related to vitamin A) in the eyes of crustaceans (Fisher and Kon 1959; Wolf 1984) and some insects (Goldsmith and Warner 1964; Stavenga and Arikawa 2006). In addition, vitamin A is a known degradation product of carotenes particularly through oxidation processes (Morgan 2004c). Another intriguing finding was a molecule similar to testosterone in mammals that was later identified as ecdysone present in various samples of *L. decemlineata* (50% of neutral decayed samples), *T. velutinus* (100% of neutral and 50% of acidic decayed samples) and *P. apterus* (100% of samples regardless of the treatment). Ecdysone is regarded as the growth and moulting hormone in insects (Karlson 1963; Clayton 1964), has also been considered as a possible precursor of sex pheromones in various insect taxa (De Loof 2006). Ecdysone is widespread found in all experimental conditions of samples of *P. apterus*, this indicates that its

appearance is sample specific and not related to the conditions of decay. Analyses of specific carotenoid content during decay of *H. axyridis* reveal that the suite of carotenoid molecules does not change as decay progresses, but the relative abundance of these is altered towards lower molecular weight compounds, especially in those samples decayed in neutral and basic media (Supplementary Fig. 3.9 and Supplementary Tables 3.2, 3.7). The photosensitivity of carotenoids has been well defined in previous studies (Carmona *et al.* 2006; Lavelli *et al.* 2007); hence, samples were kept in the dark for the duration of the experiment and storage afterwards. Alteration of carotenoid molecules in these samples must have occurred due to chemical changes prompted by the media of decay. The chemical changes observed in LC-MS and HPLC-MS are a direct result of the breakage of C–C and C–H molecular bonds of the molecules transforming into smaller molecules, subsequently the relative abundance of higher molecular weight molecules, although unaltered, increases, particularly in basic and neutrally decayed samples (Supplementary Figs. 3.9–3.13, 3.14, 3.15 and Supplementary Tables 3.2–3.7). Lastly, analyses of melanin content during decay of *H. axyridis*, *L. decemlineata* and *T. velutinus* reveal that all taxa contain both eumelanin and pheomelanin molecules in their cuticles after 60 days of decay (Supplementary Table 3.10). Calculations of absolute proportions were not completed due to lack of weight information for each sample after freeze-drying. Assuming that the sample weights are similar, in all cases the relative abundance of melanins are higher in those samples decayed in acidic conditions, these results are correlatable with other structural and chemical results which conclude that the rate of decay in insect cuticles is higher in basic pH experimental conditions.

## 6. CONCLUSION

Chemical decay produces a suite of structural and chemical modifications in the cuticles of insect taxa. Laboratory controlled experimental decay indicates that basic pH media of decay (pH 8 and pH 10) result on faster rates of degradation of the microstructural properties of the cuticle in all taxa analyzed. Rates of decay are further accelerated when the region of the cuticle is non melanin-rich regardless of the taxonomic relations. The presence of various bacteria in the surface of treated cuticles indicates that the proliferation of these is aided by the conditions of the media of decay and the pigment content of the cuticle, bacteria being more numerous and diversified in basic conditions of decay and in non melanin-rich regions of the cuticle. Furthermore, the chemical component of the cuticle shows larger alteration on those experiments under basic conditions of decay, where the relative abundance of trace elemental chemistry and organic molecules contained in the cuticle are greatly affected. Data acquired strongly suggests that alkalinity of the media and composition of cuticular pigments are important environmental and biological biases, thus **hypotheses 1 (*contrasting environments of decay, particularly that of pH conditions, affect the rate of decay*) and 2 (*regions of varying pigment are affected distinctively through chemical decay when the conditions of decay are identical*) are accepted**, This bias significantly increases rates of decay for specific orders of insect taxa, promoting the loss of valuable information and greatly reducing the prospects for fossilisation of organic molecules contained within the cuticle of these insects, thus **hypotheses 6 (*the association between certain trace elements and pigment molecules persists during decay*) and 7 (*distinctive trace elements or sets of elements can be used as biomarkers for the original pigmentary content in the cuticle of fossil insects*) are**

**rejected.** Numerous fossil insects are recovered from sedimentary rocks deposited in palaeolacustrine settings, the record of insect palaeocommunities is known to be biased towards small and allochthonous taxa regardless of the chemical and physical conditions in the sedimentary basin. The environmental and biological constraints revealed on these analyses add further biases that affect our understanding of insect palaeocommunities and the relations of their components with the environment. The results of these analyses, suggest that biological (i.e. pigment content and distribution) and environmental (i.e. alkalinity of the media) controls are important drivers decay and are the promoters of crucial biases to take into consideration when describing fossil palaeocommunities regardless of the dominant taxon.

## 7. FUTURE WORK

Further analysis will reveal the impact of these biological and environmental biases in other orders of insects and recognise similar biases in the fossil record of other organisms, potentially elucidating processes of decay and by extension, fossilisation, in all organisms from deep time.

**LIST OF FIGURES AND TABLES:**

**Fig. 3.1:** Light microscopy images of colour changes occurring in specimens of *Harmonia axyridis* during 180 days of decay.

**Fig. 3.2:** Light microscopy images of colour changes occurring in specimens of *Leptinotarsa decemlineata*, *Goliathus goliathus*, *Typocerus velutinus* and *Pyrrhocoris apterus* during 60 days of decay.

**Fig. 3.3:** Scatterplots representing pH conditions against final maximum lambda ( $\lambda_{\text{max}}$ ) of differently coloured regions on the cuticle of *Harmonia axyridis*, *Leptinotarsa decemlineata*, *Goliathus goliathus*, *Typocerus velutinus* and *Pyrrhocoris apterus*.

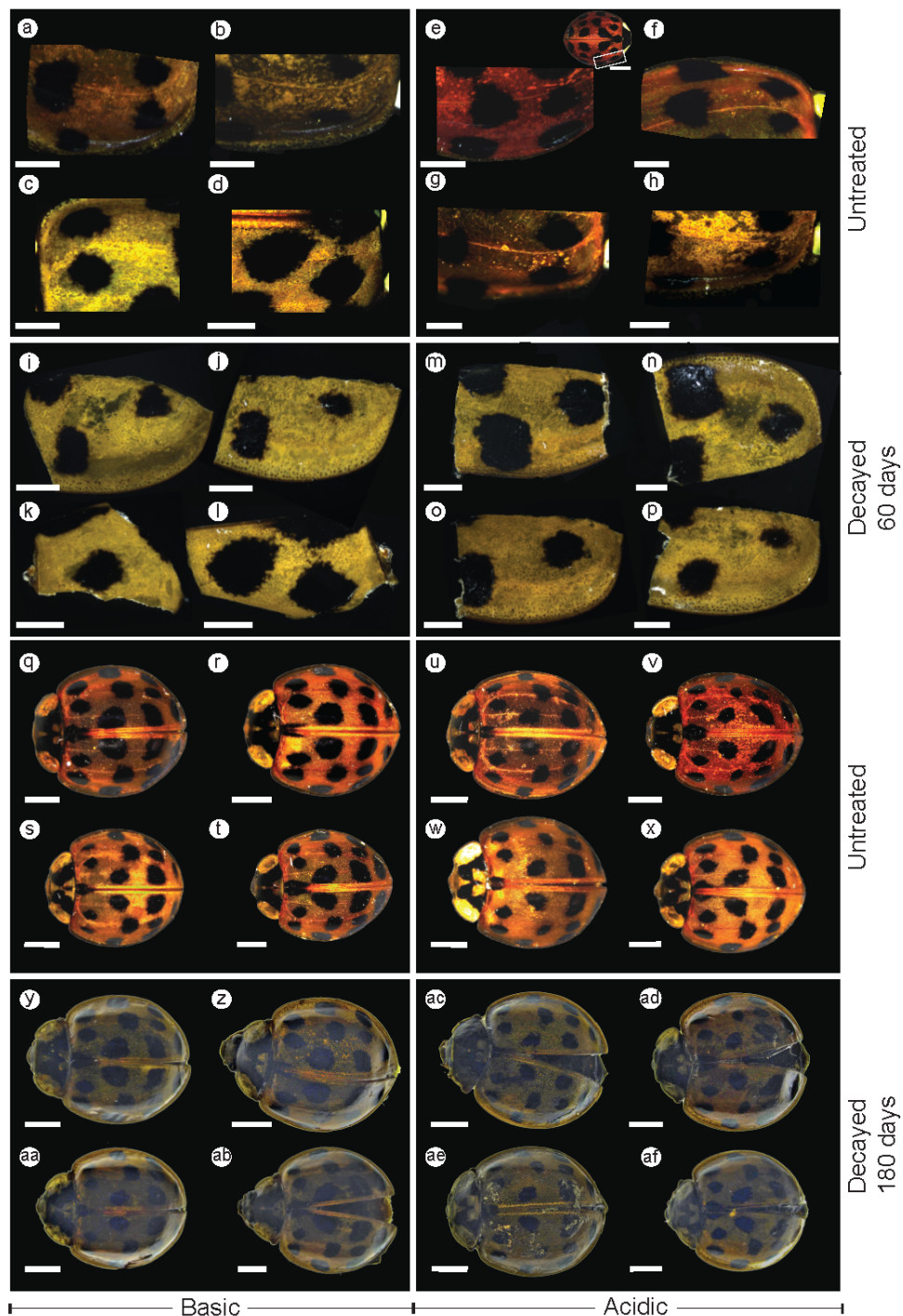
**Fig. 3.4:** Scanning electron micrographs of cuticular ultrastructure of *Harmonia axyridis* during 180 days of decay

**Fig. 3.5:** Trace element chemistry in cuticles of *Harmonia axyridis* cuticles during 60 days of decay.

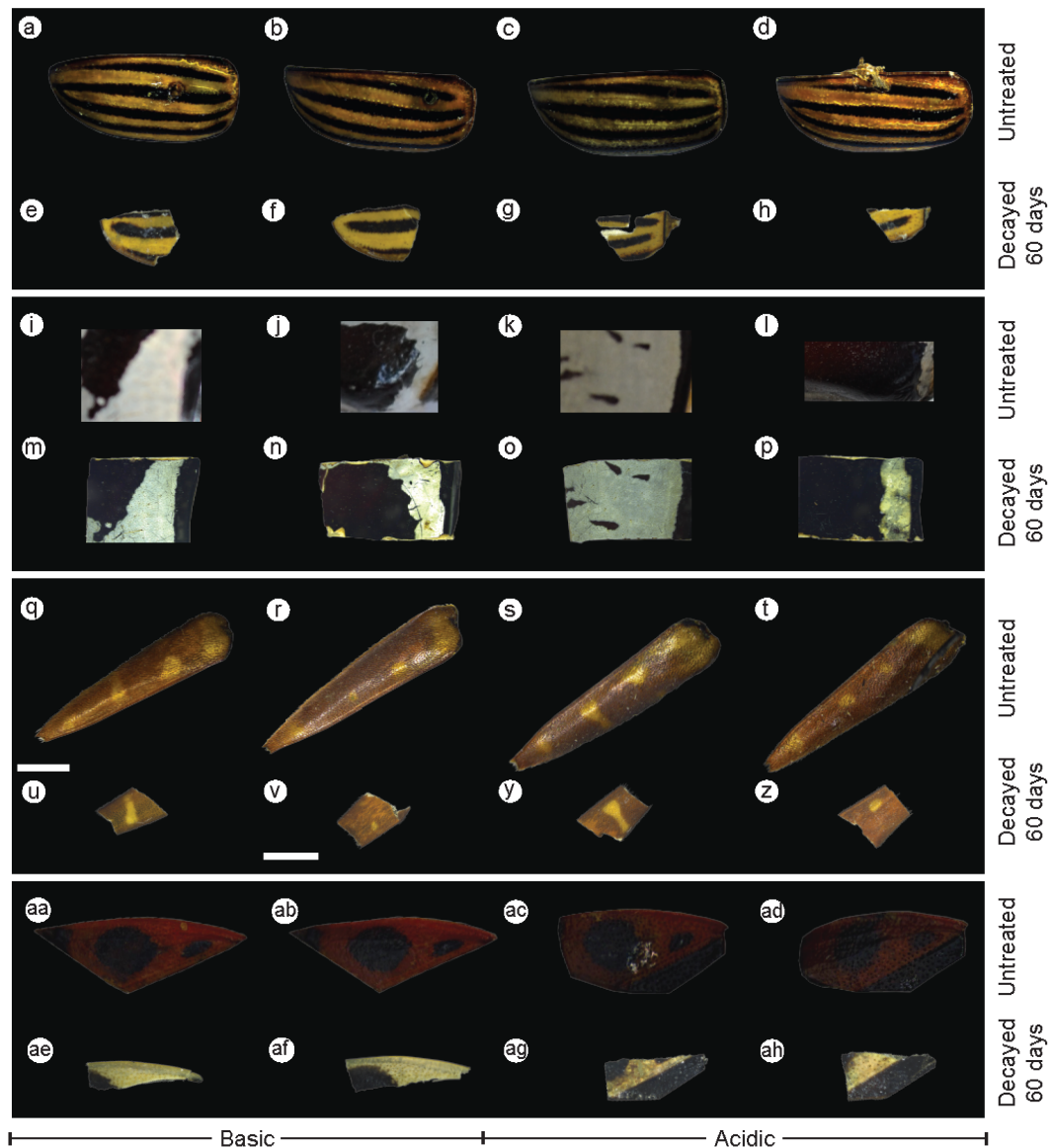
**Fig. 3.6:** Gas chromatography-mass spectrometry chromatograms and linear discriminant analysis displaying variations during decay of the largest molecular peaks from representative analyses.

**Table 3.1:** Conditions of decay and number of specimens decayed per taxon analyzed.

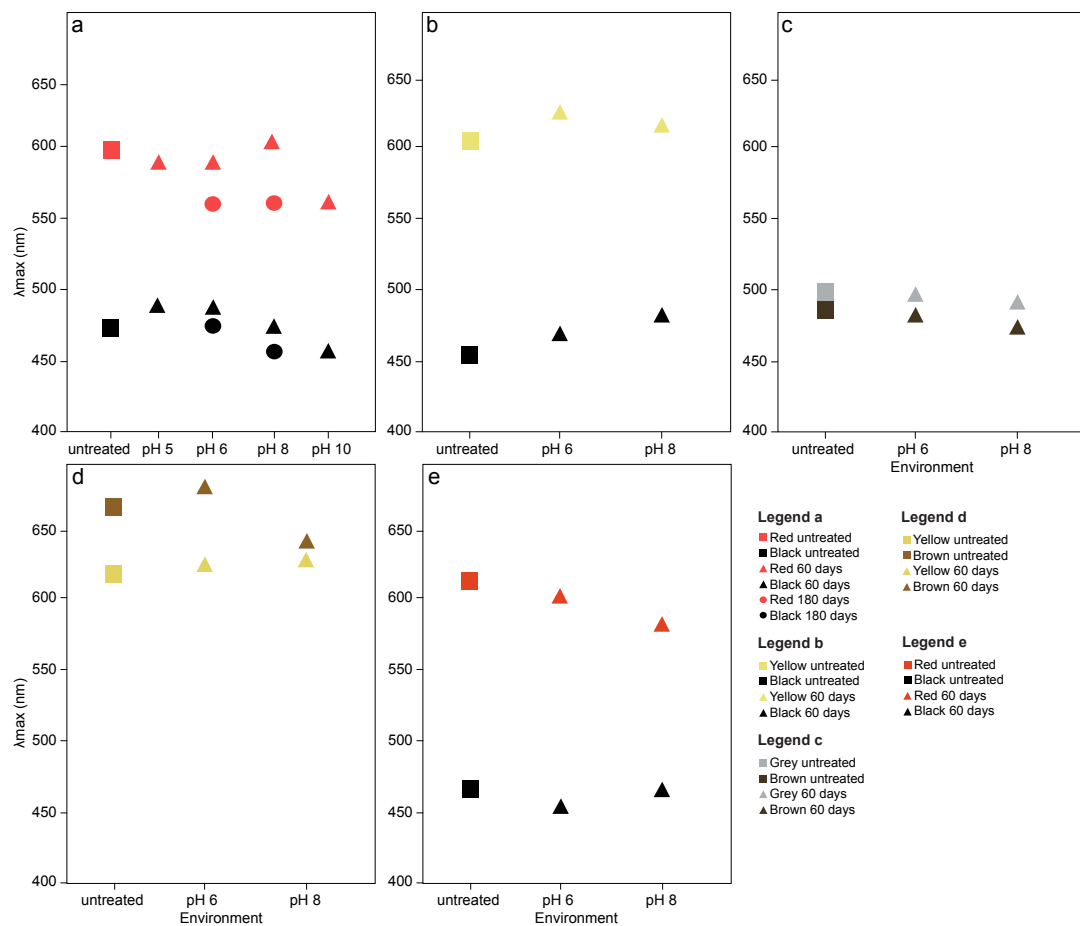




**Fig. 3.1. Light microscopy images of colour changes occurring in specimens of *Harmonia axyridis* during 180 days of decay. a–h and q–x Optical images of untreated specimens. i–p Optical images of cuticles after 60 days of treatment. i–l Cuticles decayed in basic media (pH 8); m–p cuticles decayed in acidic media (pH 6). Inset in e shows entire specimen, with region detailed in e indicated. y–af Optical images of cuticles after 180 days of treatment. y–ab Cuticles decayed in basic media (pH 8); ac–af cuticles decayed in acidic media (pH 6). Scale bars: a–p 500  $\mu\text{m}$ , inset in e 2 mm, q–f 1 mm.**



**Fig. 3.2.** Light microscopy images of colour changes occurring in specimens of *Leptinotarsa decemlineata* (a–h), *Goliathus goliathus* (i–p), *Typocerus velutinus* (q–z) and *Pyrrhocoris apterus* (aa–ah) during 60 days of decay. a–d, i–l, q–t and aa–ad Optical images of untreated specimens. e–h, m–p, u–z and ae–ah Optical images of cuticles after 60 days of treatment. e–f, m–n, u–v and ae–af Cuticles decayed in basic media (pH 8); g–h, o–p, y–z, ag–ah Cuticles decayed in acidic media (pH 6). Scale bars: 1 mm.



**Fig. 3.3.** Scatterplots representing pH conditions against final maximum lambda ( $\lambda_{max}$ ) of differently coloured regions on the cuticle of *Harmonia axyridis* (a), *Leptinotarsa decemlineata* (b), *Goliathus goliathus* (c), *Typocerus velutinus* (d) and *Pyrhocoris apterus* (e).



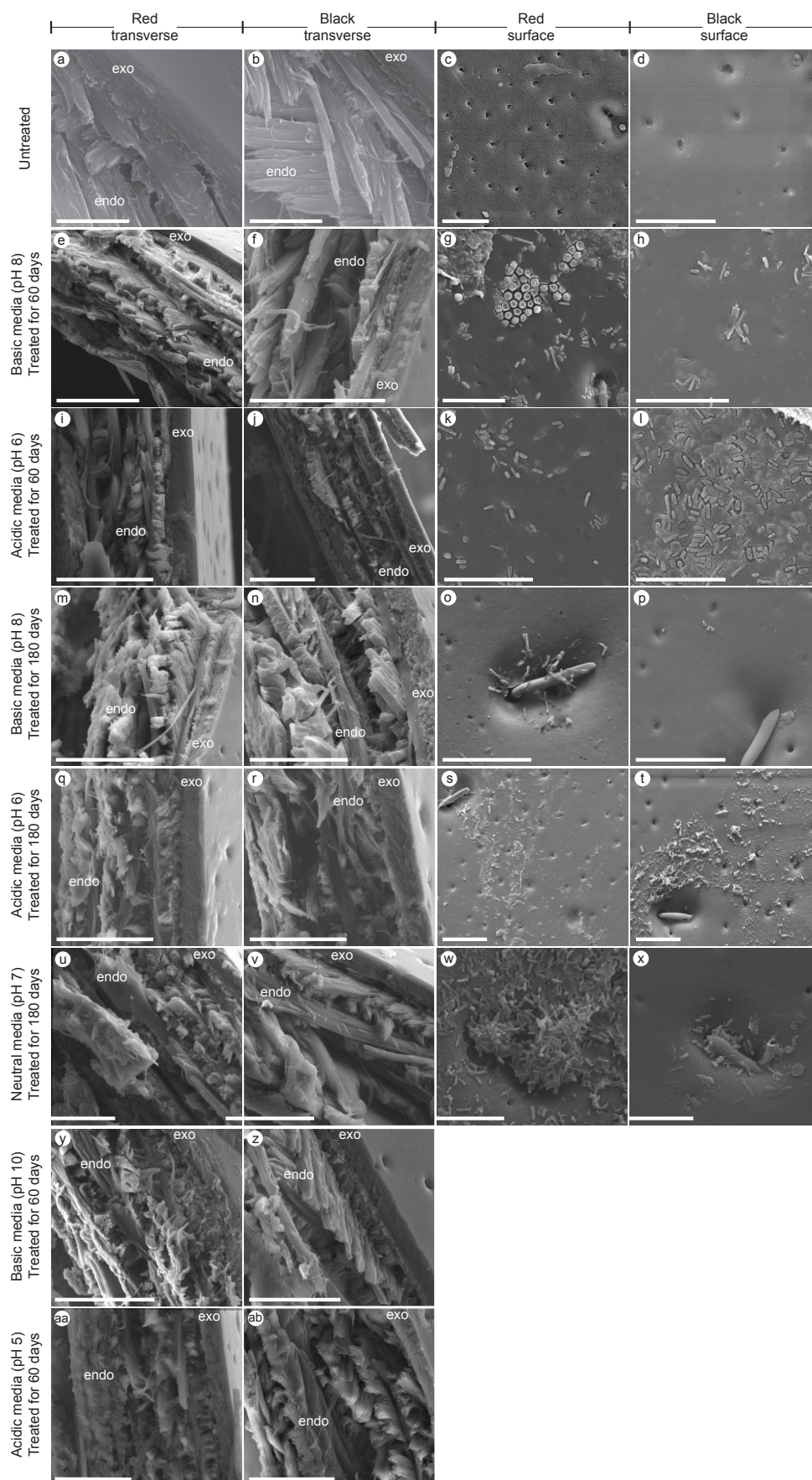
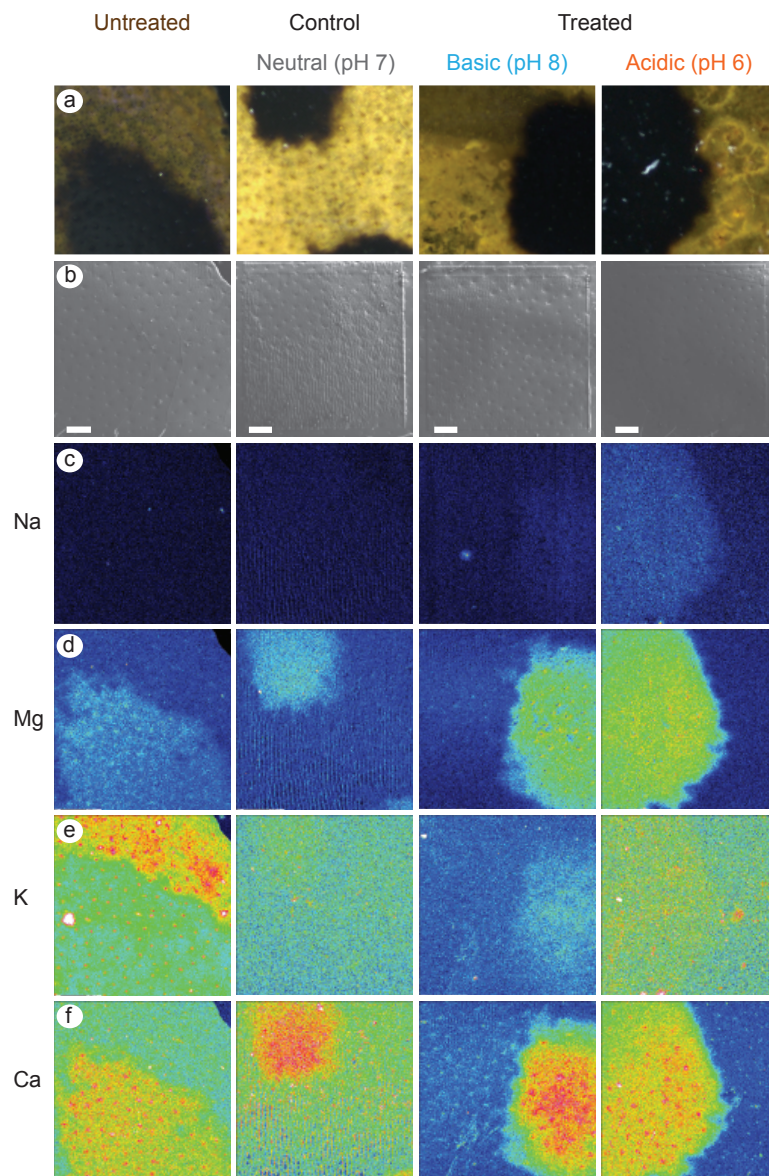
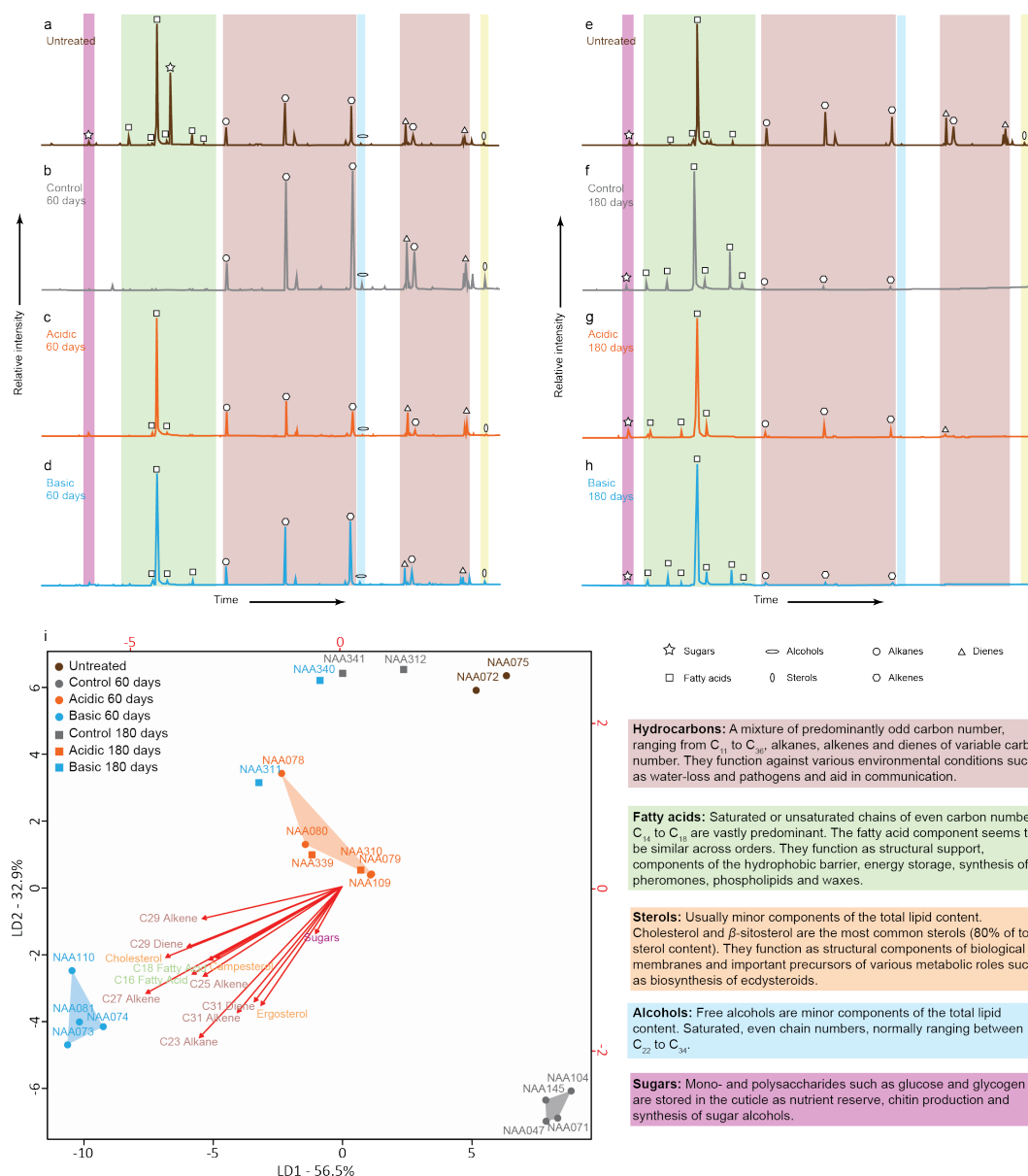


Fig. 3.4. Caption overleaf.

**Fig. 3.4 (previous page). Scanning electron micrographs of cuticular ultrastructure of *Harmonia axyridis* during 180 days of decay. a–d** Untreated cuticles; **a** internal red region, **b** internal black region, **c** external red region, **d** external black region. **e–h** Cuticles treated in basic media (pH 8) for 60 days; **e** internal red region, **f** internal black region, **g** external red region, **h** external black region. **i–l** Cuticles treated in acidic media (pH 6) for 60 days; **i** internal red region, **j** internal black region, **k** external red region, **l** external black region. **m–p** Cuticles treated in basic media (pH 8) for 180 days; **m** internal red region, **n** internal black region, **o** external red region, **p** external black region. **q–t** Cuticles treated in acidic media (pH 6) for 180 days; **q** internal red region, **r** internal black region, **s** external red region, **t** external black region. **u–x** Cuticles treated in neutral media (pH 7) for 180 days; **u** internal red region, **v** internal black region, **w** external red region, **x** external black region. **y–z** Cuticles treated in basic media (pH 10) for 60 days; **y** internal red region, **z** internal black region. **aa–ab** Cuticles treated in acidic media (pH 5) for 60 days; **aa** internal red region, **ab** internal black region. Note prominent endocuticular chitin bundles (e.g., **m**, **n**) and exocuticular laminations (e.g., **f**, **n**) in treated (**e–f**, **i–j**, **m–n**, **q–r**, **u–v**, **y–z**, **aa–ab**) relative to untreated (**a–b**) cuticles. Scale bars: 10  $\mu\text{m}$ .



**Fig. 3.5. Trace element chemistry in cuticles of *Harmonia axyridis* cuticles during 60 days of decay. a** Light microscopy images of the dorsal surface of the cuticles. **b** Scanning electron micrographs of regions in **a**. **c–f** Elemental maps of regions in **a–b**. All elemental maps are EPMA maps. Scale bars: 100  $\mu\text{m}$ .



**Fig. 3.6. Gas chromatography-mass spectrometry chromatograms (a–h) and linear discriminant analysis (i) displaying variations during decay of the largest molecular peaks from representative analyses. a, e** Untreated specimens. **b, e** Specimen decayed in neutral pH conditions for 60 days (**b**) and 180 days (**f**). **c, g** Specimen decayed in acidic pH condition for 60 days (**c**) and 180 days (**g**). **d, h** Specimen decayed in basic pH conditions for 60 days (**d**) and 180 days (**h**).

| Species                          | pH 4 | pH 6 | pH 7 | PH 8 | pH 10 |
|----------------------------------|------|------|------|------|-------|
| <i>Harmonia axyridis</i>         | 13   | 28   | 12   | 28   | 13    |
| <i>Leptinotarsa decemlineata</i> | 0    | 6    | 2    | 6    | 0     |
| <i>Goliathus goliathus</i>       | 0    | 18   | 6    | 18   | 0     |
| <i>Typocerus velutinus</i>       | 0    | 6    | 2    | 6    | 0     |
| <i>Pyrrhocoris apterus</i>       | 0    | 6    | 2    | 6    | 0     |

**Table 3.1. Conditions of decay and number of specimens decayed per taxon analyzed.**



## CHAPTER 4

# Colouration bias in the fossil record of insects

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### ABSTRACT

Extant insects exhibit some of the most striking colouration in the animal kingdom, usually arranged in elaborate patterning in their cuticles and wings. Although colour in fossil insects is far from being as diverse as in their extant relatives, patternings highly resemble those found in extant insects. This is most likely the result of fossilisation processes, not a direct translation of colour variability in the past. Time and type of transport are recognized as the most destructive factors of any fossil-to-be organism. Anatomical and mechanical characteristics of the body, such as morphology and permeability, are important factors influencing the total duration of transport. Type of pigment and spatial distribution on the cuticle have never been considered as possible biases in the amount of breakage induced to insect cuticles during transport. Here, the analysis the distribution of breakages in cuticles of *Harmonia axyridis* (n = 16) is conducted with various contents of recalcitrant (melanin) and labile (carotenoid) pigments distributed in variable numbers of spots on the cuticle. Cuticles were decayed in basic (pH 8) and neutral (pH 7) conditions for 6 months and tumbled for 38 days. Data indicate that the rate of fracturing is statistically significant more likely to occur in regions of the cuticle

where two pigments meet. In addition, cuticles with black background containing one or two large red/orange spots reached mechanical fracture faster than cuticles with red/orange background and variable numbers of small black spots. Cuticles with red/orange background and up to two small black spots did not reach total breakage of cuticle at the end of the experiment. These data show a previously unthought-of bias during transport and deposition of insects, which will aid in the interpretation of fossil insect assemblages and their environment of deposition as well as the preservation potential of patterned insect fossils.

## 1. PREVIOUS WORK

### 1.1. Introduction

Fossil insects are found in a great variety of host sediments (Duncan 1997; McCobb *et al.* 1998; Engel and Grimaldi 2004; Smith 2012; Barling *et al.* 2015; Anderson and Smith 2017). All deposits containing fossilized insects are considered Konservat-Lagerstätten with exceptional preservation of soft tissues (Smith 2012; Karr and Clapham 2015; Anderson and Smith 2017). Insects are usually preserved disarticulated and incomplete (Duncan 1997; Barling *et al.* 2015; Anderson and Smith 2017). As discussed in Chapter 3, this is result of three main biases: i) nature of tissues and pre-burial processes; ii) lithology and environment of deposition; iii) position of the body respective to the sediment at the time of burial. Where fossil insects are preserved both articulated or disarticulated, it is frequent to find patterning preserved in their cuticles, this is often displayed as monotonal colours; usually varying tones of brown and black (Bechly 2010; McNamara 2013; Barling *et al.* 2015). In extant insects and other animals, patternings are produced by pigments (and sometimes structures) stored in the

epithelium that reflect light in different wavelengths and function primarily in intra- and interspecific communication (Kemp *et al.* 2005; Bezzerides *et al.* 2007; Bezzerides and Loofbourrow 2014; Skelhorn and Rowe 2016). Despite efforts on elucidating the taphonomic processes involved in the fossilisation of arthropods during varying conditions of decay (Sansom 2014; Butler *et al.* 2015), transport (Duncan *et al.* 2003) and mineralisation (Briggs and Kear 1994), the diversity of pigment content in the cuticle has never been considered as a determining factor in the preservation potential of arthropods, especially in insects.

## **1.2. Labile vs recalcitrant**

Insects have a soft tissue exoskeleton or cuticle which functions as a protective agent against external factors and provides body support (Hadley 1984; Andersen 2009a, b). Cuticles are composed of a series of overlying layers of chitin, protein and various lipids (Locke 1965, 2001; Andersen 2009a, b). Morphology and sturdiness of insect cuticles vary among species, sex, ontogeny and laterally from location to location in the cuticle (Andersen 2009a, b; Neville 2012b; Moussian 2013). All insect cuticles are sclerotized to some extent, which stabilizes proteinaceous structures and aids in durability and sturdiness of the cuticle. The degree of sclerotisation and chitin content are often considered as a factor for the degree of preservation of an insect cuticle (Briggs 1999; Andersen 2010). Melanin is a known sclerotisation factor, which provides sturdiness to the insect cuticle (Fuzeau-Braesch 1972; Vincent and Hillerton 1979; Sugumaran 1998; Andersen 2010), the result is that patterned cuticles containing spatial partitioning of melanin (e.g. dark spots on a red background) have a heterogeneous response to decay and potentially transport (personal observation). Generally, melanin is considered to

posses the most recalcitrant molecular structure of all pigments, it is unreactive and insoluble (not in alkalis) and is formed by irregular polymers, which are difficult to break down in nature (Hackman 1984; Ghiradella 2009; Morgan 2004b; Hu *et al.* 2013). Other mechanical properties of the cuticle, such as the thickness and elasticity, are important contributors to the preservation potential of insect cuticle. Coleoptera are usually the most numerous group of insects recovered from fossil assemblages (Labandeira and Sepkoski 1993; Labandeira 1994). This is not only a direct reflection of the living dominance of this class of insects, but rather it reflects their durability on transport and the robustness of their cuticle (Duncan 1997; Coram 2003). Other insects orders with more labile tissues, such as Lepidoptera, are possibly under-represented in the fossil record and when found are commonly disarticulated and incomplete (Labandeira 1994).

### **1.3. Decay**

Decay commences on death, continues through burial, and it may occur concurrently with processes of mineralisation and organic preservation (Briggs and Kear 1994; Briggs and Wilby 1996). The amount of decay a carcass undergoes is dependant on various factors such as the composition and resistance of the tissues (Briggs and Kear 1994), the completeness of the protective external barrier of the body (Davis 2014), the conditions in the environment of decay (Allison 1988), and the timeframe over which decay occurs (Sansom 2014). The effects of pigment content in decay process are examined in Chapter 3.

#### 1.4. Transport

One of the most degrading factors after death of an organism is transport before it reaches its final resting place (Briggs 1995). The degree of disarticulation and fragmentation of any animal carcass is a direct relation to the amount of pre-burial decay the nature and to a less extent the distance of transport experienced (Allison 1986; Briggs 1995; Enright *et al.* 2021). Thus, other factors such as the shape and composition of the tissue (Chave 1964), decay (Duncan 1997), predation (Trewin and Welsh 1976) and compaction (Shinn *et al.* 1977) are also of importance in the degree of preservation of animal parts. Generally, well-preserved soft-bodied fossils are considered to have undergone little transport (Whittington 1971; Stürmer and Bergström 1973; Allison 1986); however, pre-transport processes, particularly decay, have a greater impact in the potential for preservation of unmineralized organisms (Allison 1986; Duncan *et al.* 2003; Enright *et al.* 2021). Transport in terrestrial environments can occur via various means, in the case of insects, the bodies are transported in two main media, aerial and aqueous (lacustrine or riverine) and they are usually combined in this order (Karr and Clapham 2015). The carcass' residence time in the media of transport before deposition is highly dependant on the morphological dynamics, permeability and mass of the body but is also dependant of external factors such as substrate, nature of airborne sedimentation and oxygen content in the water, which affects the density of the medium (Martínez-Delclòs and Martinell 1993; Briggs 1995; Smith *et al.* 2006; Tian *et al.* 2020).

### 1.5 Pigment content in *Harmonia axyridis*

Diverse disparity of pigments is found in insect cuticles. These pigments are deposited and stored in the cuticle exclusively or in combination with colour structures to form patternings that aid in communication. Carotenoids have been reported in *H. axyridis* from non-black regions of the cuticle (Bezzerrides *et al.* 2007); the concentration of carotenoids is correlated positively with the hue and brightness of the colour (Bezzerrides *et al.* 2007). The high plasticity displayed by the patterning of *H. axyridis* is a result of a phenotypic interaction such as seasonal pigment concentrations (Michie *et al.* 2010; Michie *et al.* 2011), defence mechanism against predators (Bezzerrides *et al.* 2007) or sexual dimorphism such as in the content and extent of melanic facial patterning (Bezzerrides and Loofbourrow 2014). In addition, melanin molecules have been characterized in black regions of the cuticle (Chapter 3). Melanin has been previously reported in the preserved cuticle of fossil insects (Labandeira *et al.* 2016); additionally, the recalcitrant nature of this molecule has been argued to allow the preservation of coloured wings in fossil dragonflies (Martínez-Declòs *et al.* 2004).

The content of pigments as a hardening factor of insect cuticles has never been investigated from a biomechanical perspective; however, heterogeneous endocuticle sclerotisation and distribution of cuticular components have been proven to have a large effect in the mechanical properties of insect cuticle (Rajabi *et al.* 2017; Wang *et al.* 2019). On the other hand, biomechanics of insect flight, cuticular adaptations and evolution associated with flying insects have been largely explored in extant and fossil insects (e.g. Evans and Sanson 2005; Parle 2015; Parle and Taylor 2017; Alexander 2018; Stamm *et al.* 2021). Here, the possible

biases caused by uneven distribution of pigment content in the cuticle of *H. axyridis* during the time of transportation prior to burial are explored.

## 2. AIM, SCOPE AND JUSTIFICATION OF EXPERIMENTS

Tumbling experiments on decayed cuticles of *H. axyridis* were conducted to investigate the effect of pigment content and amount of patterning in the cuticle of insects on the consequences of transport, resultant damage produced in the cuticle and possible biases generated in the insect fossil record.

These key issues are considered for the first time by analysing a single variable, that of transport, which has been overlooked as a possible bias in the fossilisation of insect cuticle. It is critical that any morphological disparities other than those being tested or variability in experimental conditions in all experiments are minimal, aside from differences in pigment content and amount of patterning, which was the only variable to test. This analysis provides insights into a taphonomic bias that constrain the fossilisation potential of insects with patterned cuticles.

This chapter addresses the following hypotheses as formulated in Chapter 1.

*Hypothesis 1:* Contrasting environments of decay, particularly that of pH conditions, affect the rate of physical and chemical decay of insect cuticles differently.

*Hypothesis 2:* Regions of varying pigment content in the cuticle of insects (i.e. patterned cuticles) are affected distinctively through chemical decay when the conditions of decay are identical.

*Hypothesis 3:* Differently coloured regions of the cuticle of insects (i.e. containing distinct pigment molecules) behave disparately under the same transport conditions in a fluid medium.

*Hypothesis 4:* The pigmentary patterning and spatial distribution of the associated pigment molecules on the cuticle of extant insects plays a strong effect on their preservation potential and suggests a biological bias in the fossil record of insects.

### 3. MATERIALS AND METHODS

#### 3.1. Samples.

*H. axyridis* is the object of this study due to its smooth elytra lacking irregularities, the high convexity of the cuticle, the simplicity of the pattern composition and the high phenotypic plasticity on the amount and placement of patterning (see Supplementary Fig. 4.1). Specimens ( $n = 16$ ), display dark yellow to bright orange background with black spots of variable number and size ( $n = 8$ ) or black background with large orange spots ( $n = 8$ ). Specimens were grouped on the grounds of presence/absence and area of patterning in four groups (Fig. 4.1): group 1 consisted of elytra with black background and one single orange spot in the centre ( $n = 4$ ); group 2 were elytra also with black background but with two large orange spots ( $n = 4$ ); group 3 was formed by elytra with red background with up to two small black spots ( $n = 4$ ); group 4 consisted of elytra with red background and with at least eight small black spots ( $n = 4$ ). All specimens analyzed were collected from a single commercial supplier (Blades Biological, UK) with a common origin and diet.



Body orientation of insects for measurements and descriptions was positioned with the convex side of the insect's body facing upwards and the anterior side of the insect placed away from the viewer.

### **3.2. Image acquisition and editing.**

Digital images of all specimens were obtained using a Leica MSV266 stereomicroscope and a Leica DFC 290 HD camera with a polarizing filter. Stacks of images were combined onto a single file using CombineZP.Ink for Windows (<https://combinezp.software.informer.com/>).

### **3.3. Decay experiment.**

Specimens were euthanized via freezing and stored at -40°C prior to the start of the experiments. Frozen specimens (n = 16) were transferred to individual glass vials with a solution of circa 5 ml double distilled water, potassium hydroxide (KOH) and hydrochloric acid (HCl) adjusted to one of the following conditions: three specimens from each group were treated at pH 8 (n = 12) and one specimen per group was treated as control at pH 7 (n = 4). Conditions of decay were selected in the basis of the results presented in chapter 3; these results indicate that insects treated in alkaline media display more advanced decay than samples decayed in acidic conditions after 180 days of decay. Vials were placed in a constant temperature incubator at 25°C ± 1°C in the dark. Experiment was terminated at 180 days.

### 3.4. Tumbling experiment.

Decayed specimens were placed in individual plastic vials with 2.5 g of siliceous 1-2 mm sand (collected in Inchydoney Beach with coordinates 51°59'67.89"N, 8°86'41.46"W and sieved to desired grain size) and 14 ml of double distilled water. Plastic vials containing samples were introduced in a rotabarrel tumbling machine of 660 mm inner diameter and 860 mm outer diameter with surrounding padding material and left to tumble at 40 rpm. Samples were inspected for changes at the following durations of tumbling: 41, 86, 109.5, 136.75, 161, 201.25, 230.5, 298.25, 329, 375.5, 537.95, 588.35, 634.35, 726.65 and 799.65 hours. Experiments were terminated after 920.65 hours (38.36 days). Insoluble residues were transferred to filter papers and dried at 15°C overnight. Fragments of cuticle were picked from sediment residues using a Leica MSV266 stereomicroscope and petri dish lined with gridded paper. Digital images were obtained using a Leica DFC 290 HD camera with a polarizing filter and edited as described in section 3.1.2 of this chapter.

### 3.5. Degradation–Quality scores.

In this text *fissured* refers to fracture without displacement of the two adjacent regions of the cuticle, *fractured* refers to displacement of two adjacent regions of the cuticle and *fragmented* refers to loss of cuticle and transformation into a series of smaller fragments. Changes in fragmentation of individual elytra throughout tumbling experiment were recorded in a quantitative manner by assigning pre-set scores for different categories of cuticle degradation. Increasing score indicates larger fragmentational change via mechanical degradation during tumbling experiment (Table 4.1).

*0. Cuticle is visually undamaged.* Cuticle is undamaged not showing any fractures or fragmentation.

*1. Cuticle is whole but visually fissured.* There is a superficial crack in the cuticle, which has not reached through the total thickness of the cuticle.

*2. Cuticle is whole and visually fractured.* There is a visual fracture in the cuticle, which has reached through the total thickness of the cuticle, but there has been no displacement of the opposite sides.

*3. Cuticle is fractured and fragmented in up to two fragments.* There is a visual fracture in the cuticle, which has reached through the total thickness of the cuticle, and there has been displacement of the opposite sides creating a fragmentation separating the cuticle in two fragments.

*4. Cuticle is fractured and fragmented between three and five fragments.* There are visual fractures in the cuticle, which have reached through the total thickness of the cuticle, and there has been displacement of the opposite sides creating a fragmentation separating the cuticle in three–five fragments.

*5. Cuticle is fractured and fragmented between six and eight fragments.* There are visual fractures in the cuticle, which have reached through the total thickness of the cuticle, and there has been displacement of the opposite sides creating a fragmentation separating the cuticle in six–eight fragments.

*6. Cuticle is fractured and fragmented in more than eight fragments.* There are visual fractures in the cuticle, which have reached through the total thickness of the cuticle, and there has been displacement of the

opposite sides creating a fragmentation separating the cuticle in more than eight fragments.

### **3.6. Length of fracture and area of elytron fragments.**

Images of fragments from individual samples were printed and relocated manually on their original position to reconstruct the elytron's morphology before fragmentation. Images were then re-scaled to real size and length of fractures and areas of fragments in each coloured region on the cuticle were calculated using ImageJ64 (Schneider *et al.* 2012). Portions of fractures and area of individual fragments were separated into three distinct groups: i) black fractures separate two regions of the cuticle displaying black visual colour and black fragments contain only black cuticle on their total surface area; ii) red fractures separate two regions of the cuticle displaying red/orange visual colour and red fragments contain only red/orange cuticle on their total surface area; iii) mixed fractures separate two regions of the cuticle displaying black and red/orange visual colour and mixed fragments contain black and red/orange cuticle on their total surface area.

### **3.7. Data analysis.**

Trends in the data were assessed using Kruskal-Wallis rank test and Box-Whisker plot in the packages “stats” and “graphics” in R (R CORE TEAM 2013). These were contrasted using parametric analysis; analysis of variance (ANOVA) and post hoc testing (Holm-Bonferroni and Tukey 95% confidence tests) of the normalized data using the package “stats” in R (R CORE TEAM 2013).

## 4. RESULTS

### 4.1. Experimental decay.

Results of experimental decay are extensively described in Chapter 3. Mechanical breakage was observed on one elytron after decay (BB2), any other elytra included in the analysis did not display mechanical breakage prior to tumbling.

### 4.2. Tumbling.

One elytron from the sample BBS3 was fissured, prior to the commence of the tumbling experiment (Table 4.2); this fissure was considered to have formed during decay, consequently, the sample was removed from further statistical analyses. All other samples lacked any visible structural damage prior commence of the tumbling experiment. The first fissured elytron (RBS4) was detected at 136.75 hours (Tables 4.2, 4.3). At 375.5 hours samples were retrieved from the vials to digitally record breakage; it was observed that elytra with more colour spots (i.e. pronounced spatial distribution of pigment molecules in the cuticle) were succumbing to breakage faster than those with more homogeneous colour distribution in the cuticle (Tables 4.2, 4.3). By the end of the experiment (920.65 hours), three samples were fragmented in more than five fragments (RBS4, BB3 and BBS1) and 11 samples were fragmented in less than five fragments (RB1, RB2, RBS3, RBS4, BB1, BB2, BB3, BB4, BBS1, BBS2, BBS3) (Fig. 4.1 and Tables 4.2 and 4.3). Group 3 contains the samples with the most homogeneous colouration in the cuticle (i.e. red/orange background with up to two small black spots in the periphery), none of these samples progressed from taphonomic stage 3 after the tumbling experiment was terminated (Fig. 4.2 and Tables 4.2, 4.3). None of the samples from group 2 (i.e. black background with two orange spots in the

centre) progressed from taphonomic stage 5 after the tumbling experiment was terminated (Fig. 4.2 and Tables 4.2, 4.3). Whilst samples of groups, 1 (black background with a large orange spot in the centre) and 4 (red/orange background with at least 8 small black spots distributed all over the surface) reached taphonomic stage 6 at the termination of the experiment (Fig. 4.2 and Tables 4.2, 4.3).

Results of the tumbling experiment are summarized in Figs. 4.1, 4.2, Tables 4.2 and 4.3, Supplementary Text and Supplementary Table 4.1; NOTE: Supplementary files mentioned in this chapter are located in Appendix 4).

Tumbling experiments of patterned cuticles in a taxon with high phenotypic plasticity on pigment content allow to test for variations in mechanical properties of the cuticle associated with visual colour and pigment content. Results suggest that highly patterned elytra are more prone to breakage, thus supporting hypotheses 2 and 3. Additionally, elytra with large, continuous melanin-rich regions (i.e. dark background) are more likely to break than those elytra with smaller melanin-rich regions (i.e. dark spots on a red background) and when decay occurred under alkaline conditions, thus supporting hypotheses 1, 2 and 3.

**Hypotheses 1 (*contrasting environments of decay, particularly that of pH conditions, affect the rate of decay*), 2 (*regions of varying pigment are affected distinctively through chemical decay when the conditions of decay are identical*) and 3 (*differently coloured regions of the cuticle of insects behave disparately under the same transport conditions in a fluid medium*) are accepted.**

### 4.3. Statistical analysis.

Analyses of the dataset concluded a non normal distribution (see Supplementary Text, Supplementary Figs. 4.1, 4.2). Non-parametric analysis of variance, box-and-whisker diagrams of length of fracture show that the degree of dispersion and skewness in the data are larger in fractures of the cuticle displaying one visual colour than in those fractures separating two different colours (Fig. 4.3a). In addition, the skewness of the length of fracture data in fractures where two colours meet is negative (Fig. 4.3a), this indicates that fractures in this region of the cuticle are statistically significant smaller than those fractures separating two regions of the same colour (Fig. 4.3a). To the contrary, dispersion and skewness in the area of fragments is larger in those fragments containing two colours than in fragments with only one colour; furthermore, the skewness of the three parameters (i.e. black, red and mixed) is positive (Fig. 4.3b). This indicates that the area of fragment where two colours meet in the cuticle are statistically significant larger than those fractures separating two regions of the same colour (Fig. 4.3a); subsequently, the likelihood of these regions with mixed colouration to fragment and completely disarticulate from the rest of the insect's carcass is higher than monochromatic regions of the cuticle.

These trends were cross-tested exploring one-way ANOVA for which the data was normalized using log transformation (Supplementary Table 4.2). ANOVA analysis in length of fracture and area of fragment indicate that the means from each grouping of colours (i.e. black, red and mixed) are statistically significant from each other and the variability of data associated with different colour grouping is significant in 2-degrees of freedom (Supplementary Table 4.2). Post hoc analyses, Holm-Ponferroni and Tukey 95%, denote that the largest variability of

the data for length of fracture and area of fragment derive from regions of the cuticle where two pigments meet (i.e. mixed group of colour) and carotenoid-rich regions of the cuticle (i.e. red group of colour). Black regions of the cuticle (melanin-rich) do not contribute to the variability of the data in large quantities. Additionally, analyses of ANOVA residuals indicate that these are homogeneous (Supplementary Figs. 4.3a, 4.4a) and normal (Supplementary Figs. 4.3b, 4.4b), which allow for justification of the model. These results concur with results obtained in non-parametric testing, indicating that regions of the cuticle where two colour meet are more prone to breakage and subsequent fragmentation.

Statistical results suggest that highly patterned elytra are more prone to breakage, thus supporting hypotheses 2 and 3. Additionally, regions of the cuticle where two different pigment molecules meet (i.e. melanins-carotenoids) contribute largely to the variability of the data indicating that they are more prone for breakage than regions of the cuticle where there is only one pigment molecule concentrated (e.g. melanin), thus supporting hypotheses 3 and 4.

**Hypotheses 3 (*differently coloured regions of the cuticle of insects behave disparately under the same transport conditions in a fluid medium*) and 4 (*pigmentary patterning and spatial distribution of the associated pigment molecules on the cuticle of extant insects plays a strong role on their preservation potential and suggests a biological bias in the fossil record of insects*) are accepted.**



## 5. DISCUSSION

Analysis of the mechanical properties of extant insects during experimental transport indicates that regions of the cuticle where two different pigments meet are more inclined to breakage and subsequent disarticulation than those regions where there is unique pigment content (Figs. 4.1, 4.3 and Table 4.4). This strongly suggests that patterning and pigment content in the cuticle of insects are important biases during transport and deposition processes, which can produce extended breakage and disarticulation and subsequently confounding the taxonomical placement of fossil insects, description of palaeo-communities with insects as a dominant and/or associated taxa and concealing the true evolutionary history of the group.

It is possible that the trends shown on breakage by this data are a result of sample heterogeneity and pre- or post-mortem imperfections in the cuticle. All samples of *H. axyridis* used in this study were sourced from a single online dealer, were collected in the same ontogenetic stage and had followed a similar diet until their collection. Samples chosen were of similar size and no imperfections were observed in their cuticles prior decay experiments. Samples were euthanized upon arrival via freezing and in identical conditions. Aberle and colleagues (2017) demonstrated that freezing of insect samples have no effect on the biomechanical properties of the cuticle, and show no significant differences respective to fresh samples in resistance to breakage or Young's modulus. Homogeneity of samples collected and previous published experiments are not consistent with breakage controls being other than differences in pigment content.

Other authors have tried to recreate physical conditions of transport as faithfully as possible in order to reproduce past environments through tumbling

experiments in the field (Driscoll 1967) and in laboratory conditions (Kuenen 1959; Kuenen 1960; Duncan 1997). However, it is important to elucidate mechanical properties and duration of transport, the aim of this study was to achieve breakage in cuticles of insects in a timely manner and not necessary to recreate the exact conditions of transport for a particular fossil insect occurrence. The author acknowledges that 1–2 mm sand size would not be possible natural agent of abrasion in cuticles of *H. axyridis* under normal sedimentary conditions (i.e. riverine and lacustrine) but would be acceptable during flood events, when most transport would probably occur. Nonetheless, these cuticles proved to be highly resistant to breakage; in trial experiments, any grain size smaller to 1 mm did not produce any effect in the cuticle after 168 hours of tumbling. The sturdiness of these cuticles seems to be a combination of the mechanical properties and the saturation of water (personal observation), which aids in flexibility of the exoskeleton (Smith *et al.* 2006). However, observations during these experiments indicate that pigment content, in this case the absence of melanin molecules, may aid in the flexible nature of the cuticle assisting in resistance against breakage (Fig. 4.1). Groups 1 and 2 (elytra with black background and large red spots in the centre) endured a much higher rate of fracture and disarticulation than groups 3 and 4 (red background with varying number of small black spots). In groups 1 and 2, 7 out of 8 elytra were fractured and disarticulated at the end of the experiment, whilst in groups 3 and 4, 5 out of 8 elytra did not fracture for the duration of the tumbling experiment (Fig. 4.1).

Density graphs and quantile plots indicate that area of fragments dataset is not normally distributed (Supplementary Text and Supplementary Figs. 4.1, 4.2). In length of fracture dataset, two of the parameters (black and red) are different to

normal distributions while the last parameter (mixed) is not. Due to the distributions of these datasets being different to that of a normal distribution, the sample size of the populations being small (length  $n = 208$ ; area  $n = 101$ ) and independent from each other then the use of non-parametric testing is justified. The author acknowledges that non-parametric tests are less powerful than parametric tests but when the skew is small and  $p < 0.05$  (Table 4.4) the result of non-parametric testing is warranted. In addition, parametric testing of previously normalized data yielded similar results (Supplementary Table 4.2). Kruskal-Wallis Rank Test concludes that differences among groups (i.e. black, red and mixed) are statistical significant ( $p < 0.05$ ) (Table 4.4). This dataset shows that the length of fractures are statistically significant smaller in regions of the cuticle separating two different colours (Fig. 4.3). It is important to note that these measurements yielded 206 fractures (98 in black, 99 in red and 9 in mixed colour regions of the cuticle) (Supplementary Table 4.1) but the nature of the patterning in *H. axyridis* generates larger areas dominated by a single colour in the cuticle in all samples (Fig. 4.1). Number of fragments is more numerous in regions of the cuticle with two distinct colours ( $n = 49$ ) against red ( $n = 31$ ) and black ( $n = 41$ ) regions (Supplementary Table 4.1), but more interesting is the positive skewness of the data, indicating that larger and more numerous fragments are statistically significant in these regions (Fig. 4.3).

## 6. CONCLUSION

Insects are notorious for the colourful patternings in their cuticles, fossil insects, however, display monotonal patternings regardless of the sedimentary conditions and processes of fossilisation. In addition, fossil taxa related to greatly

coloured living relatives (e.g. Lepidoptera and many Coleoptera taxa) are found disarticulated and incomplete in the fossil record. This laboratory controlled tumbling experiment indicates that when morphologically identical cuticles are introduced in randomized transport conditions, the rate of degradation varies between samples in direct relation to the pigment content of the cuticle and the pH conditions of the media of decay. Cuticles predominantly containing one type of pigment molecule (i.e. melanin) show statistically higher resistance to breakage than those cuticles sharing a spatial distribution of pigment molecules, particularly when decay occurred in alkaline conditions. Thus **hypotheses 1 (*contrasting environments of decay, particularly that of pH conditions, affect the rate of decay*), 2 (*regions of varying pigment are affected distinctively through chemical decay when the conditions of decay are identical*) and 3 (*differently coloured regions of the cuticle of insects behave disparately under the same transport conditions in a fluid medium*) are accepted**. In addition, cuticles predominantly containing melanin are less resistant than cuticle predominantly containing carotenoids, thus **hypothesis 4 (*pigmentary patterning and spatial distribution of the associated pigment molecules on the cuticle of extant insects plays a strong role on their preservation potential and suggests a biological bias in the fossil record of insects*) is accepted**. This is presumably an effect associated with the elasticity of the cuticle, which is more sclerotized (and presumably less elastic) in regions of the cuticle containing melanin. This dataset indicates that breakage of cuticles of *H. axyridis* is dependant on the amount of patterning and content of specific pigments; heterogeneously coloured regions of the cuticle, where two or more pigments meet, form a plane of weakness through which it will be likely to fracture during transport. Although a small number of samples and a lack of

taxonomic variation this experiment shows that contrasting pigment content in the cuticle of *H. axyridis* creates the source for a fossilisation bias during transport of cuticles to the sedimentary environment for fossilisation and the fossil record of insects is biased towards non-patterned insects. This biological bias will potentially have a detrimental effect in assessing taxonomical placement of fossil insects, evaluation of palaeo-communities and ecological relationships among insect and any other associated taxa, and elucidating the true evolutionary history of the group and estimating abundance of insect taxa in specific intervals during the entire fossil record of the group. These results will also aid in understanding ecological relations of extant insect communities and extinction rates as well as increase our knowledge on the mechanical properties of a natural material that could be developed in other fields with large impacts in human quality of life, such as biotechnology or medical engineering.

## 7. FUTURE WORK

Although this study characterises a new biological bias in transport and potential of fossilisation of insects, other characteristics remain unexplored. Further experiments should include morphological variation of cuticles as well as spatial zonation of pigments and intraspecific sexual and seasonal variability of pigment content.

**LIST OF FIGURES AND TABLES:**

**Fig. 4.1:** Optical images of *Harmonia axyridis* prior, during and after termination of tumbling experiments

**Fig. 4.2:** Time required for mechanical fracture of *Harmonia axyridis* individual cuticles.

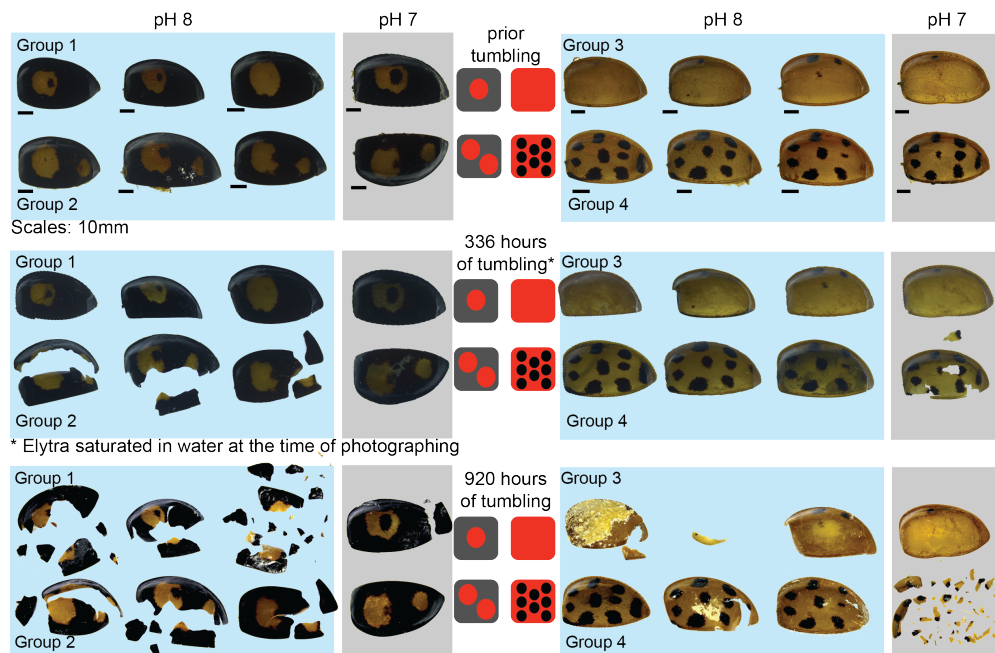
**Fig. 4.3:** Box-and-whisker diagrams of length of fracture and area of individual fragments.

**Table 4.1:** Taphonomic scores and the features they represent in *Harmonia axyridis* cuticles.

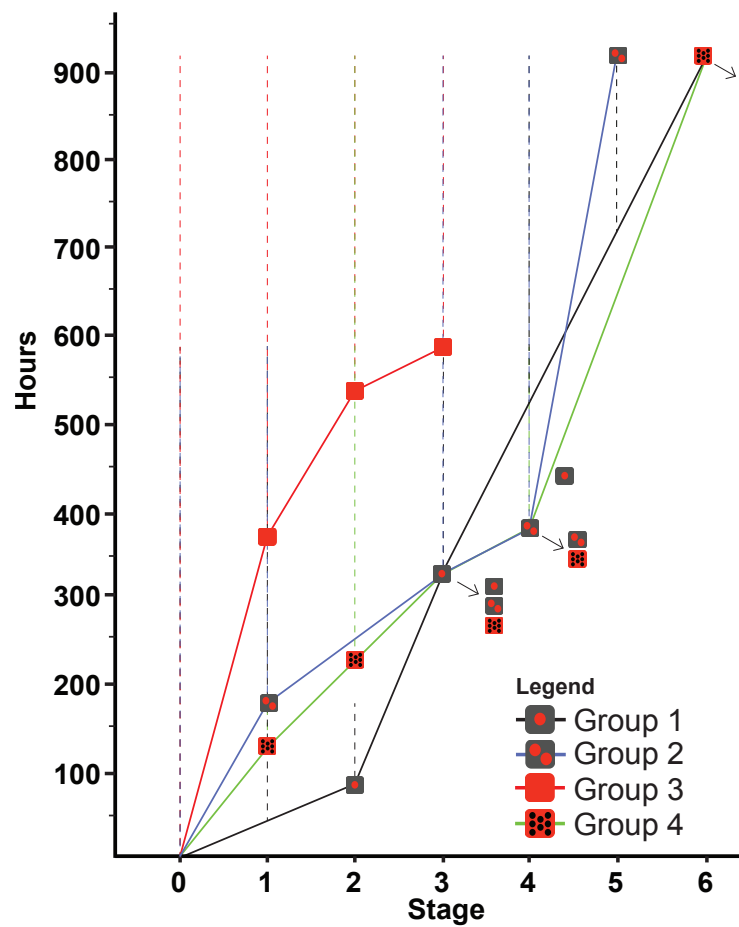
**Table 4.2:** Observations on samples for the duration of the tumbling experiment.

**Table 4.3:** Stages of degradation of cuticles of *Harmonia axyridis* throughout tumbling experiment.

**Table 4.4:** Results of statistical analysis of length of fracture and area of fragments following tumbling experiments in *Harmonia axyridis*.

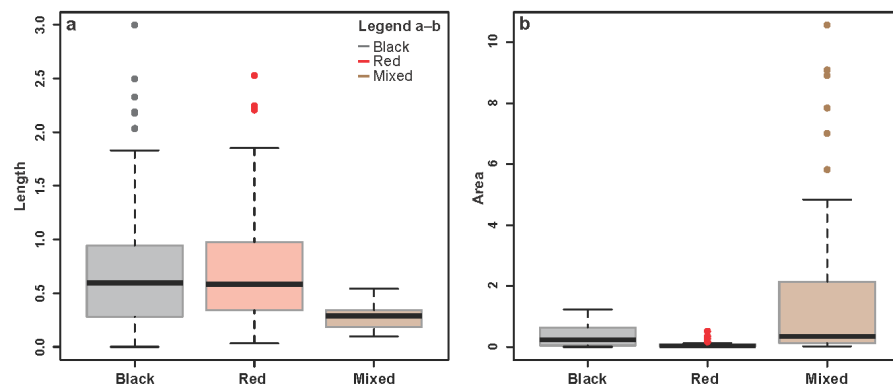


**Fig. 4.1. Optical images of *Harmonia axyridis* prior, during and after termination of tumbling experiments.** Groups 1 and 2 are formed by elytra with predominantly black background and one or two large orange spots respectively. Groups 3 and 4 are composed by elytra with predominantly red/orange background and up to two or up to eight small black spots respectively. Samples from groups 1 through 4 represented over blue background were decayed for 180 days in alkaline media (pH 8), samples over a grey background were decayed in neutral media (pH 7). Elytra were saturated in water during the acquisition of optical images at 336 hours of tumbling. All samples are to scale, 5mm.



**Fig. 4.2.** Time required for mechanical fracture of *Harmonia axyridis* individual cuticles. Dashed lines represent error bars. To be used in conjunction with Tables 4.1, 4.2 and 4.3.





**Fig. 4.3. Box-and-whisker diagrams of length of fracture (a) and area of individual fragments (b). b; see a for key.**

| Score | Meaning                                                            |
|-------|--------------------------------------------------------------------|
| 0     | Cuticle is visually undamaged.                                     |
| 1     | Cuticle is whole but visually cracked.                             |
| 2     | Cuticle is whole and visually fractured.                           |
| 3     | Cuticle is fractured and fragmented in up to two pieces.           |
| 4     | Cuticle is fractured and fragmented between two and five pieces.   |
| 5     | Cuticle is fractured and fragmented between five and eight pieces. |
| 6     | Cuticle is fractured and fragmented in more than eight pieces.     |

**Table 4.1. Taphonomic scores and the features they represent in *Harmonia axyridis* cuticles.**

| Hours  | Observations |                                                                                       |  |
|--------|--------------|---------------------------------------------------------------------------------------|--|
| 0      | RB1          | Right elytron tumbling. White biofilm, white/yellow in colour in and around the head. |  |
|        | RB2          | Right elytron tumbling.                                                               |  |
|        | RB3          | Right elytron tumbling.                                                               |  |
|        | RB4          | Right elytron tumbling. Right elytron malformation on the centre.                     |  |
|        | RBS1         | Right elytron tumbling. Head detached.                                                |  |
|        | RBS2         | Right elytron tumbling.                                                               |  |
|        | RBS3         | Right elytron tumbling.                                                               |  |
|        | RBS4         | Right elytron tumbling. Bacterial growths.                                            |  |
|        | BB1          | Right elytron tumbling. Head detached.                                                |  |
|        | BB2          | Right elytron tumbling. Head detached.                                                |  |
|        | BB3          | Right elytron tumbling. Head detached.                                                |  |
|        | BB4          | Right elytron tumbling.                                                               |  |
|        | BBS1         | Right elytron tumbling. White biofilm in the vial.                                    |  |
|        | BBS2         | Right elytron tumbling. White biofilm. White moho.                                    |  |
|        | BBS3         | Right elytron tumbling. Head detached. Left and right elytron fissured.               |  |
|        | BBS4         | Left elytron tumbling. Right elytron fissured.                                        |  |
| 41     | All          | Nothing to note.                                                                      |  |
| 86     | RBS1         | *                                                                                     |  |
|        | BB3          | *                                                                                     |  |
|        | BBS2         | Elytron fractured, longitudinal.                                                      |  |
| 109.5  | BBS1         | **                                                                                    |  |
|        | BBS3         | **                                                                                    |  |
| 136.75 | RBS4         | Elytron fissured.                                                                     |  |
|        | BB4          | **                                                                                    |  |
|        | BBS1         | Fractured along longitudinal.                                                         |  |
| 161    | BBS3         | Elytron fissured.                                                                     |  |
| 201.25 | All          | Nothing to note.                                                                      |  |
| 230.5  | All          | Nothing to note.                                                                      |  |
| 298.25 | All          | Nothing to note.                                                                      |  |
| 329    | RBS4         | Fragmented in two fragments.                                                          |  |
|        | BBS1         | Fragmented in two fragments.                                                          |  |
|        | BBS2         | Fragmented in two fragments.                                                          |  |
|        | BBS3         | Fragmented in two fragments.                                                          |  |
| 375.5  | RB2          | Elytron fissured.                                                                     |  |
|        | RB4          | Elytron fissured.                                                                     |  |
|        | RBS4         | Fragmented in three fragments. Elytron fractured.                                     |  |
|        | BB2          | Elytron fissured.                                                                     |  |
|        | BBS3         | Fragmented in three fragments.                                                        |  |
|        | BBS4         | Sediment cementing in elytron's surface.                                              |  |
| 537.95 | RB1          | *                                                                                     |  |
|        | RB2          | *                                                                                     |  |
|        | RBS4         | Fractured longitudinal. Fragmented in two fragments at top.                           |  |
|        | BB1          | Fractured longitudinal. Fragmented two fragments.                                     |  |
|        | BB2          | Elytron fissured.                                                                     |  |
|        | BB3          | Elytron fissured.                                                                     |  |
|        | BBS1         | Fractured longitudinal. Fragmented two fragments.                                     |  |
|        | BBS2         | Fractured longitudinal. Fragmented two fragments.                                     |  |
| 588.35 | RB1          | Fragmented two fragments.                                                             |  |
|        | RB2          | ***                                                                                   |  |
|        | BBS3         | Fractured transversal.                                                                |  |
| 634.35 | All          | Nothing to note.                                                                      |  |
| 726.65 | All          | Nothing to note.                                                                      |  |
| 799.65 | All          | Nothing to note.                                                                      |  |
| 920.65 | RB1          | Sediment cementing in elytron's surface.                                              |  |
|        | RB3          | Elytron fissured.                                                                     |  |
|        | RBS2         | Sediment cementing in elytron's surface.                                              |  |
|        | RBS3         | Fractured in centre. Sediment cementing in elytron's surface.                         |  |
|        | RBS4         | Fragmented in more than eight fragments.                                              |  |
|        | BB1          | Fragmented in eight fragments.                                                        |  |
|        | BB2          | Fragmented in two fragments. Elytron heavily fissured.                                |  |
|        | BB3          | Fragmented in more than eight fragments. Sediment cementing in elytron's surface.     |  |
|        | BB4          | Fractured in three fragments.                                                         |  |
|        | BBS1         | Fragmented in three fragments.                                                        |  |
|        | BBS2         | Fragmented in five fragments.                                                         |  |

**Table 4.2 (previous page). Observations on samples for the duration of the tumbling experiment.** RB, predominantly orange background; RBS, predominantly orange background with spots; BB, black background with one orange spot per elytron; BBS, black background with two orange spots per elytron.  
\* Vial was refilled with double distilled water to 14 ml; \*\* Vial was substituted for a new one and refilled with double distilled water to 14 ml; \*\*\* Vial and sample were discarded after loss of part of the sample and sediment.

| Hours        | RB1 | RB2 | RB3 | RB4 | RBS1 | RBS2 | RBS3 | RBS4 | BB1 | BB2 | BB3 | BB4 | BBS1 | BBS2 | BBS3 | BBS4 |
|--------------|-----|-----|-----|-----|------|------|------|------|-----|-----|-----|-----|------|------|------|------|
| <b>86</b>    | 0   | 0   | 0   | 0   | 0    | 0    | 0    | 0    | 0   | 0   | 0   | 0   | 0    | 2    | 1    | 0    |
| <b>136.8</b> | 0   | 0   | 0   | 0   | 0    | 0    | 0    | 1    | 0   | 0   | 0   | 0   | 2    | 2    | 1    | 0    |
| <b>161</b>   | 0   | 0   | 0   | 0   | 0    | 0    | 0    | 1    | 0   | 0   | 0   | 0   | 2    | 2    | 1    | 0    |
| <b>329</b>   | 0   | 0   | 0   | 0   | 0    | 0    | 0    | 3    | 0   | 0   | 0   | 0   | 3    | 3    | 3    | 0    |
| <b>375.5</b> | 0   | 1   | 0   | 1   | 0    | 0    | 0    | 4    | 1   | 0   | 0   | 0   | 3    | 3    | 4    | 0    |
| <b>538</b>   | 0   | 1   | 0   | 2   | 0    | 0    | 0    | 4    | 3   | 1   | 1   | 0   | 3    | 3    | 4    | 0    |
| <b>588.4</b> | 3   | 3   | 0   | 2   | 0    | 0    | 0    | 4    | 3   | 1   | 1   | 0   | 3    | 3    | 4    | 0    |
| <b>920.7</b> | 3   | 3   | 1   | 2   | 0    | 0    | 2    | 6    | 5   | 3   | 6   | 4   | 4    | 5    | 4    | 0    |

**Table 4.3. Stages of degradation of cuticles of *Harmonia axyridis* throughout tumbling experiment.** To be used in conjunction with Table 4.1 and Fig. 4.2. Abbreviations as per caption to Table 4.2.

| Length       |              |       |      |                 |                |        |         |                 |
|--------------|--------------|-------|------|-----------------|----------------|--------|---------|-----------------|
|              | Shapiro-Wilk |       |      |                 | Kruskal-Wallis |        |         |                 |
|              | mean         | SD    | W    | p-value         | chi-squared    | df     | p-value |                 |
| <b>Black</b> | 0.73         | 0.601 | 0.86 | <b>2.68E-08</b> | <b>All</b>     | 10.186 | 2       | <b>6.14E-03</b> |
| <b>Red</b>   | 0.74         | 0.538 | 0.89 | <b>7.11E-07</b> |                |        |         |                 |
| <b>Mixed</b> | 0.28         | 0.127 | 0.95 | 0.618           |                |        |         |                 |

| Area         |              |       |      |                 |                |        |         |                 |
|--------------|--------------|-------|------|-----------------|----------------|--------|---------|-----------------|
|              | Shapiro-Wilk |       |      |                 | Kruskal-Wallis |        |         |                 |
|              | mean         | SD    | W    | p-value         | chi-squared    | df     | p-value |                 |
| <b>Black</b> | 0.39         | 0.401 | 0.83 | <b>1.03E-03</b> | <b>All</b>     | 30.099 | 2       | <b>2.91E-07</b> |
| <b>Red</b>   | 0.08         | 0.108 | 0.63 | <b>8.74E-08</b> |                |        |         |                 |
| <b>Mixed</b> | 1.82         | 2.823 | 0.67 | <b>9.40E-09</b> |                |        |         |                 |

**Table 4.4. Results of statistical analysis of length of fracture and area of fragments following tumbling experiments in *Harmonia axyridis*. Statistically significant results are indicated in bold font.**

## CHAPTER 5

# Synchrotron-X-ray fluorescence as a tool for interpreting the original colouration of insects

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### ABSTRACT

Colour patterning is one of the most striking adaptations of insect cuticle and is produced primarily by pigments such as melanins, carotenoids and pterins. Fossil insects can also exhibit colour patterns, but the colouration mechanisms responsible are largely unknown. Some fossil beetles preserve evidence for melanin in the form of spatial zonation of Cu, but whether this characterises melanin in other insect taxa, and whether this approach can characterise non-melanin pigments, has not been tested. Non-destructive synchrotron rapid scan-X-ray fluorescence (SRS-XRF) was used to characterise the spatial distributions of elements in the cuticles of extant and fossil insects. Linear Discriminant Analysis (LDA) of the SRS-XRF data reveals a strong taxonomic signal whereby members of the same order (and for modern insects, family) show similar chemical signatures. Within genera, cuticle regions containing different pigments have distinct chemistries, i.e., broad taxonomic trends in chemistry are overprinted by a strong pigment-specific signal. The chemistry of fossil insect cuticle differs to that

of the extant analogues and is controlled by both taxonomy and depositional context, indicating some diagenetic overprinting. Despite this, dark and light cuticle regions with different tones in individual fossils contain different suites of elements, suggesting preservation of a pigmentary component in the chemical signal, although trace elemental chemistry of individual pigment molecules is specific to the diagenetic history. These data will aid the interpretation of the original colours of fossil insects, thus informing models of the evolution of colour and its ecological functions in insects through deep time.

## 1. PREVIOUS WORK

### 1.1. Introduction

Insects are the most diverse and ecologically- and numerically dominant group of animals on Earth (Sabrosky 1953; Mayhew 2007) and have been important components of terrestrial ecosystems for the last 400 million years (Labandeira and Sepkoski 1993; Mayhew 2007). The evolutionary success of the group relates to the acquisition of a suite of novel anatomical, physiological and behavioural characters linked to flight, reproduction, small size, cuticle structure and symbiotic relationships with other organisms (Mayhew 2007; Nicholson *et al.* 2014). One of the most striking adaptations of extant insects is colour patterning, e.g. spots, bands and eyespots, that function primarily in intra- and interspecific communication. These patterns reflect spatial distributions of pigments, e.g. carotenoids (Jitariu and Petcu 1974; Czeuczuga 1981; Bezzerides *et al.* 2007), melanin (Koch *et al.* 2000a; Bezzerides *et al.* 2007; Bezzerides and Loofbourrow 2014), pterins (Pfeiler 1968; Stavenga *et al.* 2006; Wijnen *et al.* 2007), flavonoids (Morris and Thomson 1963; Wilson 1985) and ommochromes (Koch *et al.* 2000b; Stavenga *et al.* 2014) and/or



structural colours (Rutowski *et al.* 2005; Yoshioka and Kinoshita 2006; Seago *et al.* 2009). Despite recent work on the genomic basis and heritability of insect colour patterns (Nadeau *et al.* 2012; Nadeau *et al.* 2014; Riesch *et al.* 2017), many aspects of their evolutionary history, especially their origins in deep time, remain poorly understood.

## 1.2. Pigments in fossil insects

Fossil insects can also exhibit colour patterns defined by varying tones of brown to black. This fossil patterning has the potential to inform on the evolutionary history of insect colour patterns and of pigment biosynthesis in insects, especially if the original colouration mechanisms are known. Recent research has demonstrated that dark-toned cuticle regions in fossil insects can contain chemical evidence of melanin (Labandeira *et al.* 2016). To date, however, non-melanin pigments have not been reported in fossil insects. This likely reflects - at least in part - the low preservation potential of such pigments: carotenoids are degraded rapidly by UV light and are lipid soluble (McNett and Marchetti 2005), pterins are soluble in strong acids and bases (Blau *et al.* 2008; Hevener *et al.* 2009) and water (Ragone *et al.* 2012) and flavonoids are soluble in various organic solvents (Chebil *et al.* 2007). Collectively, these characteristics suggest that non-melanin pigments are unlikely to survive decay. Even where pigment molecules are degraded, however, original associations (i.e., formed *in vivo*) between pigments and elements are considered to preserve in fossils (Wogelius *et al.* 2011). A diverse suite of elements, including Cu, Zn and Ca, is associated with melanin in extant (Hong and Simon 2007) and fossil (Wogelius *et al.* 2011; Edwards *et al.* 2013) feathers. Cu has also been proposed as a biomarker for melanin in fossil insects (McNamara *et*

*al.* In prep.), but whether this constitutes part of a suite of elemental biomarkers for melanin in fossil insects (as with melanin in feathers (Wogelius *et al.* 2011)) is unknown. Non-melanin pigments, e.g. chlorophylls in cyanobacteria (Gupta 1986) and plants (Droppa and Horváth 1990; Fernandes and Henriques 1991), can also associate with distinct suites of elements, but similar signatures for non-melanin pigments in extant and fossil insects have not been reported. In summary, the abundance and distribution of diverse elements in the cuticles of extant and fossil insects and the extent to which these are linked to specific pigments have not been investigated systematically.

### 1.3. Geological setting

**1.3.1. Daohugou (now referred to as the Yanliao Biota).** Characterized by thick medium-coarse conglomerate interbedded by sandy mudstones of felsic origin and thin coal layers, the biota bearing member is overlaid by igneous rocks (Li *et al.* 2021). Continental deposition with a fresh water environment in a warm and humid to hot and dry climate possibly associated with volcanic activity (Li *et al.* 2021). The expected pH condition of the media of decay is acidic.

**1.3.2. Green River.** Characterized by limestone, siltstone, and low grade oil shale deposited in shallow- to deep-water stratified lakes (Surdam and Wolfbauer 1975). The expected conditions of the media of decay are highly saline and alkaline.

**1.3.3. Grubstake.** Characterized by grey, weakly consolidated and poorly stratified lacustrine shale less than one meter below a prominent white volcanic ash layer (Stanley *et al.* 1990; Grimaldi and Triplehorn 2008). The expected pH conditions of the media of decay are acidic.

**1.3.4. Oeningen.** Upper Freshwater Molasse is characterized by fluvatile sands and clays, and fan conglomerates (Buatois *et al.* 2020) with eventual alkaline volcanic activity (Rocholl *et al.* 2018). The expected pH conditions of the media of decay are alkaline.

**1.3.5. Old Crow River.** Characterized by organic rich sand, clays and silts deposited in a glacial meltwater lake (Morlan and Matthews 1983). The expected pH conditions of the media of decay are acidic.

**1.3.6. Rubielos de Mora.** Characterized by bituminous rhythmites finely laminated deposited in a meromictic lake located in a tectonic basin (Peñalver and Martínez-Delclós 2002) The expected pH conditions of the media of decay are alkaline.

## 2. AIM AND SCOPE OF EXPERIMENTS

Synchrotron rapid-scanning X-ray fluorescence analysis (SRS-XRF) of patterned cuticles from diverse extant insects (Supplementary Tables 5.1 and 5.2; NOTE: Supplementary files mentioned in this chapter are located in Appendix 5) are analyzed to investigate the trace element inventory of cuticles and potential controls of pigmentary colours in the cuticle.

This chapter addresses the following hypotheses as formulated in Chapter

1.

*Hypothesis 5:* Various elements or sets of elements are characteristic of distinct pigment molecules in the cuticle of modern insects prior to decay.

*Hypothesis 7:* Distinctive trace elements or sets of elements can be used as biomarkers for the original pigmentary content in the cuticle of fossil insects.

### 3. MATERIAL AND METHODS

#### 3.1. Samples.

Extant specimens analyzed ( $n = 92$ ) represent 68 species, 39 families and nine orders and met the following criteria: (i) colour pattern comprises large, sharply defined blocks, bands or spots; (ii) cuticle lacks prominent bosses, tubercles and setae (thus avoiding topographic artefacts on SRS-XRF maps); (iii) the pigment(s) responsible for producing the patterning is known for that species or closely related species in the same family; (iv) the family is known in the fossil record; (v) specimens are readily available (Supplementary Fig. 5.1, Supplementary Tables 5.1 and 5.2). Eleven specimens, comprising 10 families, were plotted separately since it was not possible to centre the data (Supplementary Tables 5.1 and 5.2).

Fossil specimens analyzed ( $n = 15$ ) represent 13 species and four orders and met the following criteria: (i) cuticle includes sharply defined blocks, bands or spots that differ in visual tone; (ii) cuticle is preserved in both dark- and light-toned regions; (iii) taxa have extant representatives at family level (Supplementary Fig. 5.2, Supplementary Tables 5.3 and 5.4). All fossils analyzed are preserved as carbonaceous impressions.

#### 3.2. Synchrotron rapid-scanning X-ray fluorescence (SRS-XRF).

All specimens of Lepidoptera, Mecoptera, Diptera, Hymenoptera and Odonata, were analyzed whole since there was no interference from the body with the elemental readings of the extended wings on both sides of the body. Elytra from Coleoptera, Hemiptera and Blattodea were dissected specimens using sterile tools and singularly analyzed using SRS-XRF at beam line 10-2 at the Stanford Synchrotron Radiation Lightsource (SSRL). The beam line used a toroidal mirror

to focus the beam and a Si (111) monochromator for energy selection. Extant and fossil specimens were mounted vertically on a steel frame holder and positioned at 45° relative to the incidence beam. All specimens were analyzed using an excitation energy of 11 keV with a 100 µm pinhole to determine the X-ray beam spot size. Mean distance from the pinhole to the specimen was ca. 100 mm. The fluorescence signal was recorded at 50 ms dwell time intervals and pixel size varied among specimens from 0.025 µm x 0.025 µm to 0.15 µm x 0.15 µm (Supplementary Table 5.1). A photon-counting single element silicon drift detector (Vortex, SII NanoTechnology USA Inc.) was used to detect fluorescent X-ray photons. X-ray energies were discriminated using a Quantum Detectors Xpress3 system. Individual maps were produced for the following elements: P, S, Cl, K, Ca, Ti, Mn, Fe, Ni, Cu and Zn. Scans were monitored during analysis using the software packages MicroScanGUI and uXAS GUI (Webb 2011).

### 3.3. Data analysis.

Synchrotron-XRF data were analyzed using Sam's Microprobe Analysis Toolkit (Webb 2011) as follows. Raw counts were converted to concentration values (µg/cm<sup>2</sup>) by calibrating against NIST traceable analytical standards. Data for certain specimens (*Aglais urticae*, *Trichius* sp., *Polyphylla fullo*, *Cerambycidae* indet., *Cicindela hybrida*, *Odynerus gracilis*, *Andrena carantonica*, *Sympetrum flaveolum*, *Pyrrhosoma nymphula*, *Aeshna caerulea* and *Cordulia aenea*) in Supplementary Figs. 5.3 and 5.4 were calibrated against different standards to the remainder of the specimens and were hence plotted separately. In all species analyzed, pigment chemistry is known in that species or in another member of the same family (Supplementary Table 5.1). Regions of interest (ROIs) were selected

for cuticle regions with different colours/pigments in extant specimens and with different tones and adjacent sedimentary matrix in fossil specimens ( $n = 1-3$  ROIs per colour). XRF spectra were produced for the same ROIs using multi channel analysis (MCA) fitting spectra function in MicroAnalysis Toolkit (Webb 2011), which uses pyMCA algorithms to fit the continuum to constrained fluorescence peaks (Solé *et al.* 2007). Peaks were fitted operating spectrometer zero, spectrometer gain and detector width to achieve the best fit. For extant specimens, when the pigment equivalent to a colour region in the cuticle was not previously described in any published literature it was assumed to be a different pigment than the adjacent coloured region in the cuticle, black regions of the cuticle without pigmentary descriptions are presumed to contain melanin. In fossil specimens, different tones in the preserved cuticle were assumed to be produced by different pigments. Mean and standard deviation values were calculated for the concentration data for each element in each ROI. Mean hex (RGB) values were calculated for each ROI using the RGB\_Measure.java plugin (Rasband 2004) for ImageJ64 (Schneider *et al.* 2012). Concentration data were centred and log-transformed to minimize variation relating to absolute concentrations. Trends in the data were assessed using Linear Discriminant Analysis (LDA), which reduces the dimensions of multivariate data into linear combinations of variables in order to maximise separation among data classes, Principal Component Analysis (PCA) and MANOVA at 95% confidence intervals in the packages “stats” and “MASS” in R (R CORE TEAM 2013).

### 3.4. Image acquisition and editing.

Digital images of all specimens were obtained using a Leica MSV266 stereomicroscope and a Leica DFC 290 HD camera with a polarising filter. Stacks of images were combined onto a single file using CombineZP.Ink for Windows (<https://combinezp.software.informer.com/>). SRS-XRF maps are shown in Fig. 5.1 and Supplementary Figs. 5.1 and 5.2 as raw concentration hdf5 files.

## 4. RESULTS

### 4.1. Elemental chemistry associated with pigment molecules in extant insect cuticle.

Element chemistry of 405 cuticle regions in extant insects was analyzed (Supplementary Fig. 5.1, Supplementary Table 5.2). SRS-XRF maps confirm that spatial distributions of certain elements correspond to visible colour patterns in each taxon (Fig. 5.1, Supplementary Fig. 5.1 and Supplementary Table 5.2).

Taxa displaying spatial partitioning of elements in SRS-XRF maps comprise 65% of Lepidoptera ( $n = 11$ ), 56% of Coleoptera ( $n = 9$ ) and 71% of Hemiptera ( $n = 5$ ) (Supplementary Fig. 5.1 and Table 5.1). For the rest of the groups analyzed (Blattodea  $n = 1$ , Mecoptera  $n = 2$ , Diptera  $n = 1$ , Hymenoptera  $n = 2$  and Odonata  $n = 7$ ), 100% of taxa show spatial partitioning (Tables 5.1, 5.2 and Supplementary Fig. 5.1). The majority of elements analyzed present spatial partitioning among different pigments in extant taxa; however, this pattern is constant intraspecifically, but not across genera or families. For instance, melanin-rich or presumably melanin-rich regions in the cuticles of some Coleoptera (Table 5.2, Supplementary Fig. 5.1s, v, y, aa, ae), Hemiptera (Table 5.2, Supplementary Fig. 5.1ak–al, ap–aq), Mecoptera (Table 5.2, Supplementary Fig. 5.1ar–as),

Hymenoptera (Table 5.2, Supplementary Fig. 5.1au–av) and Odonata (Table 5.2, Supplementary Fig. 5.1aw–bc) are typically enriched in Mn, K and especially Ca (Tables 5.1, 5.2 and Supplementary Table 5.2), but not necessarily in combination. Some Lepidoptera (Tables 5.1, 5.2 and Supplementary Fig. 5.1b, g, l–m), Blattodea (Tables 5.1, 5.2 and Supplementary Fig. 5.1aj) and Diptera (Tables 5.1, 5.2 and Supplementary Fig. 5.1at) also show enrichment of Ca and K in melanin-rich and presumably melanin-rich regions. Cu and Zn rarely show spatial partitioning, except in some Coleoptera (Tables 5.1, 5.2 and Supplementary Fig. 5.1ad), Hemiptera (Tables 5.1, 5.2 and Supplementary Fig. 5.1ak, aq), Mecoptera (Tables 5.1, 5.2 and Supplementary Fig. 5.1ar), Hymenoptera (Tables 5.1, 5.2 and Supplementary Fig. 5.1av) and Odonata (Fig. 5.1a, Tables 5.1, 5.2 and Supplementary Fig. 5.1ay, ba–bc), in which melanin-rich regions are consistently enriched in these elements (Tables 5.1, 5.2). Concentrations of P, S and Fe rarely show spatial variation, excluding some Hemiptera (Fig. 5.1c, Tables 5.1, 5.2, Supplementary Fig. 5.1ak–al, aq), Hymenoptera (Tables 5.1, 5.2, Supplementary Fig. 5.1au) and Odonata (Fig. 5.1a, Tables 5.1, 5.2 and Supplementary Fig. 5.1ay–bc) in which melanin-rich regions are consistently enriched in these elements (Tables 5.1 and 5.2). Melanin-rich regions in Lepidoptera are relatively depleted in Cl and, in some cases, Ca; these elements are enriched in cuticle regions that contain ommochromes, carotenoids and flavonoids (Tables 5.1, 5.2 and Supplementary Fig. 5.1g–h, n–q).

Only in members of Odonata the spatial partitioning is consistent across all taxa (Table 5.1 and Supplementary Fig. 5.1aw–bc). In Libellulidae, Coenagrionidae and Corduliidae Cl, K, Ca, Fe, Cu and Zn are enriched and present strong partitioning in melanin-rich regions (combined with structural colour) of the



cuticle (Table 5.1 and Supplementary Fig. 5.1ay–ba, bc). In Aeshnidae the strong partitioning limits to K, Ca, Cu and Zn in melanin-rich regions (combined with structural colour) of the cuticle (Table 5.1 and Supplementary Fig. 5.1bb). Calopterygidae only Ca shows weak partitioning in melanin-rich regions (combined with structural colour) of the cuticle (Table 5.1 and Supplementary Fig. 5.1aw–ax). MANOVA analysis show that Ti, P and Fe have the most impact on the variation of the data among Odonata while Ni, Mn, K and S have the smallest impact (Supplementary Table 5.5).

In Lepidoptera, two families display consistent spatial partitioning across taxa, Nymphalidae and Zygaenidae (Table 5.1). In Nymphalidae, Ca shows strong spatial partitioning in regions of the cuticle containing ommochromes and carotenoids (Table 5.1). MANOVA show that all elements analyzed, with the exception of Ni, have a high impact in the variation of the data in Nymphalidae (Supplementary Table 5.5). Zygaenidae displays strong spatial partitioning of Ca in regions of the cuticle containing carotenoids, flavonoids and orange and blue (possibly structural) colours (Table 5.1). All elements analyzed show a high input for the variation of the data in Zygaenidae (Supplementary Table 5.5).

For other taxa, spatial partitioning of elements is taxon-specific (Fig. 5.1, Table 5.1 and Supplementary Fig. 5.1).

In Pieridae, *Anthocaris cardamines* displays enrichment of K in regions of the cuticle containing pterins and melanins, while *Colotis regina* shows enrichment of Ca in regions containing pterins (Table 5.1, Supplementary Fig. 5.1b, d and Supplementary Table 5.2). There are statistical significant differences between group means in the mentioned taxa as determined by MANOVA ( $F(16.67) = 0.953$ ,  $p = 1.15e-4$ ; Supplementary Table 5.5). Among Papilionidae, *Papilio*

*glaucus* show strong partitioning of Ca regions containing melanins and ommochromes while *Parnassius apollonius* and *Papilio nierus* show weak partitioning of Ca in regions containing these same pigments (Table 5.1, Supplementary Fig. 5.1l–n and Supplementary Table 5.2). There are statistical significant differences between group means in the mentioned taxa as determined by MANOVA ( $F(89.32) = 1.982, p = 3.56\text{e-}14$ ; Supplementary Table 5.5).

In Coleoptera *Trichius* sp., *Brachita interrogationis* and *Leptinotarsa decemlineata* all display strong spatial partitioning of Mn in melanin-rich (/or presumably) regions of the cuticle (Table 5.1, Supplementary Fig. 5.1v, aa, ae and Supplementary Table 5.2). There are statistical significant differences between group means in the mentioned taxa as determined by MANOVA ( $F(52.10) = 1.979, p = 9.06\text{e-}9$ ; Supplementary Table 5.5). In Hemiptera *Cercopis vulnerata*, *Graphosoma lineatum* and *Cicciana obliqua* show strong partitioning of K and Ca in melanin-rich (/or presumably) regions of the cuticle (Table 5.1, Supplementary Fig. 5.1ak–al, aq and Supplementary Table 5.2). There are statistical significant differences between group means in the mentioned taxa as determined by MANOVA ( $F(16.41) = 1.875, p = 1.23\text{e-}9$ ; Supplementary Table 5.5).

Both members of Panorpidae, *Panorpa* sp. and *Panorpa lugubris*, are enriched in Ca in regions of the cuticle containing melanin (Table 5.1, Supplementary Fig. 5.1ar–as and Supplementary Table 5.2). Taxa not displaying spatial partitioning comprises 31% (n = 17) of the total (Table 5.1, Supplementary Fig. 5.1a, c, e, f, j, k, t–u, w–x, z, af, ag, ah–ai, am, ao and Supplementary Table 5.2).

Trace elemental partitioning associated with visual colour in the cuticle of extant insects suggests that there are specific elements or combination of elements

which are characteristic of distinct pigment molecules in the cuticle, thus supporting hypothesis 5.

**Hypothesis 5 (*various elements or sets of elements are characteristic of distinct pigment molecules in the cuticle of modern insects prior to decay*) is accepted.**

#### **4.2. Linear Discriminant Analysis of elemental chemistry associated with pigment molecules in extant insect cuticle.**

Trends in the SRS-XRF data were explored using Linear Discriminant Analysis (LDA). The results show that carotenoids and melanins occupy large overlapping regions in the chemospace; these areas also overlap regions corresponding to flavonoids, ommochromes, pterins and structural colours (Fig. 5.2a, Supplementary Figs. 5.3c, 5.5n–t and Supplementary Table 5.4). In contrast, taxonomic groups are well separated in LDA plots, especially at order-level (Fig. 5.2b). Coleoptera, Lepidoptera and Hemiptera occupy discrete regions in chemospace (Fig. 5.2b, Supplementary Figs. 5.3b, 5.5b, d, e and Supplementary Table 5.4). Regardless of visible colour, cuticles are relatively enriched in S in Lepidoptera (Fig. 5.2b, Supplementary Figs. 5.3b and 5.5e), K and Mn in Coleoptera (Fig. 5.2b, Supplementary Figs. 5.3b and 5.5b), and Ni and Ca in Hemiptera (Fig. 5.2b, Supplementary Figs. 5.3b and 5.5d).

This order-level control on cuticle chemistry is superimposed by a pigment-related control: within individual families, cuticles rich in different pigments usually plot separately in LDA plots (Fig. 5.3, Table 5.4, Supplementary Text and Supplementary Figs. 5.3c, 5.5n–t). See Table 5.4 and Supplementary Text for a detailed description of these results.

These results are supported by Principal Component Analysis (PCA), which (unlike LDA) maximizes variance in datasets independently of designated classes (Supplementary Figs. 5.3, 5.6, Supplementary Table 5.6). LDA and PCA analysis of unlogged data produce similar results as explained above (Supplementary Figs. 5.4, 5.8 and Supplementary Table 5.6).

In summary, there are clear trends in the distribution of elements among extant taxa: melanin-rich regions show relatively elevated concentrations in some, or all, of Ca and Mn (57.1%, 42.9% of taxa, respectively); carotenoid-rich regions are usually enriched in Cl, Ca and P (62.5% of taxa), ommochrome-rich regions are enriched in K, S and Mn (50% of taxa), flavonoid-rich regions are enriched in K and Ca (50% and 25% of taxa, respectively), pterin-rich regions are enriched in Ca and Fe (all taxa) and regions lacking pigment are enriched in Cl, Ca and K (50% of taxa). Additionally, specimens of the same order and family sourced from different collections and highly diverse times of death present similar elemental chemistry associated with pigments contained in the cuticle (Supplementary Fig. 5.4).

MANOVA analysis of the SRS-XRF data shows that Ni, Ti, Ca and K have the most impact on the variance of the data (Supplementary Table 5.5). In extant insects, carotenoids can be discriminated from melanins on the basis of significantly higher concentrations of P (Supplementary Table 5.5). Flavonoids show significantly higher concentrations of Ca than those found in melanins and carotenoids (Supplementary Table 5.5). Melanins show significantly higher concentrations of Mn than those found in ommochromes (Supplementary Table 5.5). Ommochromes show lower concentrations of K than those found in cuticles containing flavonoids (Supplementary Table 5.5). Finally, pterins show the highest

concentrations of Ca across all pigments and are the only ones in which the concentration of Fe is statistically significant (Supplementary Table 5.5).

For PCA and unlogged results, refer to Supplementary Text, Supplementary Figs. 5.3, 5.4, 5.7, 5.8 and Supplementary Table 5.6.

Multivariate analyses of trace elemental chemistry in the cuticle of extant insects suggest that there are specific elements or combination of elements which are characteristic of distinct pigment molecules in the cuticle, thus supporting hypothesis 5. However, these sets of elements are strongly taxonomically controlled and are highly variable across specific pigment molecules, contradicting hypothesis 7.

**Hypothesis 5 (*various elements or sets of elements are characteristic of distinct pigment molecules in the cuticle of modern insects prior to decay*) is accepted but hypothesis 7 (*distinctive trace elements or sets of elements can be used as biomarkers for the original pigmentary content in the cuticle of fossil insects*) is rejected.**

#### **4.3. Elemental chemistry associated with pigment molecules in fossil insect cuticle.**

Analysis of 125 regions of interest in 15 fossil insects representing 13 species and four orders (Supplementary Tables 5.3 and 5.4) was completed following the procedure described above. All fossils analyzed show spatial partitioning of some elements, although the suites of elements vary among specimens (Table 5.5 and Supplementary Table 5.7).

In summary, dark-toned regions in fossil insects are typically enriched in Cu, Ti and Ca (85%, 77% and 38% of specimens, respectively), whereas light-

toned regions are often enriched in K, Zn and Fe (77%, 69% and 15% of specimens, respectively). When the data is constrained by insect order, the chemical signature of the sediment is enriched in K, Zn and Fe in comparison to the fossil samples regardless of the deposition and diagenetic histories (Fig. 5.4b, d, g, h). For a detailed account of these results see Table 5.5 and Supplementary Text.

Trace elemental partitioning associated with visual colour in the cuticle of fossil insects suggests that there are specific elements or combination of elements which are characteristic of distinct visual colours (presumably disparate pigment molecules) in the fossil cuticle, thus supporting hypothesis 5. However, these combination of trace elements are strongly taxonomically controlled and the diagenetic overprint seems to greatly affect lighter regions of the fossil cuticle (presumably lacking melanin), contradicting hypothesis 7.

**Hypothesis 5 (*various elements or sets of elements are characteristic of distinct pigment molecules in the cuticle of modern insects prior to decay*) is accepted but hypothesis 7 (*distinctive trace elements or sets of elements can be used as biomarkers for the original pigmentary content in the cuticle of fossil insects*) is rejected.** However, melanin appears to strongly bind to specific trace elements (i.e. Mg and Ca) *in vivo* and the association is maintained during decay, see Chapter 3.

#### **4.4. Linear Discriminant Analysis of SRS-XRF data from fossil insects.**

LDA plots of the fossil data show a strong signal linked to fossil locality.

Specimens from Grubstake and Old Crow River are relatively enriched in Ti, fossil from Grubstake are enriched in Ti and Cu, those from Oeningen, in Ca, those from Green River and Rubielos de Mora, in Fe and K and those from Daoughou in K

and Zn (Fig. 5.4a, c, e, f). This signal likely reflects differences in the diagenetic history of fossils from different localities: the data for the sediment and fossils from each locality plot close to each other (Fig. 5.4a, c and Supplementary Fig. 5.9a, b). Despite this diagenetic signal, however, the fossil data also show a taxonomic signal at order level, as in the extant insects: fossil Coleoptera, Hemiptera, Neuroptera and Diptera occupy distinct regions of the LDA plot (Fig. 5.4b, d, g, h). There is no obvious age-related trend in the data (Supplementary Fig. 5.9a, b).

Remarkably, LDA plots reveal that spatial distributions of certain elements in fossils correspond to visible differences in cuticle tone (Fig. 5.4, Supplementary Fig. 5.9 and Supplementary Table 5.7). For instance, dark cuticle regions in fossil insects from Rubielos de Mora, Oeningen and Green River show similar enrichment in S, Ca and Ti, whereas dark regions in specimens from Daohugou and Grubstake are enriched in K and Fe. Dark regions in fossil insects from Old Crow River are enriched in Ca only. Light cuticle regions are enriched in K, Ti and Fe (and in some specimens Zn and/or Cu) in specimens from Rubielos de Mora, Oeningen, Daohugou and Green River but in Ti, S and Cu in specimens from Grubstake and Old Crow River (Fig. 5.4a, c, e–f and Supplementary Table 5.7).

Grouping the data by order reveals shared features among different fossil orders (Fig. 5.4b, d). When the plots exclude sediment, K and Zn are consistently enriched in dark (melanin-rich) cuticle regions in all Hemiptera and Coleoptera analyzed; when the plots include sediment, S, Ca and Ti are consistently associated with dark cuticle regions in all taxa except Hymenoptera. There is minor overlap among fossil orders in the chemospace (YPM IP454454 (Coleoptera) and YPM YPM522483 (Hemiptera) in Fig. 5.4b, d), however; these were collected at the

same fossil locality (Green River). Similarly, extant orders show minor overlapping in the chemospace (Fig. 5.2b). Chemical overlapping in fossil insects is most likely a reflection of the combination of the original biological characteristics and the chemistry acquired through diagenesis.

PCA plots show a similar chemical signals (Supplementary Fig. 5.9h–l and Supplementary Table 5.6). LDA and PCA analysis of unlogged data produce similar results (Supplementary Table 5.6). However, the same trends identified in LDA are difficult to identify in unlogged PCA plots as the data are clustered tightly (Supplementary Fig. 5.9e–j and Supplementary Table 5.6).

Multivariate analyses of trace elemental chemistry in the cuticle of fossil insects suggest that there are specific elements or combination of elements which are characteristic of distinct pigment molecules in the cuticle, thus supporting hypothesis 5. However, these sets of elements are strongly taxonomically controlled and are highly variable across specific pigment molecules, contradicting hypothesis 7.

**Hypothesis 5 (*various elements or sets of elements are characteristic of distinct pigment molecules in the cuticle of modern insects prior to decay*) is accepted but hypothesis 7 (*distinctive trace elements or sets of elements can be used as biomarkers for the original pigmentary content in the cuticle of fossil insects*) is rejected.**

#### **4.5. Comparison of elemental chemistry in extant and fossil insect taxa.**

Data acquired show that members of individual extant insect families cluster tightly in chemospace (Fig. 5.3 and Supplementary Figs. 5.3a and 5.5f–m). The area of chemospace occupied by each family is independent of sample size, i.e. the



number of specimens analyzed per family (Pearson's  $r = 0.71$ ,  $p = 0.0002$ ,  $n = 22$  (LDA plots);  $r = 0.59$ ,  $p = 0.0041$ ,  $n = 22$  (PCA plots); Fig. 5.3, Supplementary Figs. 5.3a, g and 5.5f–m and Supplementary Table 5.8), thus supporting hypothesis 5.

Comparison of concentration data from the extant and fossil insects analyzed reveals that Zn, Cu, K and S show similar concentrations in both extant and fossil samples. Other elements, e.g. Fe and Ca, are present in higher concentrations – up to two orders of magnitude higher – in fossil samples (Supplementary Table 5.9), hypothesis 7 is not supported by these results. Collectively, data show that colour patterning is defined by different suites of elements in fossil and extant insects. Melanin-rich regions in Hemiptera are enriched in S, K and Ca in extant specimens but in Cu and Zn in fossil members of the group (Tables 5.2 and 5.3 and Supplementary Figs. 5.1ak–aq and 5.2a–e). Similarly, melanin-rich regions in Coleoptera are enriched in K, Ca, Mn and Zn in extant specimens but in Cu, Zn and Ti in fossils (Tables 5.2 and 5.3 and Supplementary Figs. 5.1r–ai and 5.2f–I). Melanin-rich regions in fossil Hymenoptera are enriched in Ti and Cu and Zn; however, extant specimens are enriched in Cl, K, Ca and Fe (Tables 5.2 and 5.3 and Supplementary Figs. 5.1au–av and 5.2n). Dark (presumably melanin-rich) regions of the cuticle in fossil Hemiptera (e.g. RR1-12-07-10 and RR1-12-07-44) are enriched in Ti and Ca (Fig. 5.4d, h), but melanin-rich regions of the cuticle in extant Hemiptera are enriched in Mn, K (and Ca) when grouped by order (Fig. 5.2b, d). The chemical differences between fossil and extant insects may not be attributed solely to taxonomic factors but likely reflect a sedimentary control: when the fossil data are grouped by order (Fig. 5.4d), the sediment data (in orange) plot separately from the fossils from the

same locality. In contrast, when the sediment data (in orange) are grouped by fossil locality (Fig. 5.4c), they cluster tightly with the data from the fossils from the same locality. These results support hypothesis 5; conversely, the high variability of elemental concentrations among different fossil sites contradicts hypothesis 7.

**Hypothesis 5 (*various elements or sets of elements are characteristic of distinct pigment molecules in the cuticle of modern insects prior to decay*) is accepted, whilst hypothesis 7 (*distinctive trace elements or sets of elements can be used as biomarkers for the original pigmentary content in the cuticle of fossil insects*) is rejected.**

MCA fitting spectra show well-constrained k-shell peaks for all elements of interest in extant (Supplementary Figs. 5.10–5.38) and fossil specimens (Supplementary Figs. 5.39–5.49).

## 5. DISCUSSION

Analysis of extant insects reveals that cuticle regions with different colouration mechanisms possess distinct elemental signals at the level of individual species and genera/families (Supplementary Figs. 5.3 and 5.5). This strongly suggests that elemental chemistry is a proxy for colour in extant insects.

It is possible that the broad, order-level, trends in cuticular trace element chemistry in the extant insects (Supplementary Figs. 5.3 and 5.5) may relate to diet, in particular, plant-derived pigments such as carotenoids (Bezzarides *et al.* 2007; Heath *et al.* 2013) and flavonoids (Morris and Thomson 1963; Erlund 2004). This dataset, however, *Nezara robusta* (Hemiptera: Pentatomidae) and *Pieris rapae* (Lepidoptera: Pieridae) both feed primarily, or exclusively, on members of the Brassicaceae (Schuh and Slater 1995; Panizzi *et al.* 2000; Agrawal and Kurashige

2003; Van Dam *et al.* 2005); carotenoid-rich regions in *P. rapae* (Supplementary Fig. 5.5m, v) are enriched in Cl and Ni and pterin-rich regions in *N. robusta* (Supplementary Fig. 5.5l, v) are enriched in Fe and Mn. These data are not consistent with a dietary controls on cuticular chemistry; alternatively, the broad chemical differences among insect orders may reflect the evolution of phylogenetically distinct pathways for pigment chemical synthesis involving elements. This hypothesis is supported by known variation in the biosynthesis of certain pigments in insects (Ziegler and Harmsen 1970; McGraw 2006), e.g. butterflies, flies and roaches synthesize pterins via different chemical pathways (Ziegler and Harmsen 1970). In addition, specimens of the same order and family sourced from different collections have similar chemistry (Supplementary Fig. 5.4), suggesting that specimen provenance does not exert a strong control on the results.

This study shows that chemical signals of pigments defining colour patterning are not produced by the same trace elements in modern representatives of the same taxonomic group, although they are comparable at family level, and they may differ in fossil representatives of the same group. In particular, not all elements associated with melanin-rich regions in extant insects are enriched in dark-toned regions in their fossil relatives. This suggests that (i) the dark-toned regions in these fossils did not contain melanin *in vivo*; (ii) the dark-toned regions in these fossils did contain melanin *in vivo* but differed in chemistry to their extant relatives; or (iii) the original trace element inventory of the cuticle has been altered during diagenesis. In insects, dark toned colouration is not restricted to melanin molecules; for example, dark browns can be achieved via other pigment molecules, such as ommochromes (Stavenga *et al.* 2014) and flavonoids (Wilson 1987). Elemental chemistry in regions of the cuticle that are known to contain melanin,

have been proven to differ within taxa of the same order (this study; Table 5.1), additionally, in vertebrates melanin stored within different internal organs contains distinct chemical signature in the same specimen (Rossi *et al.* 2019). Diagenesis is proven to have a strong effect in the chemistry of fossils whether the preserved tissue is mineralized or not (e.g. Kohn *et al.* 1999; Xingyue *et al.* 2011; Colleary *et al.* 2015).

Although some elements in fossil insects may be derived, at least in part, from diagenetic processes (e.g. Cu (McNamara *et al.* In prep.; Rogers *et al.* 2021; Rossi *et al.* 2021)) and S (McNamara *et al.* 2016b)), these may still act as markers for certain pigments if they preferentially associate with specific binding sites that form in the pigment molecule during decay and/or diagenesis. Based on the distribution of certain elements in extant taxa, this study suggests an originally melanin-rich composition, especially in fossil Coleoptera, Hemiptera and Hymenoptera. In contrast, enrichment of light-toned cuticle regions in fossils in S, Ti and Fe in fossil Coleoptera, Hemiptera and Neuroptera and K and Ca in fossil Hymenoptera suggest an original composition rich in light colour producing pigments (e.g. carotenoids and flavonoids). In Coleoptera, original melanin-rich regions are enriched in K, Ca, Mn and Zn while dark toned regions in the fossils (presumably melanin-rich) are enriched in Zn, K and Ca. In Hemiptera, original melanin-rich regions are enriched in S, K and Ca while dark toned regions in fossil specimens (presumably melanin-rich) are enriched in Zn and K. In Hymenoptera, original melanin-rich regions are enriched in Cl, K, Ca and Fe while in dark toned regions (presumably melanin-rich) in fossil specimens are enriched in Fe and Ti.

Cu and Zn appear to be elements of great importance related to the spatial distribution of pigments in fossil insects, their ionisation energies are excessively

close (7.73 keV for Cu and 7.58 keV for Zn), thus minute differences can not be accounted for at the 11 keV excitation energy used for the analysis. Despite this, the fossil insects differ in chemistry to their respective host sediments, commonly the proportion of elements is higher in fossil samples (Supplementary Figs. 5.1 and 5.2), and show chemical variation between light- and dark-toned cuticle regions (Fig. 5.4 and Supplementary Figs. 5.2 and 5.9); however, when the data is grouped by formation the signal of the host sediment is not different than that of the fossils regardless of their origin or age (Fig. 5.4c, f, Supplementary Fig. 5.9a–d). This almost certainly reflects differences in the elemental composition of host sediments of the various formations sampled

## 6. CONCLUSION

The chemical variation in extant specimens reported here particularly at order and family levels precludes the possibility of chemically characterising pigment molecules in the fossil record of insects using XRF methods, for many taxa, thus **hypothesis 5 (*various elements or sets of elements are characteristic of distinct pigment molecules in the cuticle of modern insects prior to decay*) is accepted.** Elements present in fossils in much higher concentrations than in modern insects may reflect partial overprinting of the original chemistry by diagenetic processes. Alternatively, original chemical components may be retained in the fossils. Regardless of the original pigment content of the insect cuticles, this research suggests that the chemical signal associated with distinct coloured regions in the preserved cuticle of fossil insects is dependant on the chemical components of the host sediment, thus **hypothesis 7 (*distinctive trace elements or sets of elements can be used as biomarkers for the original pigmentary content in the cuticle of***

***fossil insects*) is rejected.** Nonetheless, the original pigmentary components of the cuticle could potentially be determined in specific circumstances where the combination of the diagenetic overprint and the chemical intra-taxonomical variation are minimal (e.g. melanin molecules in Odonata, also see Chapter 3).

## 7. FUTURE WORK

Future experimental studies will reveal how the intrinsic trace element inventory of cuticles of diverse insects alters during decay and diagenesis, thus providing tighter constraints on interpretations of the original colouration of fossil insects and its ecological functions through time.

**LIST OF FIGURES AND TABLES:**

**Fig. 5.1:** Synchrotron rapid-scanning X-ray fluorescence maps for selected elements and specimens.

**Fig. 5.2:** Linear Discriminant Analysis of concentrations of elements in the cuticle of extant insects.

**Fig. 5.3:** Linear Discriminant Analysis of concentrations of elements in the cuticle of extant insects grouped by family.

**Fig. 5.4:** Linear Discriminant Analysis of concentrations of elements in fossil insect cuticles and associated sedimentary matrix, grouped by fossil locality and order.

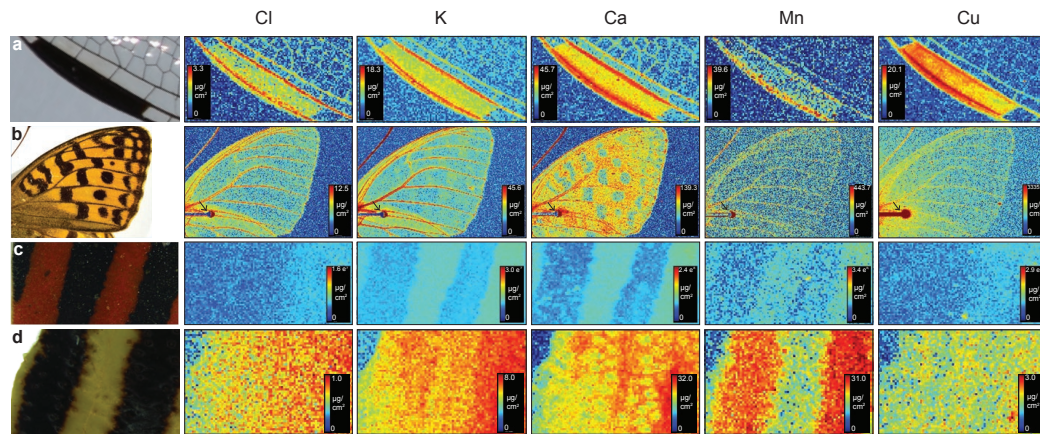
**Table 5.1:** Elements with high concentration and associated pigments and/or visual colours

**Table 5.2:** Elements showing spatial partitioning in extant samples analyzed using synchrotron-rapid scanning X-ray fluorescence.

**Table 5.3:** Elements showing spatial partitioning in fossil samples analyzed using synchrotron-rapid scanning X-ray fluorescence.

**Table 5.4:** Elements showing enrichment in distinct coloured regions of the cuticle of extant insects containing disparate pigment molecules as interpreted from multivariate analyses.

**Table 5.5:** Elements showing enrichment in distinct coloured regions of the cuticle of fossil insects containing presumably disparate pigment molecules as interpreted from multivariate analyses.



**Fig. 5.1.** Synchrotron rapid-scanning X-ray fluorescence maps for selected elements and specimens. **a** *Libellula quadrimaculata* (Odonata); **b** *Argynnis adippe* (Lepidoptera); **c** *Graphosoma lineatum* (Hemiptera); **d** *Leptinotarsa decemlineata* (Coleoptera). Black arrows in **b** indicate the specimen-mounting pin. Progressively higher concentrations towards the right-hand side of the maps in **c** and **d** are an artefact resulting from curvature of the cuticle. Scales: blue to red represents low to high concentration.



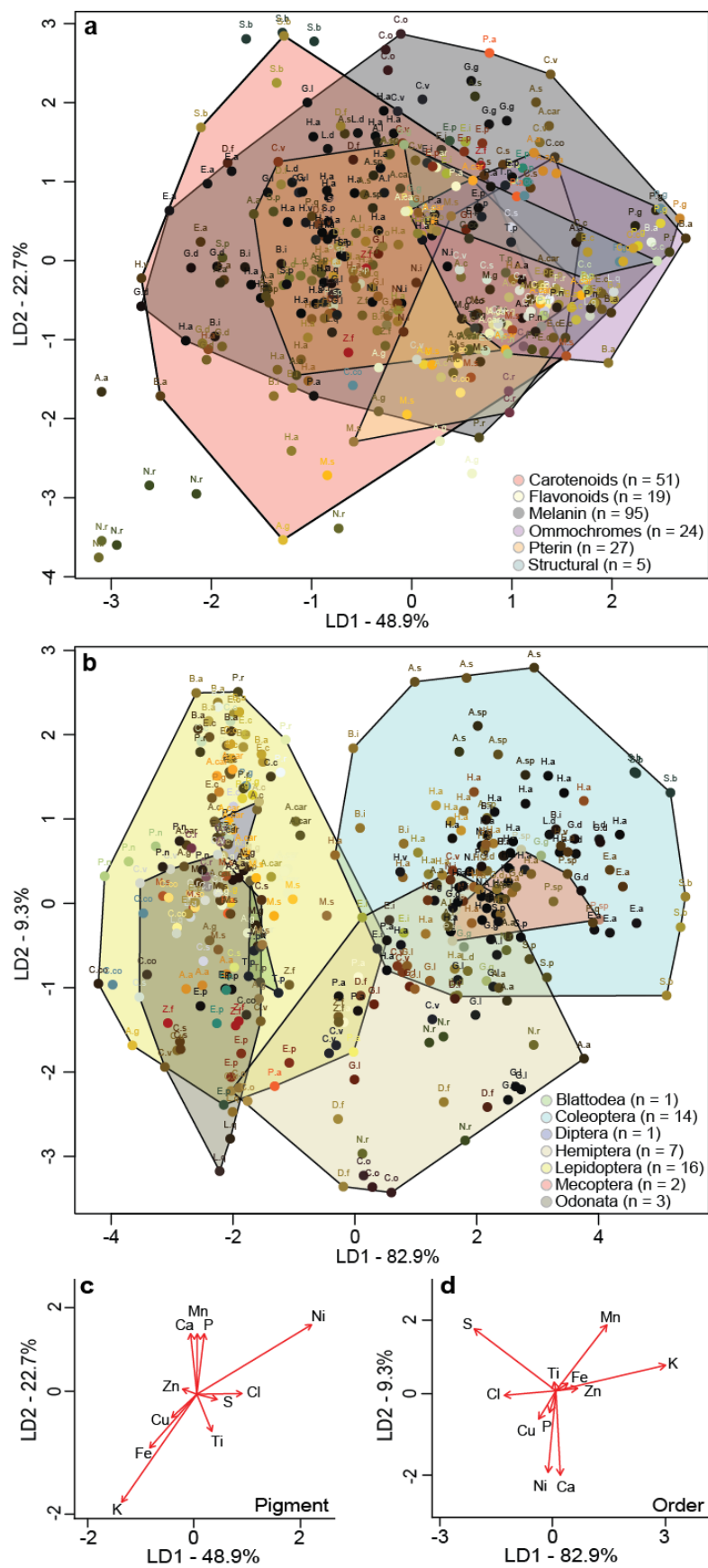


Figure 5.2. Caption overleaf.

**Fig. 5.2 (previous page). Linear Discriminant Analysis of concentrations of elements in the cuticle of extant insects. a, b** LDA plots of coloration mechanism (a) and order (b). **c–d** Biplot vectors showing the contribution of each element to variation in the dataset. A.a, *Argynnis adippe*; A.ac, *Aelia acuminata*; A.c, *Asilus crabroniformis*; A.car, *Anthocharis cardamines*; A.g, *Abraxas grossulariata*; A.l, *Aphodius luridus*; A.s, *Aphodius sticticus*; A.sp, *Aphodius* sp.; B.a, *Bicyclus anynana*; B.i, *Brachyta interrogationis*; C.c, *Cupido comyntas*; C.co, *Chalcosia coliadoideas*; C.o, *Cicciana obliqua*; C.r, *Colotis regina*; C.s, *Calopteryx splendens*; C.v, *Calopteryx virgo*; C.vu, *Cercopis vulnerata*; D.f, *Deselvana fulvofasciata*; E.a, *Epilachna argus*; E.c, *Epargyreus clarus*; E.i, *Evacanthus interruptus*; E.p, *Erasmia pulchella*; G.d, *Gonioctena decemnotata*; G.g, *Goliathus goliathus*; G.l, *Graphosoma lineatum*; H.a, *Harmonia axyridis*; H.v, *Hippodamia variegata*; L.d, *Leptinotarsa decemlineata*; L.q, *Libellula quadrimaculata*; M.s, *Macroglossum stellatarum*; M.g, *Melanargia galathea*; N.i, *Nicrophorus investigator*; N.r, *Nezara robusta*; P.a, *Parnassius apollonius*; P.g, *Papilio glaucus*; P.l, *Panorpa lugubris*; P.n, *Papilio nireus*; P.q, *Phyllobrotica quadrimaculata*; P.r, *Pieris rapae*; P.sp, *Panorpa* sp.; S.b, *Sternotomis bohemanii*; S.p, *Saperda populnea*; T.p, *Therea petiveriana*; Z.f, *Zygaena filipendulae*.

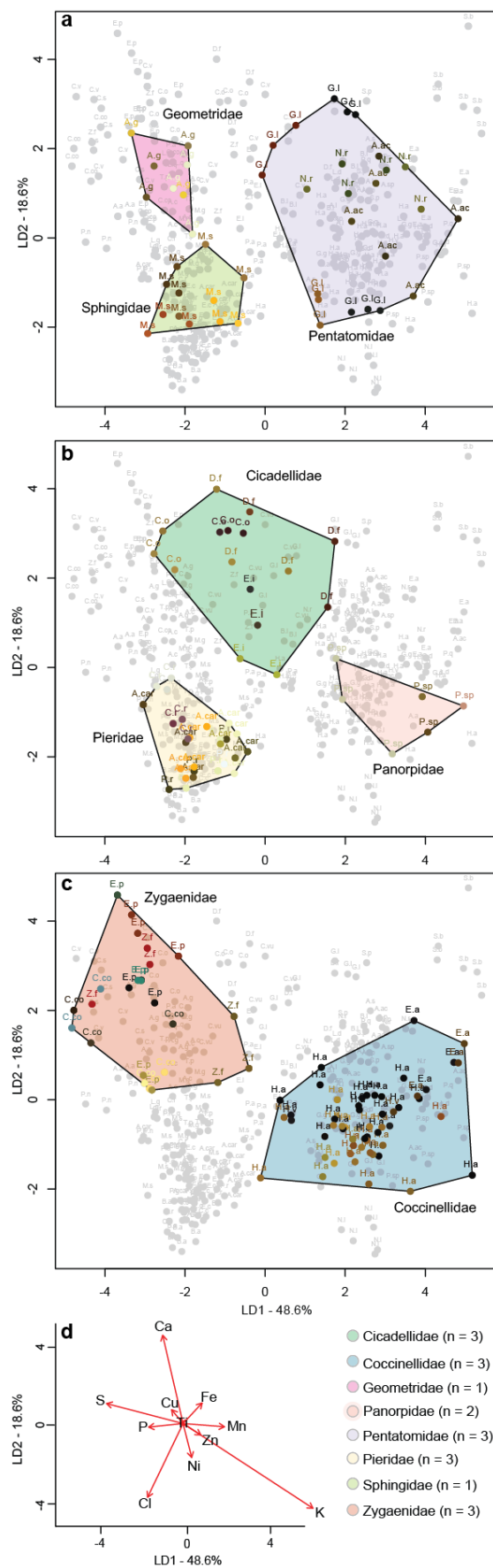
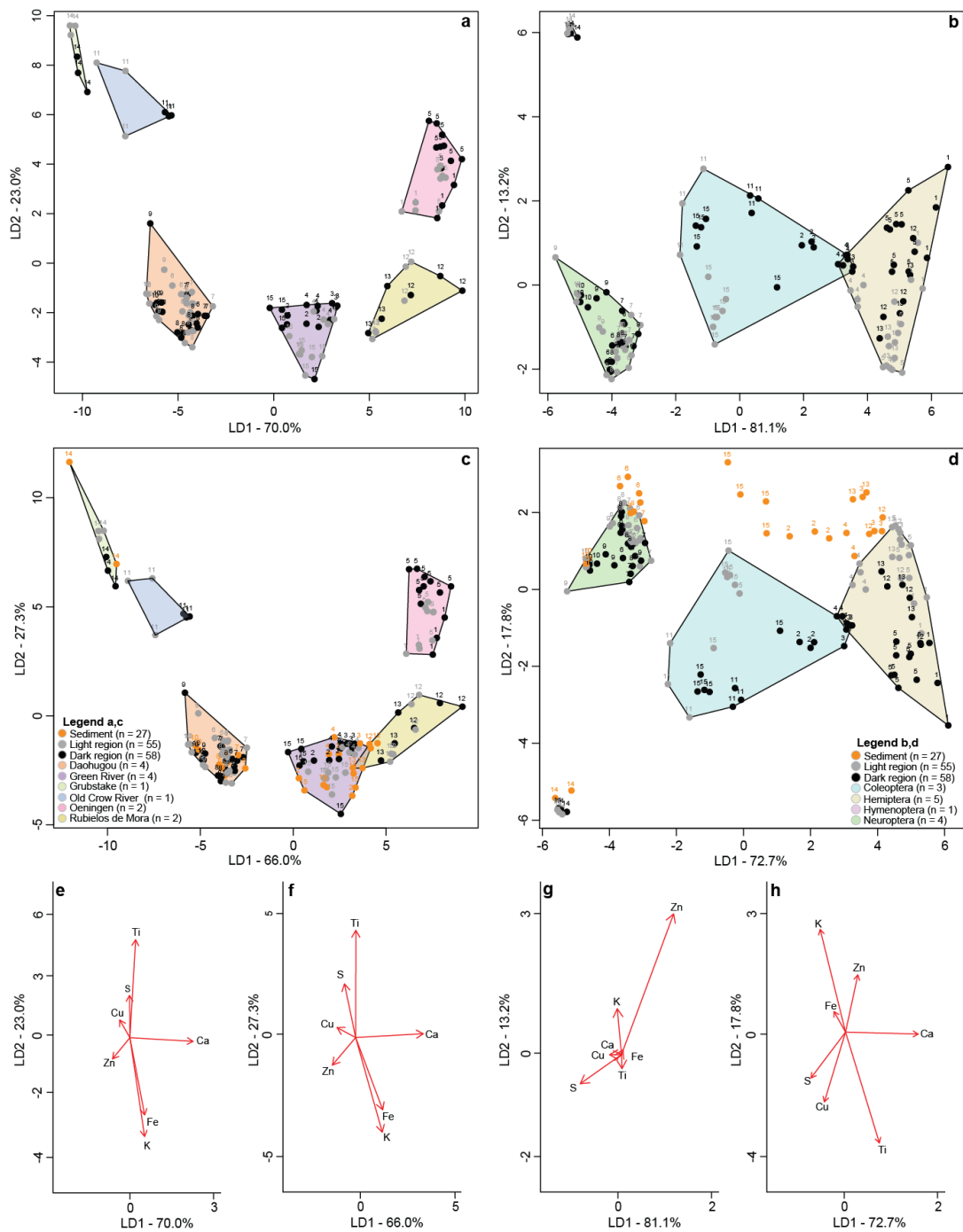


Figure 5.3. Caption overleaf.

**Fig. 5.3 (previous page). Linear Discriminant Analysis of concentrations of elements in the cuticle of extant insects, with data grouped by family. a** Cicadellidae, Geometridae and Sphingidae. **b** Panorpidae, Pentatomidae and Pieridae. **c** Coccinellidae and Zygaenidae. **d** Biplot vectors showing the contribution of each element to variation in the dataset. Abbreviations as per caption to Fig. 5.2.



**Fig. 5.4. Linear Discriminant Analysis of concentrations of elements in fossil insect cuticles and associated sedimentary matrix.** Data grouped by fossil locality (**a, c**; please see **c** for key) and order (**b, d**; please see **d** for key). Biplots in **e–h** correspond to LDA plots in **a–d**, respectively; vectors show the contribution of each element to variation in the dataset. 1, MCZ 14431; 2, YPM IP390330; 3, YPM IP454454; 4, YPM IP522483; 5, MCZ 14426; 6, NIGPAS NND11010; 7, NIGPAS NND11021-1; 8, NIGPAS NND11021-2; 9, NIGPAS NND11023; 10, NIGPAS NND140539; 11, OCB6; 12, RR1-12-07-10; 13, RR1-12-07-44; 14, UAF GS7; 15, UCM 51311a.

| taxon                             |    | concentration (µg/cm <sup>2</sup> ) |             |              |                         |                                                                |           |             |             |           |             |             |  |
|-----------------------------------|----|-------------------------------------|-------------|--------------|-------------------------|----------------------------------------------------------------|-----------|-------------|-------------|-----------|-------------|-------------|--|
|                                   |    | P                                   | S           | Cl           | K                       | Ca                                                             | Ti        | Mn          | Fe          | Ni        | Cu          | Zn          |  |
| Lepidoptera                       |    |                                     |             |              |                         |                                                                |           |             |             |           |             |             |  |
| Sphingidae                        |    |                                     |             |              |                         |                                                                |           |             |             |           |             |             |  |
| <i>Macroglossum stellatarum</i>   | a  |                                     |             |              |                         |                                                                |           |             |             |           |             |             |  |
| Pieridae                          |    |                                     |             |              |                         |                                                                |           |             |             |           |             |             |  |
| <i>Anthocharis cardamines</i>     | b  |                                     |             |              | P - orange<br>O - white |                                                                |           |             |             |           |             |             |  |
| <i>Pieris rapae</i>               | c  |                                     |             |              |                         |                                                                |           |             |             |           |             |             |  |
| <i>Colotis regina</i>             | d  |                                     |             |              |                         | S - blue                                                       |           |             |             |           |             |             |  |
| Geometridae                       |    |                                     |             |              |                         |                                                                |           |             |             |           |             |             |  |
| <i>Abraxas grossulariata</i>      | e  |                                     |             |              |                         |                                                                |           |             |             |           |             |             |  |
| Nymphalidae                       |    |                                     |             |              |                         |                                                                |           |             |             |           |             |             |  |
| <i>Bicyclus anynana</i>           | f  |                                     |             |              |                         | O - brown<br>C - orange<br>O - orange                          |           |             |             |           |             |             |  |
| <i>Melanargia galathea</i>        | g  |                                     |             |              |                         |                                                                |           |             |             |           |             |             |  |
| <i>Argynnis adippe</i>            | h  |                                     |             |              | M - brown               |                                                                |           |             |             |           |             |             |  |
| <i>Aglais urticae</i>             | i  |                                     |             |              |                         | S - blue                                                       |           |             |             |           |             |             |  |
| Lycaenidae                        |    |                                     |             |              |                         |                                                                |           |             |             |           |             |             |  |
| <i>Cupido comyntas</i>            | j  |                                     |             |              |                         |                                                                |           |             |             |           |             |             |  |
| Hesperiidae                       |    |                                     |             |              |                         |                                                                |           |             |             |           |             |             |  |
| <i>Epargyreus clarus</i>          | k  |                                     |             |              |                         |                                                                |           |             |             |           |             |             |  |
| Papilionidae                      |    |                                     |             |              |                         |                                                                |           |             |             |           |             |             |  |
| <i>Parnassius apollonius</i>      | l  |                                     |             |              |                         | M - black<br>O - blue<br>O - green                             |           |             |             |           |             |             |  |
| <i>Papilio glaucus</i>            | m  |                                     |             |              |                         |                                                                |           |             |             |           |             |             |  |
| <i>Papilio nireus</i>             | n  |                                     | O - green   |              | O - green               | O - green                                                      |           |             |             |           |             |             |  |
| Zygaenidae                        |    |                                     |             |              |                         |                                                                |           |             |             |           |             |             |  |
| <i>Zygaena filipendulae</i>       | o  |                                     | C - red     |              | C - red                 | C - red                                                        |           |             |             |           |             |             |  |
| <i>Erasmia pulchella</i>          | p  |                                     |             | N/A - orange |                         | F - brown<br>N/A - orange<br>S - blue<br>F - brown<br>S - blue |           |             |             |           |             |             |  |
| <i>Chalcocis coliadoides</i>      | q  |                                     |             |              |                         |                                                                |           |             |             |           |             |             |  |
| Coleoptera                        |    |                                     |             |              |                         |                                                                |           |             |             |           |             |             |  |
| Scarabaeidae                      |    |                                     |             |              |                         |                                                                |           |             |             |           |             |             |  |
| <i>Goliathus goliathus</i>        | r  |                                     |             | S - white    |                         | S - white                                                      | S - white |             |             | S - white |             |             |  |
| <i>Aphodius sp.</i>               | s  |                                     |             |              |                         |                                                                |           | N/A - black |             |           |             |             |  |
| <i>Aphodius sticticus</i>         | t  |                                     |             |              |                         |                                                                |           |             |             |           |             |             |  |
| <i>Aphodius luridus</i>           | u  |                                     |             |              |                         |                                                                |           |             |             |           |             |             |  |
| <i>Trichius sp.</i>               | v  |                                     |             |              |                         |                                                                |           | M - black   |             |           |             |             |  |
| <i>Polyphylla fullo</i>           | w  |                                     |             |              |                         |                                                                |           |             |             |           |             |             |  |
| Coccinellidae                     |    |                                     |             |              |                         |                                                                |           |             |             |           |             |             |  |
| <i>Harmonia axyridis</i>          | x  |                                     |             |              |                         | M - black                                                      |           |             |             |           |             |             |  |
| <i>Epilachna argus</i>            | y  |                                     |             |              | M - black               | M - black                                                      |           |             |             |           |             |             |  |
| <i>Hippodamia variegata</i>       | z  |                                     |             |              |                         |                                                                |           |             |             |           |             |             |  |
| Cerambycidae                      |    |                                     |             |              |                         |                                                                |           |             |             |           |             |             |  |
| <i>Brachyta interrogationis</i>   | aa |                                     |             |              |                         |                                                                |           | N/A - black |             |           |             |             |  |
| <i>Saperda populnea</i>           | ab |                                     |             |              |                         | N/A - green                                                    |           |             |             |           |             |             |  |
| <i>Sternotomis bohemani</i>       | ac | C - yellow                          | C - yellow  | C - yellow   | C - yellow              | C - yellow                                                     |           |             |             |           |             |             |  |
| Cerambycidae indet.               | ad |                                     |             |              |                         |                                                                |           |             |             |           |             | N/A - black |  |
| Chrysomelidae                     |    |                                     |             |              |                         |                                                                |           |             |             |           |             |             |  |
| <i>Leptinotarsa decemlineata</i>  | ae |                                     |             |              | M-black                 |                                                                |           | M - black   |             |           |             |             |  |
| <i>Goniocenta decemnotata</i>     | af |                                     |             |              |                         |                                                                |           | M-black     |             |           |             |             |  |
| <i>Phyllotreta quadrimaculata</i> | ag |                                     |             |              |                         |                                                                |           |             |             |           |             |             |  |
| Silphidae                         |    |                                     |             |              |                         |                                                                |           |             |             |           |             |             |  |
| <i>Nicrophorus investigator</i>   | ah |                                     |             |              |                         |                                                                |           |             |             |           |             |             |  |
| Carabidae                         |    |                                     |             |              |                         |                                                                |           |             |             |           |             |             |  |
| <i>Cicindela hybrida</i>          | ai |                                     |             |              |                         |                                                                |           |             |             |           |             |             |  |
| Blattodea                         |    |                                     |             |              |                         |                                                                |           |             |             |           |             |             |  |
| Corydiidae                        |    |                                     |             |              |                         |                                                                |           |             |             |           |             |             |  |
| <i>Therea petiveriana</i>         | aj |                                     |             |              |                         | N/A - white                                                    |           |             |             |           |             |             |  |
| Hemiptera                         |    |                                     |             |              |                         |                                                                |           |             |             |           |             |             |  |
| Cercopidae                        |    |                                     |             |              |                         |                                                                |           |             |             |           |             |             |  |
| <i>Cercopis vulnerata</i>         | ak | N/A - black                         | N/A - black | N/A - black  | N/A - black             | N/A - black                                                    |           |             | N/A - black |           | N/A - black | N/A - black |  |
| Pentatomidae                      |    |                                     |             |              |                         |                                                                |           |             |             |           |             |             |  |
| <i>Graphosoma lineatum</i>        | al | M - black                           |             |              | M - black               | M - black                                                      |           | M - black   |             |           |             |             |  |
| <i>Aelia acuminata</i>            | am |                                     |             |              |                         |                                                                |           |             |             |           |             |             |  |
| <i>Nezara robusta</i>             | an |                                     |             |              |                         |                                                                |           |             |             |           |             |             |  |
| Cicadellidae                      |    |                                     |             |              |                         |                                                                |           |             |             |           |             |             |  |
| <i>Evacanthus interruptus</i>     | ao |                                     |             |              |                         |                                                                |           | M - brown   |             |           |             |             |  |
| <i>Deselvana fulvofasciata</i>    | ap |                                     |             | N/A - yellow | N/A - yellow            | N/A - yellow                                                   |           | M - brown   |             |           |             |             |  |
| <i>Cicciana obliqua</i>           | aq | M - brown                           |             |              | M - brown               | M - brown                                                      | M - brown | M - brown   |             | M - brown | M - brown   | M - brown   |  |

|                                 |    |           |           |           |             |           |           |           |           |             |           |           |              |
|---------------------------------|----|-----------|-----------|-----------|-------------|-----------|-----------|-----------|-----------|-------------|-----------|-----------|--------------|
| <b>Mecoptera</b>                |    |           |           |           |             |           |           |           |           |             |           |           |              |
| <b>Panorpidae</b>               |    |           |           |           |             |           |           |           |           |             |           |           |              |
| <i>Panora sp.</i>               | ar |           |           | M - brown | M - brown   | M - brown |           | M - brown |           |             |           | M - brown |              |
| <i>Panorpa lugubris</i>         | as |           |           |           | M - brown   | M - brown |           |           |           |             |           |           |              |
| <b>Diptera</b>                  |    |           |           |           |             |           |           |           |           |             |           |           |              |
| <b>Asilidae</b>                 |    |           |           |           |             |           |           |           |           |             |           |           |              |
| <i>Asilus crabroniformis</i>    | at |           |           |           |             |           |           | M - brown |           |             |           |           |              |
| <b>Hymenoptera</b>              |    |           |           |           |             |           |           |           |           |             |           |           |              |
| <b>Vespidae</b>                 |    |           |           |           |             |           |           |           |           |             |           |           |              |
| <i>Odynerus gracilis</i>        | au |           |           |           | N/A - brown |           |           |           |           | N/A - brown |           |           |              |
| <b>Andrenidae</b>               |    |           |           |           |             |           |           |           |           |             |           |           |              |
| <i>Andrena carantonica</i>      | av |           |           |           | M - brown   |           |           |           |           |             |           | M - brown | M - brown    |
| <b>Odonata</b>                  |    |           |           |           |             |           |           |           |           |             |           |           |              |
| <b>Calopterygidae</b>           |    |           |           |           |             |           |           |           |           |             |           |           |              |
| <i>Calopteryx virgo</i>         | aw |           |           |           |             |           |           | M - brown |           |             |           |           |              |
|                                 |    |           |           |           |             |           |           | S - brown |           |             |           |           |              |
| <i>Calopteryx splendens</i>     | ax |           |           |           |             |           |           | M - brown |           |             |           |           |              |
|                                 |    |           |           |           |             |           |           | S - brown |           |             |           |           |              |
| <b>Libellulidae</b>             |    |           |           |           |             |           |           |           |           |             |           |           |              |
| <i>Libellula quadrimaculata</i> | ay | M - brown | M - brown | M - brown | M - brown   | M - brown |           | M - brown | M - brown |             | M - brown | M - brown |              |
|                                 |    | S - brown | S - brown | S - brown | S - brown   | S - brown |           | S - brown | S - brown |             | S - brown | S - brown |              |
| <i>Sympetrum flaveolum</i>      | az | M - brown | M - brown | M - brown | M - brown   | M - brown |           |           |           |             |           |           |              |
|                                 |    | S - brown | S - brown | S - brown | S - brown   | S - brown |           |           |           |             |           |           |              |
| <b>Coenagrionidae</b>           |    |           |           |           |             |           |           |           |           |             |           |           |              |
| <i>Pyrrhosoma nymphula</i>      | ba | M - brown | M - brown | M - brown | M - brown   | M - brown |           |           | M - brown |             | M - brown | M - brown |              |
|                                 |    | S - brown | S - brown | S - brown | S - brown   | S - brown |           |           | S - brown |             | S - brown | S - brown |              |
| <b>Aeshnidae</b>                |    |           |           |           |             |           |           |           |           |             |           |           |              |
| <i>Aeshna caerulea</i>          | bb |           |           |           | M - brown   | M - brown |           |           | M - brown |             | M - brown | M - brown |              |
|                                 |    |           |           |           | S - brown   | S - brown |           |           | S - brown |             | S - brown | S - brown |              |
| <b>Corduliidae</b>              |    |           |           |           |             |           |           |           |           |             |           |           |              |
| <i>Cordulia aenea</i>           | bc | M - brown | M - brown | M - brown | M - brown   | M - brown | M - brown | M - brown | M - brown | M - brown   | M - brown | M - brown |              |
|                                 |    | S - brown | S - brown | S - brown | S - brown   | S - brown | S - brown | S - brown | S - brown | S - brown   | S - brown | S - brown |              |
| <b>total partitioning</b>       |    | 8         | 8         | 10        | 20          | 29        | 3         | 11        | 7         | 2           | 7         | 9         |              |
| <b>strong partitioning</b>      |    | 0         | 2         | 4         | 12          | 18        | 0         | 2         | 4         | 0           | 4         | 5         |              |
| <b>Carotenoids</b>              |    | 1         | 2         | 1         | 2           | 3         | 0         | 0         | 0         | 0           | 0         | 0         | <b>Total</b> |
| <b>Flavonoids</b>               |    | 0         | 0         | 0         | 0           | 2         | 0         | 0         | 0         | 0           | 0         | 0         | 9            |
| <b>Melanins</b>                 |    | 6         | 4         | 5         | 13          | 15        | 2         | 9         | 4         | 2           | 6         | 0         | 2            |
| <b>Ommochromes</b>              |    | 0         | 1         | 0         | 2           | 4         | 0         | 0         | 0         | 0           | 0         | 7         | 66           |
| <b>Pterins</b>                  |    | 0         | 0         | 0         | 1           | 0         | 0         | 0         | 0         | 0           | 0         | 0         | 14           |
| <b>Not described</b>            |    | 1         | 1         | 3         | 3           | 5         | 0         | 2         | 2         | 0           | 1         | 2         | 1            |
| <b>Structural</b>               |    | 4         | 4         | 5         | 4           | 12        | 2         | 2         | 5         | 1           | 4         | 4         | 20           |
|                                 |    |           |           |           |             |           |           |           |           |             |           |           | 47           |

C - carotenoids  
 F - flavonoids  
 M - melanins  
 O - ommochromes  
 P - pterins  
 N/A - not described pigment  
 S - structural  
 Strong partitioning  
 Weak partitioning

**Table 5.1 (previous page). Elements with high concentration and associated pigments and/or visual colours.**



| taxon                 | colour mechanisms                                 | trace elements showing partitioning  |
|-----------------------|---------------------------------------------------|--------------------------------------|
| <b>Lepidoptera</b>    |                                                   |                                      |
| <b>Sphingidae</b>     | carotenoids and flavonoids                        | none                                 |
| <b>Pieridae</b>       | melanins, pterins, flavonoids and carotenoids     | Cl, K, Ca,                           |
| <b>Geometridae</b>    | melanins and carotenoids                          | K                                    |
| <b>Nymphalidae</b>    | melanins, carotenoids, flavonoids and ommochromes | K, Ca, Fe                            |
| <b>Lycaenidae</b>     | carotenoids, flavonoids and structural colour     | Ca                                   |
| <b>Hesperiidae</b>    | flavonoids                                        | K, Ca                                |
| <b>Papilionidae</b>   | melanins, carotenoids, flavonoids and ommochromes | S, Cl, K, Ca                         |
| <b>Zygaenidae</b>     | carotenoids and flavonoids                        | S, Cl, K, Ca                         |
| <b>Coleoptera</b>     |                                                   |                                      |
| <b>Scarabaeidae</b>   | melanins and structural colour                    | Cl, K, Ca, Ti, Fe                    |
| <b>Coccinellidae</b>  | melanins and carotenoids                          | K, Ca, Mn, Fe, Zn                    |
| <b>Cerambycidae</b>   | carotenoids                                       | K, Ca, Mn, Zn                        |
| <b>Chrysomelidae</b>  | melanins and carotenoids                          | K, Ca, Mn, Zn                        |
| <b>Silphidae</b>      | AKH                                               | Ca, Zn                               |
| <b>Carabidae</b>      | melanins                                          | none                                 |
| <b>Blattodea</b>      |                                                   |                                      |
| <b>Corydiidae</b>     | AKH                                               | Ca                                   |
| <b>Hemiptera</b>      |                                                   |                                      |
| <b>Cercopidae</b>     | pterins                                           | P, S, Cl, K, Ca, Fe                  |
| <b>Pentatomidae</b>   | AKH, pterins                                      | K, Ca, Mn                            |
| <b>Cicadellidae</b>   | melanins                                          | S, K, Ca, Zn                         |
| <b>Mecoptera</b>      |                                                   |                                      |
| <b>Panorpidae</b>     | melanins and pterins                              | P, Cl, K, Ca, Ti, Mn, Fe, Ni, Cu, Zn |
| <b>Diptera</b>        |                                                   |                                      |
| <b>Asilidae</b>       | melanins and AKH                                  | Cl, K, Ca, Mn                        |
| <b>Hymenoptera</b>    |                                                   |                                      |
| <b>Vespidae</b>       | pterins                                           | K, Ca, Fe                            |
| <b>Andrenidae</b>     | melanins                                          | K, Ca, Cu, Zn                        |
| <b>Odonata</b>        |                                                   |                                      |
| <b>Calopterygidae</b> | melanins, AKH and structural colour               | P, S, Cl, K, Ca, Mn, Fe, Cu, Zn      |
| <b>Libellulidae</b>   | melanins, AKH and structural colour               | P, S, Cl, K, Ca                      |
| <b>Coenagrionidae</b> | melanins, AKH and structural colour               | P, S, Cl, K, Ca, Mn, Fe, Cu, Zn      |
| <b>Aeshnidae</b>      | melanins and structural colour                    | K, Ca, Fe, Cu, Zn                    |
| <b>Corduliidae</b>    | melanins and structural colour                    | P, S, Cl, K, Ca, Mn, Fe, Ni, Cu, Zn  |

**Table 5.2. Elements showing spatial partitioning in extant samples analyzed using synchrotron-rapid scanning X-ray fluorescence.** AKH, adipokinetic hormone/red pigment-concentrating hormone.

| <b>taxon</b>          | <b>elements associated to partitioning</b> |
|-----------------------|--------------------------------------------|
| <b>Hemiptera</b>      |                                            |
| <b>RR1-12-07-10</b>   | Cu, Zn                                     |
| <b>RR1-12-07-44</b>   | Cu, Zn                                     |
| <b>YPM IP522483</b>   | Ti, Cu, Zn                                 |
| <b>MCZ14431</b>       | Ti, Fe, Cu                                 |
| <b>MCZ14426</b>       | Cu                                         |
| <b>Coleoptera</b>     |                                            |
| <b>YPM IP454454</b>   | none                                       |
| <b>YPM IP390330</b>   | Cu, Ca                                     |
| <b>UCM_51311a</b>     | Cu                                         |
| <b>OCB6</b>           | Ca, Ti, Fe, Cu, Zn                         |
| <b>Neuroptera</b>     |                                            |
| <b>CNU NND11021-1</b> | S, Ca, Fe, Cu, Zn                          |
| <b>CNU NND11021-2</b> | K, Ca, Fe, Cu                              |
| <b>CNU NND11010</b>   | K, Ca, Ti, Fe, Cu, Zn                      |
| <b>CNU NND11023</b>   | K, Ca, Cu, Zn                              |
| <b>CNU NND140539</b>  | K, Ca, Fe, Cu, Zn                          |
| <b>Hymenoptera</b>    |                                            |
| <b>UAF_GS7</b>        | Ti, Cu, Zn                                 |

**Table 5.3. Elements showing spatial partitioning in fossil samples analyzed using synchrotron-rapid scanning X-ray fluorescence.**

| taxon                 | Melanin                                           | Melanin    | Carotenoids | Flavonoids | Pterins   | Ommochromes | Unidentified | Colourless |
|-----------------------|---------------------------------------------------|------------|-------------|------------|-----------|-------------|--------------|------------|
| <b>Lepidoptera</b>    |                                                   |            |             |            |           |             |              |            |
| <b>Sphingidae</b>     | carotenoids and flavonoids                        | Ca, S      | Cl, P       | K          |           |             |              |            |
| <b>Pieridae</b>       | melanins, pterins, flavonoids and carotenoids     | Cl, P, S   |             |            |           |             | K, Mn, Ca    |            |
| <b>Geometridae</b>    | melanins and carotenoids                          | Cl, P, S   |             |            |           |             | K, Mn, Ca    |            |
| <b>Nymphalidae</b>    | melanins, carotenoids, flavonoids and ommochromes | Ca, Fe     | Ca, S       |            | S         |             |              |            |
| <b>Lycaenidae</b>     | carotenoids, flavonoids and structural colour     | Ca, P      |             | Mn         |           |             |              |            |
| <b>Hesperiidae</b>    | flavonoids                                        |            |             | Ca, S      |           |             | Cl, P        |            |
| <b>Papilionidae</b>   | melanins, carotenoids, flavonoids and ommochromes | S, Mn, P   | Ca, Cl      |            |           | K, Mn       |              |            |
| <b>Zygaenidae</b>     | carotenoids and flavonoids                        | S, P       | Ca, S, P    |            | K, Cl, Ni |             |              |            |
| <b>Coleoptera</b>     |                                                   |            |             |            |           |             |              |            |
| <b>Scarabaeidae</b>   | melanins and structural colour                    | Cl, P      |             |            |           |             |              |            |
| <b>Coccinellidae</b>  | melanins and carotenoids                          | Ca, Mn, Fe | Cl, Ni, P   |            |           |             |              |            |
| <b>Cerambycidae</b>   | carotenoids                                       | Cl, P      | Fe, S       |            |           |             |              |            |
| <b>Chrysomelidae</b>  | melanins and carotenoids                          | K, Mn, Fe  | Cl, Ca, S   |            |           |             |              |            |
| <b>Silphidae</b>      | AKH                                               |            |             |            |           |             | Cl, S        |            |
| <b>Carabidae</b>      | melanins                                          | P, Cl      |             |            |           |             | S, Fe        |            |
| <b>Blattodea</b>      |                                                   |            |             |            |           |             |              |            |
| <b>Corydidae</b>      | AKH                                               | Ca, S      |             |            |           |             | K, Cl, Ni    |            |
| <b>Hemiptera</b>      |                                                   |            |             |            |           |             |              |            |
| <b>Cercopidae</b>     | pterins                                           | Cl, P, K   |             |            | Ca, Fe    |             |              |            |
| <b>Pentatomidae</b>   | AKH, pterins                                      | Ca, K      |             |            |           |             | Mn, S, Cl    |            |
| <b>Cicadellidae</b>   | melanins                                          | Ca, Mn     |             |            |           |             | K            |            |
| <b>Mecoptera</b>      |                                                   |            |             |            |           |             |              |            |
| <b>Panorpidae</b>     | melanins and pterins                              | Mn, K      |             |            | Ca, S     |             |              |            |
| <b>Diptera</b>        |                                                   |            |             |            |           |             |              |            |
| <b>Asilidae</b>       | melanins and AKH                                  | Ca, S      |             |            |           |             | K, Cl, Ca    |            |
| <b>Hymenoptera</b>    |                                                   |            |             |            |           |             |              |            |
| <b>Vespidae</b>       | pterins                                           | S, Fe, Cu  |             |            | Ca, Ti    |             |              |            |
| <b>Andrenidae</b>     | melanins                                          | K, Zn      |             |            |           |             |              | Cu, S      |
| <b>Odonata</b>        |                                                   |            |             |            |           |             |              |            |
| <b>Calopterygidae</b> | melanins, AKH and structural colour               | Ca, Mn, Fe |             |            |           |             | Cl, P        |            |
| <b>Libellulidae</b>   | melanins, AKH and structural colour               | Ca, Cu     |             |            |           |             | Cl           |            |
| <b>Coenagrionidae</b> | melanins, AKH and structural colour               | Cl, Ca, Ni |             |            |           |             | Fe, S        |            |
| <b>Aeshnidae</b>      | melanins and structural colour                    | Ca, Cu     |             |            |           |             | Fe, Ti       |            |
| <b>Corduliidae</b>    | melanins and structural colour                    | S, Fe      |             |            |           |             | P, Cl, Ca    |            |

**Table 5.4. Elements showing enrichment in distinct coloured regions of the cuticle of extant insects containing disparate pigment molecules as interpreted from multivariate analyses.**

| taxon             | dark       | cuticle |               | Sediment  |
|-------------------|------------|---------|---------------|-----------|
|                   |            | light   | no patterning |           |
| Hemiptera         |            |         |               |           |
| RR1-12-07-10      | Cu, Zn     | Zn, K   |               | K, Zn, Fe |
| RR1-12-07-44      | Cu, Zn     | Zn, K   |               | K, Zn, Fe |
| YPM IP522483      | Cu, Ti, Zn | Ca      |               | K, Zn, Fe |
| MCZ14431          | Ti, Cu     | Zn, K   |               | K, Zn, Fe |
| MCZ14426          | Ti, Cu     | Zn, K   |               | K, Zn, Fe |
| Coleoptera        |            |         |               |           |
| YPM IP454454      |            |         | Ti            | K, Zn, Fe |
| YPM IP390330      |            |         | Ti            | K, Zn, Fe |
| UCM_51311a        | Cu, Zn     | Fe      |               | K, Zn, Fe |
| OCB6              | Ca, Ti     | Fe, K   |               | K, Zn, Fe |
| Neuroptera        |            |         |               |           |
| NIGPAS NND11021-1 | Cu, Ca, Ti | Zn, K   |               | K, Zn, Fe |
| NIGPAS NND11021-2 | Cu, Ca, Ti | Zn, K   |               | K, Zn, Fe |
| NIGPAS NND11010   | Cu, Ca, Ti | Zn, K   |               | K, Zn, Fe |
| NIGPAS NND11023   | Cu, Ca, Ti | Zn, K   |               | K, Zn, Fe |
| NIGPAS NND140539  | Cu, Ca, Ti | Zn, K   |               | K, Zn, Fe |
| Hymenoptera       |            |         |               |           |
| UAF GS7           | Ca, Ti Zn  | Cu, S   |               | K, Zn, Fe |

**Table 5.5. Elements showing enrichment in distinct coloured regions of the cuticle of fossil insects containing presumably disparate pigment molecules as interpreted from multivariate analyses.**

## CHAPTER 6

# Synthesis

---

### 1. INTRODUCTION

Insects are, without a doubt, the most successful group of animals to inhabit this planet (Labandeira and Sepkoski 1993; Mayhew 2007). They are colonisers of virtually every terrestrial niche in extant environments (Erwin 1991); this accomplishment is similarly evident in the fossil record, which has transformed insects into ideal environmental indicators in fossil settings (Mayhew 2007). Their success as a group can be traced to a series of morphological adaptations, which gave them valuable advantages over their competitors (Duncan 1997; Mayhew 2007). Perhaps the most remarkable adaptation is the variation of colourful patterning in their cuticles, which aids in intra- and interspecific communication (see Chapter 1). The evolution of pigmentation of cuticles not only opened the door to the biosynthesis of new pigment molecules in insects, but to new methods of acquisition and storage of pigment molecules produced by majorly plants but also other animals.

The fossil record of soft-bodied organisms was often considered largely incomplete and poorly understood in terms of fossilisation pathways. With novel and critical techniques applied to all types of Konservat-Lagerstätten throughout the last two decades (Briggs and McMahon 2016; Purnell *et al.* 2018), paleontological workers have been more and more interested in analysing these

unique “windows to the past”. Konservat-Lagerstätten offer not only exceptional taxonomical details of organisms otherwise never seen, but irreplaceable chemical information which can inform us of biological circumstances never thought to be possible. Although soft-bodied organisms, insects possess an excellent fossil record (Labandeira and Sepkoski 1993), especially those taxa with widespread and greater sclerotisation (see chapter 3). In addition, the taphonomical conditions necessary for the preservation of members of this group and individual types of fossilisation have been extensively researched and constrained in the past (e.g. Martínez-Delclòs and Martínell 1993; Duncan 1997; McCobb *et al.* 1998; Martínez-Delclòs *et al.* 2004).

This thesis represents the first taphonomic study of the potential for preservation of various pigments stored in the cuticle of several orders of insects encompassing chemical and mechanical decay of insect cuticle and trace element distribution in the cuticle of extant and fossil insects. Experiments and techniques for analysis used to answer these key issues were varied; accordingly, the results of this thesis are varied with each chapter focussing on a different topic resolved for the taphonomy of pigmentary colours in insects. The experiments in chapter 3 examine the decay conditions and rate of decay of five species of insects (*H. axyridis*, *L. decemlineata*, *G. goliathus*, *T. velutinus* and *P. apterus*) with two distinct pigments spatially distributed in their cuticles and relate the changes in chemistry and ultrastructure of the cuticle to biochemical changes that occurred during decay. The experiments in chapter 4 scrutinise the rate of mechanical decay and connection to spatial distribution of pigment content in the cuticles of experimentally decayed samples of *H. axyridis*. Collectively, chapters 3 and 4 uncover important environmental and biological biases acting in the fossilisation of

potentially all insect taxa and by extension other animal groups with similar morphological characteristics. In chapter 5, characterisation of trace element chemistry associated with spatial distribution of various pigments in the cuticle of 68 extant taxa is compared to trace element chemistry in the cuticle of 13 fossil taxa from six famous fossil localities. These analyses uncover a series of possible elemental markers characterising the appearance of previously undescribed pigments in the fossil record of insects. While the results of this thesis are applicable to fossil insects, provide a valuable first attempt on the aspects of taphonomical processes involved in the preservation of pigments with the possibility of extrapolation to other animal groups.

## **2. DECAY: COLOUR CHANGE AND TRANSPORT**

### **2.1. Introduction**

Experimental decay of soft-bodied animals has been extensively explored by other authors (e.g. Allison 1986; McCobb *et al.* 1998; Duncan *et al.* 2003; McCoy *et al.* 2018). Previous publications have concentrated their efforts in describing morphological changes in the insect's body during decay and transportation, though, geochemical variations in certain molecules have also been analyzed, the degradation of pigments contained in the cuticle has never been investigated. This is the first study concentrating efforts in analysing and describing decay related to pigment content in the cuticle (chapters 3 and 4).

### **2.2. Conditions of chemical decay**

The pH of the media of decay has been considered an indirect control of decay for various decades (Allison 1988; Wilby *et al.* 1996). Acidic media were thought to

retard decay due to the early mineralisation of certain authigenic minerals (Briggs and Wilby 1996; Briggs 2003). In chapter 3, processes involved in the decay of individual cuticles of five patterned insect taxa is explored. The experimental media had introduced double distilled water, which was adjusted to desired pH by addition of potassium hydroxide (KOH) and hydrochloric acid (HCl). Decay occurred at a constant  $25^{\circ}\text{C}\pm 1^{\circ}\text{C}$  in the dark. In this circumstances, and without sediment introduced, samples decayed in basic conditions were observed to degrade slower than those samples in acidic conditions.

### **2.3. Microstructural decay in the cuticle**

During decay processes, arthropod cuticle has been found to loose thickness and interconnecting protein matrix amongst chitin bundles in the endocuticle (Briggs and Kear 1994; Gupta *et al.* 2009). Qualitative ultrastructural loss of protein matrix is documented in chapter 3. The rate of internal cuticle degradation in samples decayed in basic conditions is higher than those samples decayed in acidic conditions. In addition, dark regions of the cuticle (melanin-rich) degrade at a slower rate than lighter regions of the cuticle (non melanin-rich).

### **2.4. Changes in visual colour**

Visual colour of the five species of insects analyzed was altered during decay. This observation was never accomplished before in any other experimental decay study, at least that the author is aware of. Melanin-rich regions of the cuticle suffered virtually no alteration in their visual colour, while regions of the cuticle containing other pigments aside of melanin were visibly altered after 14 days of decay (Chapter 3).



## 2.5. Decay of biomolecules

Analysis of biomolecules contained in the cuticle of extant arthropods, and insects in particular, have been previously comprehensively researched by other authors (e.g. Andersen 1979; Blomquist *et al.* 1987; Andersen 2009b; Ghiradella 2009; Arrese and Soulages 2010). In fossil arthropods, analysis of potentially surviving biomolecules has yielded numerous descriptions of chitin and its by-products (e.g. Baas *et al.* 1995; Gupta *et al.* 2009; Cody *et al.* 2011). Chitin is a very recalcitrant molecule, and is the building block for insect cuticles (Neville 2012a). Published analyses on the effects of decay in more labile biomolecules such as pigments are unknown to the author. Carotenoid content of *H. axyridis* was analyzed using high performance liquid chromatography – mass spectrometry (chapter 3). An expected suite of six major carotenoids was found to be stored in red/orange regions of the cuticle. The total lipid content of the cuticle was analyzed using gas chromatography – mass spectrometry (chapter 3). Cuticles of insects are composed in their majority by a lipid component which can aid as anchor points to pigment molecules (personal observation). In both types of analyses, higher molecular weight molecules such as sterols were found to be more abundant as decay progressed, especially in samples decayed in more alkaline conditions. In addition, the abundance of the lower molecular weight component, such as low *n*-sugars and *n*-hydrocarbons grew respectively to the large component of mid-molecular weight fatty acids found in untreated and acidic treated samples.

## 2.6. Conditions of mechanical decay

Transport virtually affects carcasses of all animals after death and it is considered an important and destructive mode of decay (Briggs 1995). In insects, transport

generally occurs either aerially and/or in an aqueous media (Karr and Clapham 2015). Historically, experiments on transport of soft-body animals have aimed to describe the degree of disarticulation of different body parts and fragmentation of the exoskeleton (Allison 1986; Briggs 1995; Duncan 1997); this is the first study in which these variables are constrained by the content and composition of pigments in the cuticle of insects. The effect of pigment type and amount of patterning has on the consequences of transport and resultant damage on the cuticle of *H. axyridis* is investigated via tumbling experiments in chapter 4

## **2.7. Fragmentation of patterned cuticles**

Observations during laboratory controlled transport experiments have revealed a common sequence of fragmentation holding true for various arthropod species (Allison 1986; Duncan 1997). Usually, legs and antennae are prone to breakage and fragmentation and do so within the first few days of decay. Insect elytra are more resistant to mechanical decay due to the right combination of mechanical properties and saturation of water (personal observation). Laboratory controlled conditions for transport of cuticles of *H. axyridis* have shown that first signs of fracturing in elytra appeared after 86 hours of tumbling but it is not until 329 hours that the first fragmentation is observed (chapter 4). Fragmentation in the cuticle appears first in samples with high amounts of patterning and consequently more variation in pigment content. At the end of the experiment, elytra with high amounts of patterning resulted quantitatively more damaged and higher degree of fragmentation was present.

### 3. TRACE ELEMENTAL DISTRIBUTION IN PATTERNED INSECTS

#### 3.1. Introduction

Spatial distributions of pigments in the cuticles of insects have been widely explored by other authors (e.g. Bezzerides *et al.* 2007; Wijnen *et al.* 2007; Bezzerides and Loofbourrow 2014; Stavenga *et al.* 2014); however, spatial distributions of trace elements and their relations to pigment content in the cuticle are poorly understood. Suits of trace elements specific for certain pigments (i.e. melanin), gained recognition when high content of copper was detected in fossil feathers apparently containing melanin (Wogelius *et al.* 2011). Since then, other authors have worked in characterising trace elemental signatures for various biomolecules in the fossil record due to their ability to bind to certain groups in the molecule during diagenesis and their survival over long periods of geological time (e.g. Gupta 1986; Fernandes and Henriques 1991; Edwards *et al.* 2011; McNamara *et al.* In prep.). This is the first study concentrating efforts in analysing the elemental inventory in the cuticles of 81 species of extant and fossil insects and constraining five specific suits of trace elements as possible biomarkers for the characterisation of five types of pigments in the fossil record of insects (chapter 5).

#### 3.2. Extant insects

Previous studies have characterized a suite of elements, including Cu, Zn and Ca, as to be in association with melanin *in vivo* (Niecke *et al.* 1999; Hong and Simon 2007). However, these associations cannot be simply transferred to all insect cuticles; for example, some aquatic insects have been found capable of binding and storing traces of heavy elements in their cuticles, such as those found in contaminants (e.g. As, Cd, Cu, Pb and Zn), as a mode of secretion of otherwise

harmful components for their bodies (Hare *et al.* 1991; Hare 1992). Of the 27 extant families of insects analyzed in this study, 25 families show spatial partitioning of at least one element associated with distribution of pigmented colour in the cuticle (chapter 5). Ca and K are the two elements showing spatial partitioning, usually more concentrated in regions of the cuticle containing melanin, in the higher proportion of families, 88.9% and 81.5% respectively. At order level the signal is partially lost except for Odonata, where all members analyzed show spatial partitioning of similar elements in melanin-rich regions of the cuticle. LDA indicates that this order-level control on cuticle chemistry is superimposed by a pigment-level control (chapter 5). Regions of the cuticle where melanin is present are generally enriched in Ca and Mn, carotenoid-rich in Cl, Ca and P, ommochrome-rich in K, S and Mn, flavonoid-rich in K and Ca, pterin-rich in Ca and Fe and regions lacking pigment in Cl, Ca and K. In addition, the impact that decay has in the trace element chemistry of cuticles of *H. axyridis* was analyzed in chapter 3. Melanin-rich regions of the cuticle are enriched in Mg and Ca prior decay, as decay progresses the enrichment of these elements increases in the dark spots of *H. axyridis*, Na and K is additionally enriched particularly in acidic conditions. Interestingly, the region enriched in K is reversed during decay (chapter 3).

### 3.3. Fossil insects

Elemental analysis has pointed to Cu as the most promising trace element characteristic for melanin in various fossil taxa, including insects (Wogelius *et al.* 2011; Edwards *et al.* 2013; McNamara *et al.* In prep.). In chapter 5 of this thesis, possible elemental biomarkers for various types of pigments are explored in 13

taxa of fossil insects with close living representatives. Dark regions of the cuticle are enriched in Cu, Zn and Ti, whereas light-toned regions are often enriched in Ca, K and S. Conversely, LDA shows a strong signal linked to fossil locality rather than fossil taxa; specimens from Grubstake and Old Crow River are enriched in Ti, taxa from Oeningen in Ca and those from Green River and Rubielos de Mora in Fe and K. In addition, this locality signal is overprinted by a pigment-level signal; dark cuticle regions in fossils from Rubielos de Mora, Daohugou and Green River show enrichment in K and Fe, whereas dark regions in fossils from Oeningen and Grubstake are enriched in Ti.

#### 4. CONCLUSIONS

Experimental decay of various taxa of insects encompassing two orders reveals that the alkalinity of the media and the content and composition of cuticular pigments are important environmental and biological biases previously unsought of during the decay of insects (chapter 3). These biases promote the loss of valuable taxonomic and chemical information and greatly reduce the prospects for fossilisation of organic molecules contained within the cuticle of insects. Melanin-rich regions of the cuticle and acidic media delay decay and favour the preservation of chemical signals and micro-structural arrangements, being considered the ideal conditions for extensive chemical and structural preservation of insects in the fossil record.

Transport prior burial offers further conditions for another type of bias in the cuticle of insects. Experimental tumbling of cuticles of *H. axyridis* reveals that the amount of patterning, content and composition of pigments are important biases concerning the rate and amount of breakage experienced by the cuticle prior deposition and burial (chapter 4). High rates of breakage provoke the loss of important taxonomical information and are possible causes for the under-representation of certain orders of insects in the fossil record of past communities. Regions of the cuticle containing a single pigment are less likely to break during transport and deposition. Boundaries where two pigments meet in the cuticle are less resistant to breakage and are more likely to be the nucleation point for a new fracture and consequently fragmentation of the cuticle.

Synchrotron X-ray fluorescence analysis of cuticles of extant insects suggest that elemental chemistry is a proxy for cuticular pigment content and visual

colour, at least, at genera and family levels (chapter 5). Furthermore, trace elemental chemistry appears to be overprinted by elemental incorporation during diagenesis, although derived, these trace elements are suitable markers for original colouration in insects. Enrichment of Zn, K and Ca consistently coincides with dark-toned regions in fossil insects independently of the order and suggests an originally melanin-rich composition in the cuticle of the insect *in vivo*. The trace elemental signals of other non-melanin molecules are largely overprinted by the chemistry of the host sediment.

The aim of this project was to reveal taphonomical processes that permit the fossilisation of pigments in the cuticles of insects. This information will not only resolve taphonomical ambiguities but will permit to answer much deeper questions throughout the evolutionary history of insects. Does seasonality disturb the potential of fossilisation of certain pigments? What is the contribution of sediment chemistry in the organic preservation of pigments? How does maturation affect the potential for preservation of pigments in insects? How did patterning evolve? When did it become a mode of communication? When and how did colour dimorphic individuals succeed in the same species? Was phenotypic plasticity equally important in the past for some insect species? etc. The results of this thesis, although conclusive, do not have the power to answer all these questions; it is for this reason, that future work is essential to fully understand processes involved in the fossilisation of insects.

## 5. PROPOSAL FOR FUTURE STUDIES

Future experimental studies should include further taxonomic groupings in order to explore the consistency of these findings in diverse divisions of the evolutionary history of insects. Groupings with large representation of fossil taxa should be ensured priority, especially if they have living relatives. In decay experiments, the duration and conditions of decay should be altered to include all possible environmental conditions and certain types of fine carbonate and pelitic sediments could be allowed into the experimental conditions (See chapter 3).

Maturation experiments should target the same insect groups previously analyzed and the inclusion of sediments containing trace elements, particularly of metallic nature, should be considered (see chapter 5). Gas chromatography-mass spectrometry analysis has proven to be a powerful tool in characterising the lipid content of extant insects; however, the limitations of the derivatisation method used were evident when analysis of large molecules such as melanin and  $\beta$ -carotene standards, which resulted unresolved (see chapter 3). In addition, a combination of extraction techniques should be used in order to characterise a larger range of organic molecules stored in the cuticle, fatty acids and straight and cyclic hydrocarbons, have proven to be of importance but also polysaccharides which most probably in these experiments are the result of the degradation of chitin. Pyrolysis gas/liquid chromatography-mass spectrometry have been effectively used for analysis for large fossilized macromolecules, including possible melanin biomarkers (e.g. Baas *et al.* 1995; Stankiewicz *et al.* 1997a; Flannery *et al.* 2001; Barden *et al.* 2011; Glass *et al.* 2012) and Time-of-Flight secondary ion mass spectrometry has yielded extraordinary examples of successful identification of



chemical signatures of melanin in the fossil record (Lindgren *et al.* 2012; Lindgren *et al.* 2017; Lindgren *et al.* 2019). These analyses will reveal the chemical variations in the pigment content of insect cuticles during decay, maturation and diagenesis.

Young's modulus analysis should be used to assign the hardness of differently coloured regions of the cuticle prior and after decay. This analysis in combination with tumbling of cuticles after decay will result in a complete picture of the effects of aqueous transport in fragmentation of patterned cuticles (chapter 4). No previous author has attempted to model the effects of aerial abrasion in the cuticle of insects. A wind tunnel with particles suspended and/or timed air-brushing of located regions of the cuticle have yet to be tested, although previous experiments with these methods promise potentially good results (Suzuki and Takahashi 1981; Neuman and Maxwell 1999; Lian-You *et al.* 2003). Insect taxa with diverse morphologies and spatial zonation of pigments should be analyzed with all methods described above to obtain widespread results throughout the class.

Synchrotron X-ray analysis have been proven to be useful in the characterisation of trace elemental signatures in the cuticle of insects (chapter 5). Future X-ray absorption spectroscopy (XAS) should aim to include further extant insect families with fossil representatives and distended excitation energies to constrain even further original chemistry of pigments preserved in the cuticle of fossil insects. X-ray absorption near-edge structure (XANES) and extended X-ray absorption fine structure (EXAFS) analysis would extend the understanding of the nanocrystalline structures of the atoms found as trace components of insect cuticle.

In the case of fossil insects, these analyses would elucidate the origin of these atomic structures, paths for acquisition during fossilisation, and whether the origin of elemental signatures is from endogenous organic compounds or the surrounding host sediment (Wogelius *et al.* 2011).

The additional information these techniques will generate, will aid in the understanding of the processes involved in the fossilisation of insects in general, and the pigments in their cuticles in particular. The further characterisation of the environmental and biological biases constraining pigment preservation in the fossil record are crucial to fully interpret fossil insects at taxonomical levels and the ecological relations these taxa may have had in the past.

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## APPENDIX 3

# Supplementary Information on Chapter 3: Environmental bias in the fossil record of insects

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### A3.1. Supplementary Text

#### A3.1.1. Microspectrophotometry

**A3.1.1.1. *Harmonia axyridis*.** Despite there being very little evidence of visible colour change, the spectra for both red and black cuticle regions was altered during decay in all experimental conditions (Fig. 3.3a, Supplementary Fig. 3.2a–d and Supplementary Table 3.1). There are systematic changes in colour hue and colour purity, but not colour intensity, during decay. In black cuticle regions, hue changes steadily to shorter wavelengths during decay ( $\lambda_{\max} = 467.26$  (untreated) – 463.40 (180 days); Fig. 3.3a, Supplementary Fig. 3.2a–b and Supplementary Table 3.1); this shift is greatest in samples decayed in basic conditions (Supplementary Table 3.1). Purity increases during decay ( $S = 356.61$  (untreated) – 366.12 (180 days); Supplementary Table 3.1); the shift is greater in samples decayed in neutral conditions ( $S = 407.26$  (180 days); Supplementary Table 3.1). Intensity decreases during decay ( $R_{\max} = 1.66$  (untreated) – 2.06 (180 days); Supplementary Table 3.1); the shift is greater in samples decayed in acidic and neutral conditions ( $R_{\max} = 2.55$  (acidic) – 3.55 (neutral); Supplementary Table 3.1). In general, the shape of the spectrum in red regions drastically changes during treatment (Fig. 3.3a, Supplementary Fig. 3.2c–d and Supplementary Table 3.1):  $\lambda_{\max}$  at 591.59  $\mu\text{m}$  disappears after treatment and the entire spectrum is shifted to shorter wavelengths. In red cuticle regions, hue shifts to shorter wavelengths during decay ( $\lambda_{\max} = 591.59$  (untreated) – 561.55 (180 days); Fig. 3.3a, Supplementary Fig. 3.2c–d and Supplementary Table 3.1); the shift is similar in all experimental conditions (Supplementary Table 3.1). Purity slightly

increases during decay ( $S = 251.41$  (untreated) –  $276.05$  (180 days); Supplementary Table 3.1); the shift is similar in all experimental conditions. Intensity slightly decreases during decay ( $R_{\max} = 1.03$  (untreated) –  $0.91$  (180 days); Supplementary Table 3.1); the shift is similar in all experimental conditions. In black cuticle regions, the shape of the spectrum is maintained during decay and  $\lambda_{\max}$  at  $467.26 \mu\text{m}$  slightly decreases during decay in all experimental conditions (Fig. 3.3a, Supplementary Fig. 3.2a–b and Supplementary Table 3.1). The spectrum in black regions flattens slightly during decay;  $\lambda_{\max}$  at  $467.26 \mu\text{m}$  shifts to longer wavelengths in specimens decayed for 60 days in all experimental conditions (Fig. 3.3a, Supplementary Fig. 3.2a–b and Supplementary Table 3.1), however, wavelengths shift to shorter values as the time of decay increases (Fig. 3.3a, Supplementary Fig. 3.2a–b and Supplementary Table 3.1). Samples decayed for 180 days show the greatest changes in hue, purity and intensity but the results are also more heterogeneous among experimental conditions (Fig. 3.3a, Supplementary Fig. 3.2a–d and Supplementary Table 3.1). Differences in intensity in red and black regions of the cuticle of *H. axyridis* increase with increasing duration and alkalinity of the treatment (Supplementary Table 3.1). Two-sample F-test of variance shows that only in those suites of tests where experimental conditions had the most disparate pH values, the null hypothesis was rejected (in bold; Supplementary Table 3.1). Red cuticle regions in samples treated in all experimental conditions for 180 days have F-values larger than the critical value (Supplementary Table 3.1), which demonstrates that the variance within the data is not randomized at 95% confidence.

**A3.1.1.2. *Leptinotarsa decemlineata*.** The spectra for both yellow and black cuticle regions showed alteration during decay in all experimental conditions (Fig. 3.3b, Supplementary Fig. 3.2e–h and Supplementary Table 3.1). There are systematic changes in colour hue and colour purity, but not colour intensity, during decay. In black cuticle regions, hue changes to longer wavelengths during decay ( $\lambda_{\max} = 457.73$  (untreated) – 466.04 (60 days, acidic) – 472.72 (60 days, basic); Fig. 3.3b, Supplementary Fig. 3.2e–f and Supplementary Table 3.1); this shift is greatest in samples decayed in neutral and basic conditions (Supplementary Table 3.1). Purity decreases slightly as decay advances ( $S = 334.46$  (untreated) – 332.67 (60 days); Supplementary Table 3.1). Intensity remains constant during the duration of the experiment ( $R_{\max} = 1.42$  (untreated) – 1.41 (60 days); Supplementary Table 3.1). In yellow cuticle regions, hue changes systematically to longer wavelengths during decay ( $\lambda_{\max} = 589.26$  (untreated) – 626.83 (60 days, acidic) – 617.44 (60 days, basic); Fig. 3.3b, Supplementary Fig. 3.2g–h and Supplementary Table 3.1). Purity decreases during decay ( $S = 264.07$  (untreated) – 228.96 (60 days); Supplementary Table 3.1). Intensity remains constant during the duration of the experiment ( $R_{\max} = 1.03$  (untreated) – 1.00 (60 days); Supplementary Table 3.1). Yellow regions of the cuticle in samples decayed in basic conditions show the greatest changes in hue, purity and intensity of the colour (Fig. 3.3b, Supplementary Fig. 3.2e–h and Supplementary Table 3.1). Differences in intensity in yellow and black regions of the cuticle in *L. decemlineata* increase with the acidity of the treatment (Supplementary Table 3.1). Two-sample F-test of variance shows that the variance of samples across all experimental conditions are

statistically significant disparate from the untreated samples (Supplementary Table 3.1). F-values are larger than the critical value regardless of the colour displayed (Supplementary Table 3.1), which demonstrates that the variance within the data is not randomized at 95% confidence.

**A3.1.1.3. *Goliathus goliathus*.** The spectra for grey and black cuticle regions showed alteration during decay in all experimental conditions (Fig. 3.3c, Supplementary Fig. 3.2i–l and Supplementary Table 3.1). There are systematic changes in colour hue, colour purity and colour intensity during decay. In brown regions of the cuticle,  $\lambda_{\max}$  at 488.85  $\mu\text{m}$  disappears after treatment and the entire spectrum is shifted to shorter wavelengths (Fig. 3.3c, Supplementary Fig. 3.2i–j and Supplementary Table 3.1). The shape of the spectrum in brown regions remains similar in both acidic (Supplementary Fig. 3.2i) and basic (Supplementary Fig. 3.2j) conditions;  $\lambda_{\max}$ , however, does not change significantly after decay ( $\lambda_{\max} = 488.85$  (untreated) – 482.81 (60 days, acidic) – 477.37 (60 days, basic); Fig. 3.3c, Supplementary Fig. 3.2i–j and Supplementary Table 3.1). This shift is greatest in samples decayed in neutral and basic conditions (Supplementary Table 3.1). Purity ( $S = 337.72$  (untreated); Supplementary Table 3.1) remains constant during decay ( $S = 332.14$  (60 days); Supplementary Table 3.1), yet the differences are noticeable in individual experimental conditions: purity increases in samples decayed in basic media ( $S = 348.53$  (60 days, basic); Supplementary Table 3.1) and decreases in those decayed in acidic media ( $S = 315.73$  (60 days, acidic); Supplementary Table 3.1). Intensity ( $R_{\max} = 1.50$  (untreated); Supplementary Table 3.1) remains



constant during the duration of the experiment in specimens decayed in basic conditions ( $R_{\max} = 1.52$  (60 days, basic); Supplementary Table 3.1) however, it decreases in specimens decayed in acidic conditions ( $R_{\max} = 1.28$  (60 days, acidic); Supplementary Table 3.1). In general, the shape of the spectrum in grey regions is shifted in both acidic (Supplementary Fig. 3.2k) and basic conditions (Supplementary Fig. 3.2l); spectra in both of the experimental conditions acquired an extra  $\lambda_{\max}$  peak towards the longest wavelengths. In grey regions of the cuticle the change of  $\lambda_{\max}$  is minimal after decay ( $\lambda_{\max} = 497.49$  (untreated) – 496.28 (60 days, acidic) – 492.27 (60 days, basic); Fig. 3.3c, Supplementary Fig. 3.2k–l and Supplementary Table 3.1). Purity ( $S = 307.69$  (untreated)) greatly increases during decay in acidic ( $S = 350.66$ ) and basic ( $S = 374.60$ ) conditions (Supplementary Table 3.1). Intensity also increases during decay ( $R_{\max} = 1.37$  (untreated) – 1.70 (60 days); Supplementary Table 3.1) with the greatest change in cuticles decayed in basic media ( $R_{\max} = 1.79$  (60 days, basic); Supplementary Table 3.1). Brown regions of the cuticle in samples decayed in basic conditions show the greatest changes in hue for the duration of the experiment (Fig. 3.3b, Supplementary Fig. 3.2e–h and Supplementary Table 3.1). The greatest changes in purity and intensity of the colour were observed in grey regions of the cuticle decayed in acidic media (Supplementary Table 3.1). Differences in intensity in grey and black regions of the cuticle in *G. goliathus* increase with the acidity of the treatment (Supplementary Table 3.1). Two-sample F-test of variance indicates that the variances of the samples treated in all experimental conditions are statistically significant different from untreated samples

(Supplementary Table 3.1). In addition, samples from brown regions of the cuticle in *G. goliathus* decayed in acidic conditions, show an F-value larger than the critical value (Supplementary Table 3.1), which demonstrates that the variance within the data is not randomized at 95% confidence.

**A3.1.1.4. *Typocerus velutinus*.** The spectra for both yellow and brown cuticle regions showed alteration during decay in all experimental conditions (Fig. 3.3d, Supplementary Fig. 3.2m–p and Supplementary Table 3.1). There are systematic changes in colour hue, colour purity and colour intensity during decay of brown regions of the cuticle (Fig. 3.3d, Supplementary Fig. 3.2m–n and Supplementary Table 3.1), these changes also stand true for yellow regions; however, the changes in colour intensity are minimal (Fig. 3.3d, Supplementary Fig. 3.2o–p and Supplementary Table 3.1). In general,  $\lambda_{\max}$  at 613.60  $\mu\text{m}$  disappears after treatment and the entire spectrum is shifted to longer wavelengths in all treatments except in black regions of the cuticle decayed in basic conditions where the shift is towards shorter wavelengths. The shape of the spectrum in brown regions of the cuticle decayed in acidic media remains similar to the untreated spectrum in both acidic (Supplementary Fig. 3.2m) yet there is a slight shift towards shorter wavelengths in specimens decayed in basic (Supplementary Fig. 3.2n) conditions. Hue values, however, do not change significantly after decay ( $\lambda_{\max} = 660.81$  (untreated) – 671.90 (60 days, acidic) – 641.32 (60 days, basic); Fig. 3.3d, Supplementary Fig. 3.2m–n and Supplementary Table 3.1); this shift is greatest in samples decayed in basic conditions (Supplementary Table 3.1). Purity greatly increases in samples decayed in acidic media, while the changes in samples decayed in basic media are

more discrete ( $S = 221.39$  (untreated) –  $260.79$  (60 days, acidic) –  $228.96$  (60 days, basic); Supplementary Table 3.1). Intensity of colour is greatly decreased during decay, specially in cuticles decayed in basic media ( $R_{\max} = 1.50$  (untreated) –  $1.05$  (60 days, acidic) –  $1.00$  (60 days, basic); Supplementary Table 3.1). The shape of the spectrum in yellow regions of the cuticle is shifted in both acidic (Fig. 3.3o) and basic conditions (Supplementary Fig. 3.2p). In yellow cuticle regions, hue changes systematically to longer wavelengths during decay ( $\lambda_{\max} = 613.60$  (untreated) –  $622.04$  (60 days, acidic) and  $628.93$  (60 days, basic); Fig. 3.3d, Supplementary Fig. 3.2o–p and Supplementary Table 3.1). Purity ( $S = 216.53$  (untreated); Supplementary Table 3.1) greatly increases in samples decayed in acidic media ( $S = 262.65$  (60 days, acidic); Supplementary Table 3.1) while in samples decayed in basic media slightly decreases ( $S = 209.05$  (60 days, basic); Supplementary Table 3.1). The greatest changes in hue and purity were observed in yellow regions of the cuticle decayed in basic media (Supplementary Table 3.1). The largest differences in intensity during decay were those encountered in brown regions of specimens decayed in basic media (Supplementary Table 3.1). Differences in intensity in yellow and brown regions of the cuticle in *T. velutinus* increase with the alkalinity of the treatment (Supplementary Table 3.1). Two-sample F-test of variance indicates that only in the samples treated under basic conditions for 60 days the null hypothesis was rejected, consequently the variance in the data is not statistically different from that in the untreated sample (in bold; Supplementary Table 3.1). The variances from all other samples treated in all experimental conditions are statistically significant different

from untreated samples (Supplementary Table 3.1). In addition, samples decayed in acidic conditions, regardless of the colour displayed, show an F-value larger than the critical value (Supplementary Table 3.1), which demonstrates that the variance within the data is not randomized at 95% confidence.

**A3.1.1.5. *Pyrrhocoris apterus*.** The spectra for both red and black cuticle regions showed alteration during decay in all experimental conditions (Fig. 3.3e, Supplementary Fig. 3.2q–t and Supplementary Table 3.1). There are systematic changes in colour hue and colour purity and colour intensity during decay. In general, the shape of the spectrum in black regions decayed in acidic media remains similar to the untreated spectrum in both acidic (Supplementary Fig. 3.2q) and basic (Supplementary Fig. 3.2r) conditions; however,  $\lambda_{\max}$  does not change significantly after decay (Fig. 3.3e, Supplementary Fig. 3.2q–r and Supplementary Table 3.1). In black cuticle regions, hue ( $\lambda_{\max} = 465.23$  (untreated); Figs. 3.3s–t, 3.4e and Supplementary Table 3.1) changes to longer wavelengths during decay in acidic media ( $\lambda_{\max} = 455.70$  (60 days, acidic); Fig. 3.3e, Supplementary Fig. 3.2s–t and Supplementary Table 3.1) and shorter wavelengths in basic media ( $\lambda_{\max} = 466.04$  (60 days, basic); Fig. 3.3e, Supplementary Fig. 3.2s–t and Supplementary Table 3.1). This shift is greatest in samples decayed in acidic conditions (Supplementary Table 3.1). Purity does not change significantly during decay in samples decayed in basic conditions ( $S = 351.30$  (untreated) –  $357.33$  (60 days, basic); Supplementary Table 3.1), in samples decayed in acidic conditions the change is more substantial ( $S = 372.11$  (60 days, acidic); Supplementary Table 3.1). Intensity increases

significantly during decay in acidic and basic conditions ( $R_{\max} = 1.58$  (untreated) – 1.76 (60 days, acidic) – 1.64 (60 days, basic); Supplementary Table 3.1), the change is greatest in acidic conditions (Supplementary Table 3.1). The shape of the spectrum in red regions is shifted and greatly altered in both acidic (Supplementary Fig. 3.2s) and basic conditions (Supplementary Fig. 3.2t):  $\lambda_{\max}$  at 610.33  $\mu\text{m}$  disappears after treatment, the entire spectrum is shifted to shorter wavelengths and intensity values increase more rapidly in lower hue values (Supplementary Fig. 3.2s–t). In red cuticle regions, hue changes systematically to shorter wavelengths during decay (610.33 (untreated) – 603.00 (60 days, acidic) and 579.93 (60 days, basic); Fig. 3.3e, Supplementary Fig. 3.2s–t and Supplementary Table 3.1). Purity greatly increases during decay ( $S = 199.23$  (untreated) – 249.15 (60 days, acidic) – 260.58 (60 days, basic); Fig. 3.3e, Supplementary Fig. 3.2s–t and Supplementary Table 3.1); this change is greatest in specimens decayed in basic conditions (Fig. 3.3e, Supplementary Fig. 3.2s–t and Supplementary Table 3.1). Intensity slightly increases in those specimens decayed in acidic media ( $R_{\max} = 1.01$  (untreated) – 1.02 (60 days, acidic); Supplementary Table 3.1) while the change is greater on those specimens decayed in basic media ( $R_{\max} = 1.05$  (60 days, basic); Supplementary Table 3.1). The greatest changes in hue and purity occurred in red regions of the cuticle decayed in basic media (Supplementary Table 3.1). Differences in intensity in black regions of the cuticle in *P. apterus* increase with the acidity of the treatment (Supplementary Table 3.1). One-sample Z-test of means show that the majority of mean values have statistically significant differences in samples treated in all experimental conditions

(Supplementary Table 3.1). All differences in cuticle colour between acidic and basic experimental conditions were statistically significant. Two-sample F-test of variance indicates that only in samples from black regions of the cuticle the null hypothesis was rejected, consequently the variance in the data is not statistically different from that in the untreated sample (in bold; Supplementary Table 3.1). The variances from all other samples treated in all experimental conditions are statistically significant different from untreated samples (Supplementary Table 3.1). In addition, samples from red regions of the cuticle, regardless of the conditions of treatment, show an F-value larger than the critical value (Supplementary Table 3.1), which demonstrates that the variance within the data is not randomized at 95% confidence.

### **A3.1.2. Electrobeam analysis.**

**A3.1.2.1. *Harmonia axyridis*.** Endocuticle and exocuticle are clearly differentiated in transverse sections (Supplementary Fig. 3.3a–b, e–f, i–j). In untreated samples, endocuticular chitin bundles are enveloped by a proteinaceous matrix and are overlaid by finely laminated exocuticle (Fig. 3.4a–b and Supplementary Fig. 3.3a–b). The surface of the exocuticle shows well-defined punctae (Fig. 3.4c–d and Supplementary Fig. 3.3c–d). After 60 days decay, endocuticular chitin bundles are more prominent than in untreated samples, particularly in red cuticle regions and for basic media, internal lamination of the exocuticle is more evident (Fig. 3.4e–f, i–j and Supplementary Fig. 3.3e–f, i–j). After 180 days decay, in red regions of the cuticle endocuticular chitin bundles are more prominent, especially in specimens degraded at pH 8 and pH 7 (Fig. 3.4m–n, u–v and

Supplementary Fig. 3.3e–f, i–j), and exocuticular lamination is prominent, especially in specimens decayed in basic media (Fig. 3.4m–n and Supplementary Fig. 3.3e–f). There is no obvious difference in the ultrastructure of black cuticle regions decayed for 60 and 180 days decay in acidic media (Fig. 3.4j, r). In contrast, in basic media (Fig. 3.4f, n) samples decayed for 180 days show less protein matrix surrounding chitin bundles.

All experimentally degraded cuticles exhibit abundant rod-shaped bacteria on the epicuticular surface, often concentrated near cuticular punctae (Fig. 3.4g, o, t, w–x and Supplementary Fig. 3.3g–h, k–l); coccoid and / or spiriliform bacteria are less abundant (Fig. 3.4g–h, k–l, o–p, s–t, w–x and Supplementary Fig. 3.3g–h, k–l). Overall, bacteria are most abundant and morphologically more diverse in red cuticle regions and in samples decayed in basic conditions (Fig. 3.4g, o and Supplementary Fig. 3.3g, k). More specifically, coccoid and spiriliform bacteria occur only in red cuticle regions in basic experiments (Fig. 3.4g, o and Supplementary Fig. 3.3g, k), whereas rod-shaped and spiriliform bacteria occur throughout the cuticular surface in all experimental conditions independently of the colour displayed (Fig. 3.4g–h, k–l, o–p, s–t, w–x and Supplementary Fig. 3.3g–h, k–l).

**A3.1.2.2. *Leptinotarsa decemlineata*.** Endocuticle and exocuticle are clearly differentiated in transverse sections (Supplementary Fig. 3.4a–b, e–f, i–j). In untreated samples, endocuticular chitin bundles are enveloped by a proteinaceous matrix and are overlaid by finely laminated exocuticle (Supplementary Fig. 3.4a–b). The surface of the exocuticle shows a small number of short punctae (Supplementary Fig. 3.4d). After 60 days decay, endocuticular chitin bundles are more prominent than in untreated samples,

particularly in yellow cuticle regions and for basic media; internal lamination of the exocuticle is more evident for basic media (Supplementary Fig. 3.4e–f, i–j).

Experimentally degraded cuticles exhibit abundant rod-shaped bacteria on the epicuticular surface (Supplementary Fig. 3.4g–h, k–l); spiriliform bacteria are less abundant (Supplementary Fig. 3.4g, k). Overall, bacteria are most abundant and morphologically more diverse in yellow cuticle regions and in basic conditions (Supplementary Fig. 3.4g–h, k–l). More specifically, spiriliform bacteria occur only in yellow cuticle regions (Supplementary Fig. 3.4g, k), whereas rod-shaped bacteria occur throughout the cuticular surface in all experimental conditions independently of the colour displayed (Supplementary Fig. 3.4g–h, k–l).

**A3.1.2.3. *Goliathus goliathus*.** The surface of the exocuticle shows irregular clusters of short setae covered by a thin layer of wax, which are located on the grey regions of the cuticle (Supplementary Fig. 3.5a). Due to these irregularities, imaging with electrobeam proved difficult to achieve; setae constantly produced interferences on the beam's path generating charging in the surface of the cuticle of *G. goliathus*. High resolution imaging of the surface or transverse sections of the cuticle of *G. goliathus* was compromised by longitudinal distortion of the resulting image (Supplementary Fig. 3.5a). In untreated samples, endocuticular chitin bundles are enveloped by a proteinaceous matrix and are overlaid by a thick, and finely laminated exocuticle. After 60 days of decay and regardless of the conditions of decay, endocuticular bundles are slightly more prominent than in untreated samples.



Bacterial growths were not observed in any surfaces of *G. goliathus* at any stage of the experiment regardless of the conditions of decay (Supplementary Fig. 3.5b–c).

**A3.1.2.4. *Typocerus velutinus*.** Endocuticle and exocuticle are clearly differentiated in transverse sections (Supplementary Fig. 3.6a–b, e–f, i–j). In untreated samples, endocuticular chitin bundles are enveloped by a proteinaceous matrix and are overlain by finely laminated exocuticle (Supplementary Fig. 3.6a–b). The surface of the exocuticle shows long, black setae (Supplementary Fig. 3.6c–d). After 60 days decay, endocuticular chitin bundles are more prominent than in untreated samples, internal lamination of the exocuticle is more evident in both experimental conditions (Supplementary Fig. 3.6e–f, i–j).

All experimentally degraded cuticles exhibit abundant rod-shaped bacteria on the epicuticular surface (Supplementary Fig. 3.6g–h, k–l); spiriliform bacteria are less abundant (Supplementary Fig. 3.6g, k–l). Overall, bacteria are most abundant and morphologically diverse in yellow cuticle regions (Supplementary Fig. 3.6g, k). Rod-shaped and spiriliform bacteria occur throughout the cuticular surface in all experimental conditions independently of the colour displayed (Supplementary Fig. 3.6g–h, k–l).

**A3.1.2.5. *Pyrrhocoris apterus*.** Endocuticle and exocuticle are clearly differentiated in transverse sections (Supplementary Fig. 3.7a–b, e–f, i–j). In untreated samples, endocuticular chitin bundles are enveloped by a proteinaceous matrix and are overlain by finely laminated exocuticle (Supplementary Fig. 3.7a–b). The surface of the exocuticle is covered by

small conical structures of ca. 1  $\mu\text{m}$  in diameter, which function as antiglare coating for the cuticle (Yoshida *et al.* 1997) and will be referred in the following text as tubercles (Supplementary Fig. 3.7c–d, g–h, k–l). After 60 days decay, endocuticular chitin bundles are more prominent than in untreated samples particularly in basic media; internal lamination of the exocuticle is more evident for basic media (Supplementary Fig. 3.7e–f, i–j).

All experimentally degraded cuticles exhibit abundant rod-shaped bacteria on the epicuticular surface filling free space between tubercles (Supplementary Fig. 3.7g–h, k–l); spiriliform bacteria are less abundant but present in all experimental conditions (Supplementary Fig. 3.7g–h, k–l). Overall, bacteria are most abundant and morphologically diverse in red cuticle regions (Supplementary Fig. 3.7g, k). Rod-shaped and spiriliform bacteria occur throughout the cuticular surface in all experimental conditions independently of the colour displayed (Supplementary Fig. 3.7g–h, k–l).

### **A3.1.3. Gas chromatography–mass spectrometry.**

**A3.1.3.1. *Harmonia axyridis*.** Chromatograms of the cuticles of *H. axyridis* ( $n = 20$ ) show that the highest concentrations of lipid molecules in the cuticle corresponding to fatty acids and straight-chained hydrocarbons such as alkanes (Fig. 3.6, Supplementary Fig. 3.9 and Supplementary Tables 3.2, 3.7). The major peak in 80% of the samples is identified as  $\text{C}_{18}$  monounsaturated fatty acid (oleic acid) (Supplementary Fig. 3.9a–b, d–h and Supplementary Tables 3.2, 3.7). Other saturated fatty acids such as  $\text{C}_{16}$  fatty acid (palmitic acid) and  $\text{C}_{18}$  fatty acid (stearic acid) are identified in

70% and 65% of the samples, respectively (Supplementary Tables 3.2, 3.7). Length of the fatty acid C–C chains ranges from 14 to 27 carbons, 88.88% of the fatty acids have an even number of carbons and 66.66% are saturated (Supplementary Tables 3.2, 3.7). The next group of molecules in terms of relative abundance are straight-chained hydrocarbons, more specifically, alkanes, alkenes, dienes and cyclic alkanes (Supplementary Fig. 3.9a–h and Supplementary Tables 3.2, 3.7). Three of these molecules are present in 100% of the samples analyzed: C<sub>27</sub> diene, C<sub>27</sub> alkene and C<sub>19</sub> alkene (Supplementary Tables 3.2, 3.7). Other hydrocarbons with a high relative abundance in the majority of samples are C<sub>25</sub> alkene, C<sub>29</sub> diene and C<sub>23</sub> alkane, which are identified in 95%, 90% and 85% of the samples respectively (Supplementary Tables 3.2, 3.7). Length of the hydrocarbon C–C chains range from 23 to 32 carbons and 81.81% of the hydrocarbons have an odd number of carbons (Supplementary Tables 3.2, 3.7). Sterols are the highest molecular weight molecules identified in the samples of *H. axyridis*, the most abundant being cholesterol (C<sub>27</sub> sterol) which is described in 75% of the samples (Supplementary Fig. 3.9a–c, e, g and Supplementary Tables 3.2, 3.7). Sugars are encountered in 55% of the samples, when present in a sample, they are usually reoccurring (Supplementary Fig. 3.9a–h and Supplementary Tables 3.2, 3.7). Small phthalate peaks are identified in 50% of the samples (Supplementary Fig. 3.9a–c, e, g and Supplementary Tables 3.2, 3.7) and four unknown molecules are noted in one sample each (Supplementary Tables 3.2, 3.7).

Untreated samples of *H. axyridis* (n = 2) show C<sub>18</sub> monounsaturated fatty acid in high relative abundance (Supplementary Fig. 3.9a–b and

Supplementary Table 3.7). Other abundant molecules in untreated samples are C<sub>25</sub> and C<sub>27</sub> alkanes, C<sub>29</sub> diene and C<sub>29</sub> alkene (Supplementary Fig. 3.9a–b and Supplementary Table 3.7). Sugars are encountered in high relative abundance in 100% of the untreated samples (Supplementary Fig. 3.9a–b and Supplementary Table 3.7). The only sterols identified in untreated samples of *H. axyridis* are molecules of cholesterol in 100% of the samples (Supplementary Fig. 3.9a–b and Supplementary Table 3.7).

After 60 days of decay, control samples of *H. axyridis* (n = 4) show C<sub>27</sub> alkene as the molecule with the highest relative abundance in 75% and C<sub>25</sub> alkene in 25% of the samples (Supplementary Fig. 3.9c and Supplementary Table 3.7). Fatty acids are rarely present (Supplementary Fig. 3.9c and Supplementary Table 3.7). Sugars are encountered in low relative abundance in 50% of the neutral decayed samples (Supplementary Fig. 3.9c and Supplementary Table 3.7). Sterols are more abundant in samples decayed in neutral pH conditions than in untreated samples, four types of sterols are identified: cholesterol (C<sub>27</sub> sterol), campesterol (C<sub>28</sub> sterol),  $\beta$ -sitosterol (C<sub>29</sub> sterol) and ergosta (C<sub>28</sub> sterol) are found in 100%, 50%, 50% and 25% of the samples respectively (Supplementary Fig. 3.9c and Supplementary Table 3.7). Acidic samples (n = 4) show C<sub>18</sub> monounsaturated fatty acid with the highest relative abundance in 75% and C<sub>25</sub> alkene in 25% of the samples (Supplementary Fig. 3.9e and Supplementary Table 3.7). Straight-chained hydrocarbons, especially alkenes, with odd numbers of C numbers ranging from 23 to 31 are very abundant in samples treated under acidic conditions (Supplementary Fig. 3.9e and Supplementary Table 3.7). Sugars are identified in only one

sample with very low relative abundance (Supplementary Fig. 3.9e and Supplementary Table 3.7). Sterols are not abundant in samples decayed in acidic pH conditions; cholesterol (C<sub>27</sub> sterol) and  $\beta$ -sitosterol (C<sub>29</sub> sterol) are both found in one sample with very low relative abundance (Supplementary Fig. 3.9e and Supplementary Table 3.7). Samples decayed in basic pH conditions (n = 4) show C<sub>18</sub> monounsaturated fatty acid with high relative abundance in 100% of the samples (Supplementary Fig. 3.9g and Supplementary Table 3.7). Straight-chained hydrocarbons, such as alkenes and dienes, with odd numbers of C numbers ranging from 23 to 31 are very abundant in all basic decayed samples (Supplementary Fig. 3.9g and Supplementary Table 3.7). Sugars are absent in all basic decayed samples (Supplementary Table 3.7). Sterols are found in relative high abundance in samples decayed in basic pH conditions; cholesterol (C<sub>27</sub> sterol), campesterol (C<sub>28</sub> sterol),  $\beta$ -sitosterol (C<sub>29</sub> sterol) and ergosta (C<sub>28</sub> sterol) are found in 100%, 50%, 25% and 50% respectively (Supplementary Fig. 3.9e and Supplementary Table 3.7).

After 180 days of decay, control samples of *H. axyridis* (n = 2) show C<sub>18</sub> monounsaturated fatty acid with the highest relative abundance in 100% (Supplementary Fig. 3.9d and Supplementary Table 3.7). Other abundant saturated fatty acids are C<sub>16</sub> and C<sub>18</sub> fatty acids (Supplementary Fig. 3.9d and Supplementary Table 3.7). Straight-chained hydrocarbons are not present in high relative abundance in samples decayed in neutral pH conditions; dienes and alkenes with odd C numbers ranging from 21 to 27 are the most abundant in these samples (Supplementary Fig. 3.9d and Supplementary Table 3.7). Sugars are encountered in low relative

abundance in 50% of the samples (Supplementary Fig. 3.9d and Supplementary Table 3.7). Sterols are absent in 100% of the samples (Supplementary Fig. 3.9d and Supplementary Table 3.7). Acidic samples ( $n = 2$ ) show  $C_{18}$  monounsaturated fatty acid with the highest relative abundance in 100% of the samples (Supplementary Fig. 3.9f and Supplementary Table 3.7). Other abundant saturated fatty acids are  $C_{16}$  and  $C_{18}$  fatty acids (Supplementary Fig. 3.9f and Supplementary Table 3.7). An interesting set of fatty acids identified in these acidic samples are  $C_{16}$ ,  $C_{17}$  and  $C_{18}$  polyunsaturated fatty acids (Supplementary Table 3.7). Straight-chained hydrocarbons, especially alkenes, with odd numbers of C numbers ranging from 23 to 29 are abundant in all acidic decayed samples (Supplementary Fig. 3.9f and Supplementary Table 3.7). Sugars are identified in high relative abundance in 100% of the samples (Supplementary Fig. 3.9f and Supplementary Table 3.7). Sterols are not found in samples decayed in acidic pH conditions (Supplementary Fig. 3.9f and Supplementary Table 3.7). Samples decayed in basic pH conditions ( $n = 2$ ) show  $C_{18}$  monounsaturated fatty acid with high relative abundance in 100% of the samples (Supplementary Fig. 3.9h and Supplementary Table 3.7). Other abundant saturated fatty acids are  $C_{16}$  and  $C_{18}$  fatty acids (Supplementary Fig. 3.9h and Supplementary Table 3.7). In these samples,  $C_{16}$  and  $C_{17}$  polyunsaturated fatty acids have also been identified (Supplementary Table 3.7). Straight-chained hydrocarbons, such as alkanes and alkenes, with odd numbers of C numbers ranging from 23 to 27 are relatively abundant in all basic decayed samples (Supplementary Fig. 3.9h and Supplementary Table 3.7). Sugars are present in low relative abundance

in only one of the samples analyzed (Supplementary Fig. 3.9h and Supplementary Table 3.7). Sterols are not identified in samples decayed in basic pH conditions (Supplementary Fig. 3.9h and Supplementary Table 3.7).

**A3.1.3.2. *Leptinotarsa decemlineata*.** Chromatograms of the cuticles of *L. decemlineata* (n = 7) show that the highest concentrations of lipid molecules in the cuticle correspond to fatty acids and cyclic hydrocarbons, probably some variety of alkane (Supplementary Fig. 3.10 and Supplementary Table 3.3). One of the largest peaks in 100% of the samples is identified as C<sub>18</sub> monounsaturated fatty acid (oleic acid) (Supplementary Fig. 3.10a–d and Supplementary Table 3.3). Other saturated fatty acids such as C<sub>16</sub> fatty acid (palmitic acid), C<sub>18</sub> fatty acid (stearic acid) and C<sub>20</sub> fatty acid (arachidic acid) are identified in 100% the former and 85.71% the latest (Supplementary Table 3.3). Length of the fatty acid C–C chains ranges from 16 to 20 carbons, 83.33% of the fatty acids have an even number of carbons and 66.66% are saturated (Supplementary Table 3.3). Cyclic alkanes with unspecified C number are present in 100% of the samples analyzed (Supplementary Fig. 3.10b–d and Supplementary Table 3.3). The other hydrocarbon identified in some of the samples (14.28%) with low relative abundance is C<sub>29</sub> alkane (Supplementary Table 3.3). Sterols are again the highest molecular weight molecules identified in the samples of *L. decemlineata*, the most abundant being cholesterol (C<sub>27</sub> sterol) which is described in 85.71% of the samples (Supplementary Fig. 3.10b–d and Supplementary Table 3.3). Sugars are encountered in 57.14% of the samples (Supplementary Fig. 3.10a–b and Supplementary Table 3.3).

Ecdysone (20-hydroxyecdysone) is found in 14.28% of the samples analyzed (Supplementary Table 3.3). A partially identified vitamin A-like molecule is found 71.43% of the samples (Supplementary Table 3.3). A small peak of phenolic acid (protocatechuic acid) is described in 14.28% of the samples of *L. decemlineata* (Supplementary Table 3.3). Small phthalate peaks are identified in 57.14% of the samples (Supplementary Fig. 3.10a–b, d and Supplementary Table 3.3) and three unknown molecules are noted in 14.28% of the samples (Supplementary Table 3.3).

The untreated sample of *L. decemlineata* ( $n = 1$ ) shows a sugar as the molecule with the highest relative abundance (Supplementary Fig. 3.10a and Supplementary Table 3.7). Other sugar molecules are reoccurring later on the chromatogram (Supplementary Fig. 3.10a and Supplementary Table 3.7). Further abundant molecules in untreated samples are fatty acids such as  $C_{18}$  monounsaturated fatty acid,  $C_{16}$  saturated fatty acid and  $C_{18}$  saturated fatty acid (Supplementary Fig. 3.10a and Supplementary Table 3.7). Hydrocarbons are identified in low relative abundance in untreated samples, these are cyclic and possibly some type of cyclic alkane (Supplementary Table 3.7). The only sterol identified in untreated samples of *L. decemlineata* is a molecule of cholesterol in very low relative abundance (Supplementary Table 3.7).

After 60 days of decay, control samples of *L. decemlineata* ( $n = 2$ ) show  $C_{18}$  monounsaturated fatty acid with the highest relative abundance in 100% of the samples (Supplementary Fig. 3.10b and Supplementary Table 3.7). The other abundant fatty acid is  $C_{16}$  saturated fatty acid (Supplementary Fig. 3.10b and Supplementary Table 3.7). Hydrocarbons



are identified in low relative abundance in the 100% of the samples decayed in neutral pH conditions, these are cyclic and possibly some type of cyclic alkane (Supplementary Fig. 3.10b and Supplementary Table 3.7). Sugars are encountered in high relative abundance in 100% of the neutral decayed samples (Supplementary Fig. 3.10 and Supplementary Table 3.7). Sterols are more abundant in samples decayed in neutral pH conditions than in untreated samples, four types of sterols are identified: cholesterol ( $C_{27}$  sterol) and  $\beta$ -sitosterol ( $C_{29}$  sterol) are found in 100% of the samples (Supplementary Fig. 3.10b and Supplementary Table 3.7). The steroid ecdysone (20-hydroxyecdysone) is identified in 50% of the samples (Supplementary Table 3.7). A molecule of benzoic acid (protocatechuic acid) is found on one of the neutral decayed samples (Supplementary Table 3.7). An interesting molecule partially identified in these samples is a cyclic molecule reminiscent of vitamin A, which is found in 50% of the samples (Supplementary Table 3.7). Acidic samples ( $n = 2$ ) show  $C_{18}$  monounsaturated fatty acid with the highest relative abundance in 100% (Supplementary Fig. 3.10e and Supplementary Table 3.7). Further abundant fatty acids are  $C_{16}$  and  $C_{18}$  saturated fatty acids (Supplementary Fig. 3.10c and Supplementary Table 3.7). Straight-chained hydrocarbons are not present in any of the samples decayed in acidic pH conditions, however, cyclic hydrocarbons, possibly some type of alkane are present in high abundance in 100% of the samples (Supplementary Fig. 3.10c and Supplementary Table 3.7). Sugars are not found in any of the acidic decayed samples (Supplementary Fig. 3.10e and Supplementary Table 3.7). Sterols are not abundant in samples decayed in acidic pH conditions;

cholesterol ( $C_{27}$  sterol) is found in one sample with very low relative abundance (Supplementary Fig. 3.10e and Supplementary Table 3.7). The same molecule identified in neutrally decayed samples is reoccurring in acidic decayed samples, this is partially identified in these samples as a cyclic molecule reminiscent of vitamin A (Supplementary Table 3.7). Samples decayed in basic pH conditions ( $n = 2$ ) show  $C_{18}$  monounsaturated fatty acid and  $C_{16}$  saturated fatty acid with high relative abundance in 100% of the samples (Supplementary Fig. 3.10d and Supplementary Table 3.7). Cyclic hydrocarbons are identified in 100% of the samples, yet they are not found in high abundances (Supplementary Fig. 3.10d and Supplementary Table 3.7). Sugars are absent in all basic decayed samples (Supplementary Table 3.7). Sterols are identified in relative low abundance in samples decayed in basic pH conditions; cholesterol ( $C_{27}$  sterol) is found in 100% of the samples (Supplementary Fig. 3.10d and Supplementary Table 3.7). The same molecule identified in neutrally and acidic decayed samples is reoccurring in samples decayed in basic pH conditions, this is partially identified in these samples as a cyclic molecule reminiscent of vitamin A found in 100% of the samples (Supplementary Table 3.7).

**A3.1.3.3. *Goliathus goliathus*.** Chromatograms of the cuticles of *G. goliathus* ( $n = 8$ ) show that the highest concentrations of lipid molecules in the cuticle corresponding to fatty acids and straight-chained hydrocarbons such as alkanes (Supplementary Fig. 3.11 and Supplementary Table 3.4). The major peaks in 100% of the samples are identified as  $C_{18}$  monounsaturated fatty acid (oleic acid) and  $C_{16}$  saturated fatty acid (palmitic acid) (Supplementary Fig. 3.11a–d and Supplementary Table 3.4).

An interesting saturated fatty acid with presence in all samples analyzed is C<sub>16</sub> saturated fatty acid methyl ester (Supplementary Table 3.4). Other fatty acids such as C<sub>18</sub> saturated fatty acid (stearic acid) and C<sub>18</sub> polyunsaturated fatty acid (linolenic acid) are identified in 87.50% and 82.50% of the samples respectively (Supplementary Table 3.4). The length of the fatty acid C–C chains ranges from 16 to 23 carbons, 80% of the fatty acid had even numbers of carbons and 60% are saturated (Supplementary Table 3.4). The next group of molecules in terms of relative abundance are straight-chained hydrocarbons, more specifically, alkanes, alkenes, dienes and cyclic alkanes (Supplementary Fig. 3.11a–d and Supplementary Table 3.4). Five of these cyclic alkanes are present in 100% of the samples analyzed (Supplementary Table 3.4). Other hydrocarbons present with a lower relative abundance are C<sub>31</sub> alkene and C<sub>38</sub> alkane identified in 37.50% and 12.50% of the samples respectively (Supplementary Table 3.4). Length of the hydrocarbon C–C chains ranges from 26 to 38 carbons and 57.14% of the hydrocarbons are composed of an even number of carbons (Supplementary Table 3.4). In general, straight-chained hydrocarbons in *G. goliathus* have higher C number than in the other samples analyzed. The highest molecular weight molecules identified in the samples of *G. goliathus* is cholesterol (C<sub>27</sub> sterol) being the only sterol identified and encountered in 100% of the samples (Supplementary Fig. 3.11a–d and Supplementary Table 3.4). Sugars with a trimethylsilyl group are described in 62.50% of the samples (Supplementary Fig. 3.11a–d and Supplementary Table 3.4). A group of very high molecular weight molecules are found in 12.50% of the samples believed to represent some variety of bacterial lipids

(Supplementary Table 3.4). Small phthalate peaks were identified in 12.50% of the samples (Supplementary Table 3.4) and three unknown molecules were noted in 25% or less of the samples (Supplementary Table 3.4).

Untreated samples of *G. goliathus* (n = 2) show C<sub>18</sub> monounsaturated fatty acid in high relative abundance (Supplementary Fig. 3.11a and Supplementary Table 3.7). Other abundant molecules in untreated samples are C<sub>16</sub> and C<sub>18</sub> saturated fatty acids (Supplementary Fig. 3.11a and Supplementary Table 3.7). An interesting fatty acid identified is C<sub>18</sub> polyunsaturated fatty acid in 50% of the samples (Supplementary Table 3.7). Cyclic hydrocarbons are found in high relative abundance in 100% of the untreated samples (Supplementary Fig. 3.11a and Supplementary Table 3.7). An additional abundant hydrocarbon is acyclic C<sub>23</sub> alkane (Supplementary Table 3.7). Sugars are encountered in high relative abundance in 100% of the untreated samples (Supplementary Fig. 3.11a and Supplementary Table 3.7). Sterols are not identified in these samples (Supplementary Table 3.7).

After 60 days of decay, samples of *G. goliathus* (n = 2) decayed in neutral pH conditions show molecules of C<sub>18</sub> monounsaturated fatty acid and C<sub>16</sub> saturated fatty acid in high relative abundances (Supplementary Fig. 3.11b and Supplementary Table 3.7). An interesting fatty acid identified is C<sub>18</sub> polyunsaturated fatty acid in 50% of the samples (Supplementary Table 3.7). Straight-chained hydrocarbons are less abundant than cyclic hydrocarbons in these samples; within this type of molecules, dienes are the most abundant. Sugars are also found in high

relative abundance in 100% of the samples (Supplementary Fig. 3.11b and Supplementary Table 3.7). Sterols are more abundant in samples decayed in neutral pH conditions than in untreated samples: cholesterol ( $C_{27}$  sterol) molecules are found in 100% of the samples (Supplementary Fig. 3.11b and Supplementary Table 3.7). Acidic samples ( $n = 2$ ) show  $C_{18}$  monounsaturated fatty acid and  $C_{16}$  saturated fatty acid with the highest relative abundance in 100% of the samples (Supplementary Fig. 3.11c and Supplementary Table 3.7). Further abundant fatty acids are  $C_{18}$  saturated fatty acid and  $C_{18}$  polyunsaturated fatty acid identified in 100% of the samples decayed in acidic conditions (Supplementary Fig. 3.11c and Supplementary Table 3.7). Abundant hydrocarbons are cyclic in nature, possibly some sort of cyclic alkane (Supplementary Fig. 3.11c and Supplementary Table 3.7). Sugar molecules are identified in very low relative abundance in only one of the samples (Supplementary Table 3.7). Sterols are not abundant in samples decayed in acidic pH conditions; an interesting compound was found to have a similar structure to oestrogen in one of the samples but could not be fully identified (Supplementary Table 3.7). Samples decayed in basic pH conditions ( $n = 2$ ) show  $C_{18}$  monounsaturated fatty acid and  $C_{16}$  saturated fatty acid with the highest relative abundance in 100% of the samples (Supplementary Fig. 3.11d and Supplementary Table 3.7). Straight-chained hydrocarbons, such as alkanes, with C numbers ranging from 26 to 35 are found in very low relative abundance, on the other hand, cyclic alkanes are identified in a higher relative abundance in samples decayed in basic conditions (Supplementary Fig. 3.11d and Supplementary Table 3.7). Sugars are found in one of the

samples decayed in basic conditions (Supplementary Fig. 3.11d and Supplementary Table 3.7). Sterols are absent in samples decayed in basic pH conditions (Supplementary Fig. 3.11d and Supplementary Table 3.7).

**A3.1.3.4. *Typocerus velutinus*.** Chromatograms of the cuticles of *T. velutinus* (n = 7) show that the highest concentrations of lipid molecules in the cuticle corresponding to fatty acids and straight-chained hydrocarbons such as alkanes (Supplementary Fig. 3.12 and Supplementary Table 3.5). The major peaks in 100% of the samples are identified as C<sub>16</sub> saturated fatty acid (palmitic acid) and C<sub>18</sub> monounsaturated fatty acid (oleic acid) (Supplementary Fig. 3.12a–d and Supplementary Table 3.5). Other saturated fatty acids such as C<sub>20</sub> fatty acid (arachidic acid) and C<sub>18</sub> fatty acid (stearic acid) are identified in 50% and 100% of the samples respectively (Supplementary Table 3.5). Length of the fatty acid C–C chains ranges from 12 to 22 carbons, 90% of the fatty acids have even number of carbons and 70% were saturated (Supplementary Table 3.5). The next group of molecules in terms of relative abundance were straight-chained hydrocarbons, more specifically, alkanes, dienes and cyclic alkanes (Supplementary Fig. 3.12a–d and Supplementary Table 3.5). None of these molecules are found in 100% of the samples: C<sub>27</sub> alkane is found in 85.71%, C<sub>29</sub> alkane in 71.43% and cyclic alkanes vary from 42.86% to 71.43% of the samples (Supplementary Table 3.5). Length of the hydrocarbon C–C chains ranges from 17 to 29 carbons and 100% of the hydrocarbons are composed of an even number of carbons (Supplementary Table 3.5). Sterols are the highest molecular weight molecules identified in the samples of *T. velutinus*, the most abundant being cholesterol (C<sub>27</sub> sterol)

which is described in 57.14% of the samples (Supplementary Fig. 3.12b–d and Supplementary Table 3.5). Ecdysone (20-hydroxyecdysone) is found in 42.86% of the samples analyzed (Supplementary Table 3.5). Sugars with a trimethylsilyl group are encountered in 28.57% of the samples, when present in a sample, they are usually reoccurring within the same sample (Supplementary Fig. 3.12a–d and Supplementary Table 3.5). *T. velutinus* is the only taxon analyzed containing an alcohol molecule (C<sub>19</sub> alcohol) in the cuticle of 28.57% of the samples (Supplementary Table 3.5). Phenolic acid (protocatechuic acid) is described in 42.88% of the samples (Supplementary Table 3.5). Small phthalate peaks are identified in 57.14% of the samples (Supplementary Fig. 3.12b–c and Supplementary Table 3.5) and four unknown molecules were noted in one sample each (Supplementary Table 3.5).

Untreated sample of *T. velutinus* (n = 1) shows C<sub>18</sub> monounsaturated fatty acid and C<sub>16</sub> saturated fatty acid with the highest relative abundance in the untreated sample (Supplementary Fig. 3.12a and Supplementary Table 3.7). Additional abundant molecules in untreated samples are C<sub>16</sub> monounsaturated fatty acid and C<sub>18</sub> polyunsaturated fatty acid (Supplementary Fig. 3.12a and Supplementary Table 3.7). Sugar molecules are encountered in very high relative abundance and are reoccurring throughout the chromatogram (Supplementary Fig. 3.12a and Supplementary Table 3.7). A molecule of benzoic acid (protocatechuic acid) is identified in this sample (Supplementary Fig. 3.12a and Supplementary Table 3.7).

After 60 days of decay, control samples of *T. velutinus* ( $n = 2$ ) show  $C_{16}$  saturated fatty acid,  $C_{18}$  saturated fatty acid and  $C_{18}$  monounsaturated fatty acid with the highest relative abundance in 100% of the samples (Supplementary Fig. 3.12b and Supplementary Table 3.7). Other abundant molecules are  $C_{19}$  and  $C_{20}$  saturated fatty acids present in 100% of the samples (Supplementary Fig. 3.12b and Supplementary Table 3.7). Hydrocarbons such as  $C_{27}$  and  $C_{29}$  alkanes and cyclic alkanes are identified in high relative abundances in the 100% of the samples (Supplementary Fig. 3.12b and Supplementary Table 3.7). Sugars are found in low relative abundance in 50% of the neutral decayed samples (Supplementary Fig. 3.12b and Supplementary Table 3.7). The steroid ecdysone (20-hydroxyecdysone) is identified in 50% of the samples (Supplementary Table 3.7). Cholesterol ( $C_{27}$  sterol) molecules are found in 100% of the samples decayed in neutral pH conditions (Supplementary Fig. 3.12b and Supplementary Table 3.7). Acidic samples ( $n = 2$ ) show  $C_{18}$  monounsaturated fatty acid and  $C_{16}$  saturated fatty acid with the highest relative abundance in 100% of the samples (Supplementary Fig. 3.12c and Supplementary Table 3.7). Straight-chained hydrocarbons, especially alkanes, with odd numbers of C numbers ranging from 25 to 29 are very abundant in acidic decayed samples, cyclic alkanes are relatively less abundant in these samples (Supplementary Fig. 3.12c and Supplementary Table 3.7). Sugars are identified in 100% of the samples with very low relative abundance (Supplementary Fig. 3.12c and Supplementary Table 3.7). A molecule of benzoic acid (protocatechuic acid) is found in one of the acidic decayed samples (Supplementary Table 3.7). Cholesterol ( $C_{27}$



sterol) molecules are identified with high relative abundance in 100% of the samples (Supplementary Fig. 3.12c and Supplementary Table 3.7). Samples decayed in basic pH conditions ( $n = 2$ ) show  $C_{18}$  monounsaturated fatty acid,  $C_{16}$  saturated fatty acid and  $C_{18}$  saturated fatty acid with the highest relative abundance (Supplementary Fig. 3.12d and Supplementary Table 3.7). Straight-chained hydrocarbons, especially alkanes, with odd numbers of C numbers ranging from 25 to 29 are very abundant in acidic decayed samples; cyclic alkanes are also abundant in these samples (Supplementary Fig. 3.12d and Supplementary Table 3.7). Sugars are present in one of the samples decayed in basic conditions (Supplementary Fig. 3.12d and Supplementary Table 3.7). A molecule of benzoic acid (protocatechuic acid) is found in one of the samples decayed in basic conditions (Supplementary Table 3.7). Sterols are not identified in any of the samples decayed in basic pH conditions (Supplementary Table 3.7).

**A3.1.3.5. *Pyrrhocoris apterus*.** Chromatograms of the cuticles of *P. apterus* ( $n = 6$ ) show that the highest concentrations of lipid molecules in the cuticle corresponding to fatty acids and cyclic hydrocarbons corresponding to some variety of alkane (Supplementary Fig. 3.13 and Supplementary Table 3.6). The largest peak in 100% of the samples is identified as  $C_{16}$  saturated fatty acid (palmitic acid) (Supplementary Fig. 3.13a–c and Supplementary Table 3.6). Other saturated fatty acids such as  $C_{18}$  fatty acid (stearic acid) and  $C_{12}$  fatty acid (linoleic acid) are identified in 100% and 50% respectively (Supplementary Table 3.6). An interesting molecule encountered in these samples is di-fatty acid, which is not found in any other taxa analyzed and in *P. apterus* is identified in 50% of the

samples (Supplementary Table 3.6). Length of the fatty acid C–C chains ranges from 12 to 18 carbons, 100% of the fatty acids had even numbers of carbons and 60% are saturated (Supplementary Table 3.6). The next group of molecules in terms of relative abundance are straight-chained hydrocarbons, more specifically, alkanes, dienes and cyclic alkanes (Supplementary Fig. 3.13a–c and Supplementary Table 3.6). Cyclic alkanes are present in variable numbers between 33.33% and 66.67% of the samples (Supplementary Table 3.6). Alkanes and alkenes have relative low C number compared with other taxa analyzed: C<sub>19</sub> alkane and alkene are found in 16.67% while C<sub>20</sub> diene in 50% (Supplementary Table 3.6). Length of the hydrocarbon C–C chains ranges from 12 to 16 carbons and 100% of the hydrocarbons are composed of an odd number of carbons (Supplementary Table 3.6). Sterols were again the highest molecular weight molecules identified in the samples of *P. apterus*, the most abundant being campesterol (C<sub>28</sub> sterol) and  $\beta$ -sitosterol (C<sub>29</sub> sterol) which are described in 50% of the samples (Supplementary Table 3.6). Ecdysone (20-hydroxyecdysone) is found in 14.28% of the samples analyzed (Supplementary Table 3.6). Sugars with a trimethylsilyl group are encountered in 83.33% of the samples (Supplementary Fig. 3.13c and Supplementary Table 3.6). A partially identified vitamin A-like molecule is found in 50% of the samples (Supplementary Table 3.6). Small phthalate and butadiene peaks were identified in 100% and 50% of the samples respectively (Supplementary Fig. 3.13a–c and Supplementary Table 3.6) and two unknown molecules were noted in 33.33% of the samples (Supplementary Table 3.6).

Samples of *P. apterus* (n = 2) decayed in neutral pH conditions show C<sub>16</sub> and C<sub>18</sub> saturated fatty acids with the highest relative abundance in 100% of the samples After 60 days of decay (Supplementary Fig. 3.13a and Supplementary Table 3.7). Di-fatty acid is identified in 50% of the samples (Supplementary Table 3.7). Straight-chained hydrocarbons are rarely present, although cyclic alkanes are identified in high relative abundance in 100% of the samples (Supplementary Fig. 3.13a and Supplementary Table 3.7). Sugars are encountered in low relative abundance in 50% of the samples decayed in neutral conditions (Supplementary Table 3.7). Sterols are found in high relative abundance: cholesterol (C<sub>27</sub> sterol), campesterol (C<sub>28</sub> sterol) and  $\beta$ -sitosterol (C<sub>29</sub> sterol) are found in 50%, 50% and 100% of the samples respectively (Supplementary Fig. 3.13a and Supplementary Table 3.7). The steroid ecdysone (20-hydroxyecdysone) is identified in 100% of the samples (Supplementary Table 3.7). A molecule similar to benzoic acid, possibly 4-ethoxy-ethyl-ester benzoic acid is identified in 100% of the samples decayed in neutral pH conditions (Supplementary Table 3.7). Acidic samples (n = 2) show C<sub>16</sub> saturated fatty acid with the highest relative abundance in 100% of the samples (Supplementary Fig. 3.13b and Supplementary Table 3.7). Further abundant molecules are C<sub>18</sub> monounsaturated fatty acid, C<sub>18</sub> saturated fatty acid and C<sub>18</sub> polyunsaturated fatty acid (Supplementary Fig. 3.13b and Supplementary Table 3.7). Di-fatty acid is identified in 50% of the samples (Supplementary Table 3.7). Cyclic hydrocarbons are found in high relative abundance in 100% of the samples (Supplementary Fig. 3.13b and Supplementary Table 3.7). Sugar

molecules are identified in only one sample with very low relative abundance (Supplementary Table 3.7). Sterols are abundant in samples decayed in acidic pH conditions; cholesterol (C<sub>27</sub> sterol), campesterol (C<sub>28</sub> sterol) and  $\beta$ -sitosterol (C<sub>29</sub> sterol) are found with high relative abundance in 50%, 50% and 100% of the samples respectively (Supplementary Fig. 3.13b and Supplementary Table 3.7). The steroid Ecdysone (20-hydroxyecdysone) is found in 100% of the samples (Supplementary Table 3.7). A molecule similar to benzoic acid, possibly 4-ethoxy-ethyl-ester benzoic acid is identified in 50% of the samples decayed in acidic pH conditions (Supplementary Table 3.7). Samples decayed in basic pH conditions (n = 2) show C<sub>16</sub> saturated fatty acid with high relative abundance in 100% of the samples (Supplementary Fig. 3.13c and Supplementary Table 3.7). Another abundant molecule is C<sub>18</sub> saturated fatty acid (Supplementary Fig. 3.13c and Supplementary Table 3.7). Hydrocarbons are not abundant in these samples; cyclic alkanes are found in small relative abundances in 100% of the samples (Supplementary Fig. 3.13c and Supplementary Table 3.7). Sugars are present in low relative abundance in 100% of the samples (Supplementary Fig. 3.13c and Supplementary Table 3.7). The steroid Ecdysone (20-hydroxyecdysone) is found in 50% of the samples (Supplementary Table 3.7). A molecule similar to benzoic acid, possibly 4-ethoxy-ethyl-ester benzoic acid is identified in 50% of the samples (Supplementary Table 3.7). Sterols are found in 50% of the samples decayed in basic pH conditions; campesterol (C<sub>28</sub> sterol) and  $\beta$ -sitosterol (C<sub>29</sub> sterol) are found in relative high abundance (Supplementary Table 3.7).

#### A3.1.4. Multivariate analysis.

**A3.1.4.1. Principal Component Analysis.** PCA results show that *H. axyrids* occupy a large, individual region in the chemospace (Supplementary Fig. 3.14a). *L. decemlineata* occupies a large region in the chemospace, which is overlapped by *G. goliathus*, *T. velutinus* and *P. apterus* (Supplementary Fig. 3.14a). When looking at individual experimental conditions *H. axyridis* displays a clear differentiation between 60 and 180 days decayed samples (Supplementary Fig. 3.14b). Samples decayed for 60 days (filled dots on the chemospace) show a wider occupancy of the area in the chemospace and are partially separated by conditions of decay (Supplementary Fig. 3.14b). There is a certain overlap among samples decayed in neutral (grey) and those samples decayed in basic (blue) conditions (Supplementary Fig. 3.14b). Similarly, there is an overlap among samples decayed in acidic (orange) conditions and untreated (brown) samples (Supplementary Fig. 3.14b). Samples decayed for 180 days (unfilled dots on the chemospace) occupy a smaller space despite the conditions of decay (Supplementary Fig. 3.14b). Samples of *L. decemlineata* were only decayed for 60 days, yet again the same overlap among samples decayed in acidic (orange) and neutral (grey) conditions can be observed in the chemospace (Supplementary Fig. 3.14c). Untreated sample (brown) of *L. decemlineata* plots in a completely different region of the chemospace, far away from all the decayed samples (Supplementary Fig. 3.14c). *G. goliathus* data is more widespread on the chemospace than data from other taxa; both basic (blue) and acidic (orange) decayed samples

overlap with samples decayed in neutral (grey) conditions and untreated samples are not clearly differentiated in the chemospace as in other taxa (Supplementary Fig. 3.14d). The chemospace of *T. velutinus* displays a clear differentiation between basic (blue) decayed and untreated (brown) samples while samples decayed in acidic (orange) and neutral (grey) conditions partially share the same space in the chemospace (Supplementary Fig. 3.14e). The chemospace in *P. apterus* displays a clear differentiation between basic (blue), acidic (orange) and neutral (grey) conditions (Supplementary Fig. 3.14f). There is no data for untreated samples of *P. apterus*. In summary, the data indicates that there is a chemical control in the cuticles of the insects analyzed related to the conditions of decay undertaken by the sample.

These taxonomical and chemical controls are driven by differences in variability of the lipid molecules found in the cuticles of these insects, especially fatty acids. For instance, in *H. axyridis* cuticles from all experimental conditions are relatively enriched in C<sub>25</sub> and C<sub>27</sub> straight-chained hydrocarbons (Supplementary Fig. 3.14a, g). Samples of all other taxa (*L. decemlineata*, *G. goliathus*, *T. velutinus* and *P. apterus*) are relatively enriched in C<sub>16</sub> fatty acids and cyclic alkanes independently of the conditions of treatment (Supplementary Fig. 3.14a, g). In terms of differences in treatment conditions, *H. axyridis* samples decayed in neutral and basic pH conditions for 60 days are relatively enriched in straight-chained hydrocarbons with odd number of C numbers ranging from 23 to 31 (Supplementary Fig. 3.14b, h). Samples decayed in acidic conditions and untreated samples are relatively enriched in high C number dienes and C<sub>18</sub>

fatty acids (Supplementary Fig. 3.14b, h). At the same time, samples of *H. axyridis* decayed for 180 days are relatively enriched in C<sub>18</sub> fatty acids independently of the conditions of treatment (Supplementary Fig. 3.14a–b, g–h). The untreated sample of *L. decemlineata* is enriched in sugar molecules and C<sub>20</sub> fatty acids (Supplementary Fig. 3.14c, i). Samples decayed in acidic and neutral pH conditions are relatively enriched in cyclic alkanes and C<sub>18</sub> fatty acids (Supplementary Fig. 3.14c, i) and samples decayed in basic conditions are enriched in C<sub>16</sub> and C<sub>18</sub> fatty acids (Supplementary Fig. 3.14c, i). Untreated samples of *G. goliathus* are relatively enriched in C<sub>18</sub> fatty acids (Supplementary Fig. 3.14d, j). Samples decayed in acidic and basic conditions are enriched in C<sub>16</sub> and C<sub>18</sub> fatty acids (Supplementary Fig. 3.14d, j) and neutrally decayed samples in cyclic alkanes and C<sub>18</sub> fatty acids (Supplementary Fig. 3.14d, j). The untreated sample of *T. velutinus* is enriched in sugar molecules and C<sub>18</sub> fatty acids (Supplementary Fig. 3.14e, k). Samples decayed in neutral conditions are enriched in alkanes, both straight-chained and cyclic, cholesterol, ecdysone and protocatechuic acid (Supplementary Fig. 3.14e, k). Acidic samples are also enriched in cholesterol, ecdysone and protocatechuic acid but accompanied by C<sub>16</sub> and C<sub>18</sub> fatty acids (Supplementary Fig. 3.14e, k). Samples of *T. velutinus* decayed in basic conditions are relatively enriched in C<sub>16</sub> fatty acids and C<sub>29</sub> alkane (Supplementary Fig. 3.14e, k). Samples of *P. apterus* decayed in neutral conditions are relatively enriched in cyclic alkanes and C<sub>16</sub> fatty acids (Supplementary Fig. 3.14f, l). Samples decayed in acidic media are enriched in ecdysone,  $\beta$ -sitosterol and C<sub>27</sub> alkane (Supplementary Fig. 3.14f, l). Samples decayed in basic conditions are

relatively enriched in C<sub>18</sub> fatty acids, C<sub>20</sub> diene and sugar molecules (Supplementary Fig. 3.14f, l).

**A3.1.4.2. Linear Discriminant Analysis.** LDA results show *H. axyridis* occupies a large region in the chemospace, which is overlapped by *L. decemlineata*, *G. goliathus*, *T. velutinus* and *P. apterus* (Supplementary Fig. 3.15a). When looking at individual experimental conditions *H. axyridis* displays a clear differentiation between 60 and 180 days decayed samples (Fig. 3.5i and Supplementary Fig. 3.15b). Samples decayed for 60 days (filled dots on the chemospace) show a wider occupancy of the area in the chemospace and are fully separated by conditions of decay (Fig. 3.5i and Supplementary Fig. 3.15b). There is no overlap among samples decayed in neutral (grey), acidic (orange) and basic (blue) conditions (Fig. 3.5i and Supplementary Fig. 3.15b). Untreated samples plot in a region closer to acidic decayed samples and generally, there is a good spread of the data in the chemospace (Fig. 3.5i and Supplementary Fig. 3.15b). Samples decayed for 180 days (unfilled dots on the chemospace) occupy a smaller space indistinctively of the conditions of decay (Fig. 3.5i and Supplementary Fig. 3.15b), samples decayed in basic and neutral conditions partially overlap while acidic samples plot separately (Fig. 3.5i and Supplementary Fig. 3.15b). Untreated samples of *L. decemlineata* (brown) plot in a different region of the chemospace than that of decayed samples (Supplementary Fig. 3.15c). There is a partial overlap among samples decayed in acidic (orange) and basic (blue) conditions and neutral (grey) decayed samples also plot closely in the chemospace (Supplementary Fig. 3.15c). *G. goliathus* data is more widespread on the chemospace than data from other



taxa; both untreated (brown) and acidic (orange) decayed samples overlap with samples decayed in neutral (grey) conditions and basic (blue) samples plot in a more defined region of the chemospace (Supplementary Fig. 3.15d). The untreated sample of *T. velutinus* (brown) is confined to a solitary region of the chemospace furtherer from samples decayed in neutral (grey) conditions than those decayed in basic (blue) or acidic (orange) conditions (Supplementary Fig. 3.15e). There is a partial overlap between samples decayed in basic and acidic conditions (Supplementary Fig. 3.15e). The chemospace in *P. apterus* displays an overlap of all samples independent of the conditions of decay (Supplementary Fig. 3.15f). There is no data for untreated samples of *P. apterus*. In summary, the data indicates that there is a chemical control in the cuticles of the insects analyzed related to the conditions of decay undertaken by the sample.

These taxonomical and chemical controls are driven by differences in variability of the lipid molecules found in the cuticles of these insects. For instance, in *H. axyridis* cuticles from all experimental conditions are relatively enriched in the majority of lipid molecules identified in these cuticles (Supplementary Fig. 3.15a, g). Samples of all other taxa (*L. decemlineata*, *G. goliathus*, *T. velutinus* and *P. apterus*) are relatively enriched in butadiene, protocatechuic acid, cholesterol and C<sub>27</sub> alkane and alkene (Supplementary Fig. 3.15a, g). In terms on differences in treatment conditions, *H. axyridis* untreated samples are relatively enriched in sugar molecules, C<sub>29</sub> alkene and C<sub>16</sub> fatty acids (Supplementary Fig. 3.15b, h). Samples decayed in basic pH conditions for 60 days are relatively enriched in C<sub>29</sub> diene, cholesterol and C<sub>27</sub> alkene (Supplementary Fig. 3.15b, h).

Samples decayed in acidic conditions are relatively enriched in high C number hydrocarbons, such as C<sub>29</sub> alkene and diene, and C<sub>16</sub> fatty acids (Supplementary Fig. 3.15b, h). At the same time, samples of *H. axyridis* decayed for 180 days are relatively enriched in sugar molecules, C<sub>29</sub> alkene and C<sub>16</sub> fatty acids (Supplementary Fig. 3.15a–b, g–h). The untreated sample of *L. decemlineata* is enriched in sugar molecules and C<sub>20</sub> fatty acids (Supplementary Fig. 3.15c, i). Samples decayed in acidic and basic pH conditions are relatively enriched in C<sub>19</sub>, C<sub>18</sub> and C<sub>16</sub> fatty acids (Supplementary Fig. 3.15c, i) and samples decayed in neutral pH conditions for 60 days are relatively enriched in C<sub>19</sub> fatty acids, cholesterol and cyclic alkanes (Supplementary Fig. 3.15c, i). Untreated samples of *G. goliathus* are relatively enriched in C<sub>19</sub> fatty acids, cholesterol, cyclic alkanes and C<sub>16</sub> fatty acids (Supplementary Fig. 3.15d, j). Samples decayed in acidic conditions are enriched in C<sub>19</sub> fatty acids and sugar molecules (Supplementary Fig. 3.15d, j). Samples decayed in basic conditions are relatively enriched in cholesterol, cyclic alkane and C<sub>16</sub> fatty acids (Supplementary Fig. 3.15d, j). Neutral decayed samples are enriched in sugar molecules, C<sub>19</sub> and C<sub>18</sub> fatty acids (Supplementary Fig. 3.15d, j). The untreated sample of *T. velutinus* is enriched in C<sub>20</sub> fatty acids, C<sub>25</sub> alkane, sugar molecules and protocatechuic acid (Supplementary Fig. 3.15e, k). Samples decayed in neutral conditions are enriched in alkanes, both straight-chained and cyclic and C<sub>20</sub> fatty acids (Supplementary Fig. 3.15e, k). Acidic samples are relatively enriched in cyclic alkanes, C<sub>16</sub> fatty acids and sugar molecules (Supplementary Fig. 3.15e, k). Samples of *T. velutinus* decayed in basic conditions are relatively enriched in cyclic alkanes, C<sub>16</sub>,

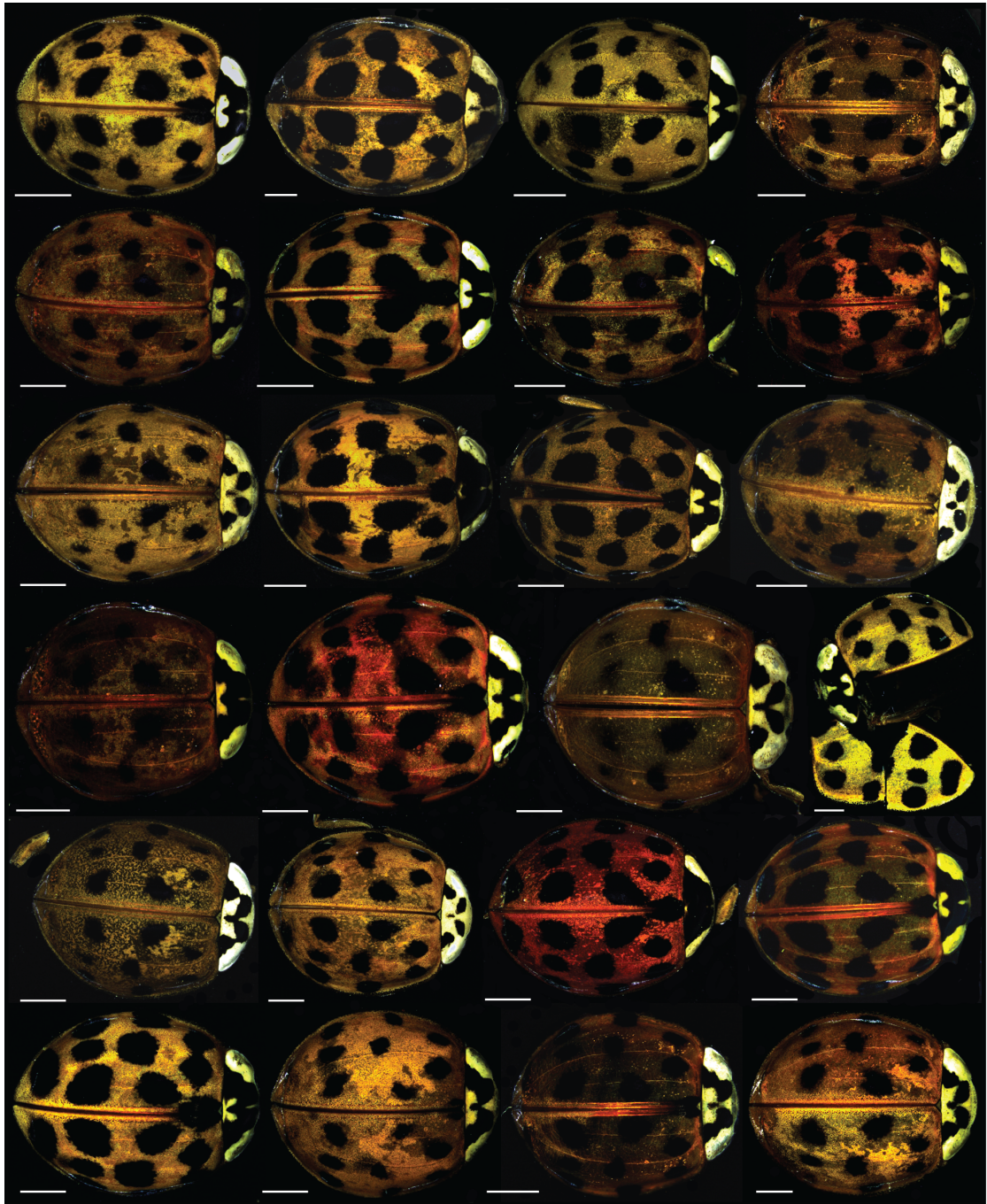
C<sub>18</sub> and C<sub>20</sub> fatty acids (Supplementary Fig. 3.15e, k). Samples of *P. apterus* decayed in neutral conditions are relatively enriched in cyclic alkanes, C<sub>27</sub> fatty acids, cholesterol, campesterol and sugar molecules (Supplementary Fig. 3.15f, l). Samples decayed in acidic media are enriched in C<sub>27</sub> alkane, C<sub>17</sub> fatty acids, ecdysone and C<sub>20</sub> diene (Supplementary Fig. 3.15f, l). Samples decayed in basic conditions are relatively enriched in C<sub>27</sub> alkane, C<sub>16</sub> fatty acids and di-fatty acids (Supplementary Fig. 3.15f, l).

**A3.1.5. High performance liquid chromatography–mass spectrometry.** After analysis of carotenoid content of cuticles of *H. axyridis*, a suite of six recurring carotenoids was detected in the samples—lycopene/β-carotene, α-carotene, β-cryptoxanthin, lutein and zeaxanthin. In the majority of samples (81%), the carotenoid with the highest concentration in the cuticle was identified as lutein (Supplementary Fig. 3.16 and Supplementary Tables 3.8, 3.9). Other carotenoids such as lycopene/β-carotene or zeaxanthin were similarly identified in numerous samples (88% and 65% respectively) but their relative abundances are slightly lesser (Supplementary Fig. 3.16 and Supplementary Tables 3.8, 3.9). In untreated samples, the carotenoid molecule with the highest relative abundance in cuticles is lutein (Supplementary Fig. 3.16a and Supplementary Table 3.9). Zeaxanthin and lycopene/β-carotene are present in much less abundance (Supplementary Fig. 3.16a and Supplementary Table 3.9). There is little dissimilarity in samples decayed in distinct media conditions after 60 days (Supplementary Fig. 3.16b–c and Supplementary Table 3.9). In all samples analyzed, lutein is the molecule with the highest relative abundance followed by lycopene/β-carotene, α-carotene, zeaxanthin and a cryptoxanthin molecule (Supplementary Fig. 3.16b–c and

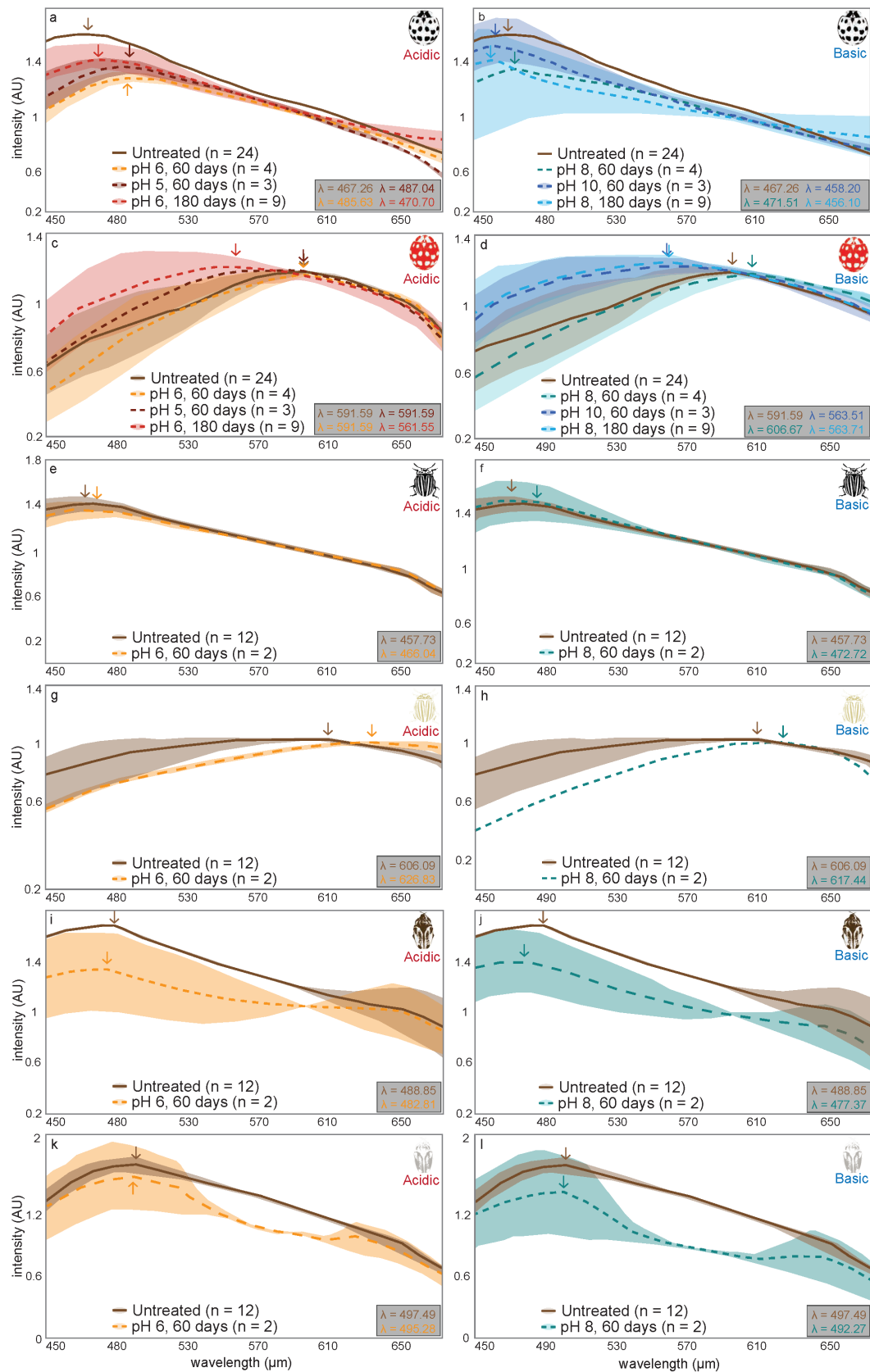
Supplementary Table 3.9). In samples decayed for 120 days, molecules with higher molecular weight are relatively more abundant; peaks of zeaxanthin and lutein combined make up for more than 50% of the total carotenoid content in all samples analyzed (Supplementary Fig. 3.16d–f and Supplementary Table 3.9).

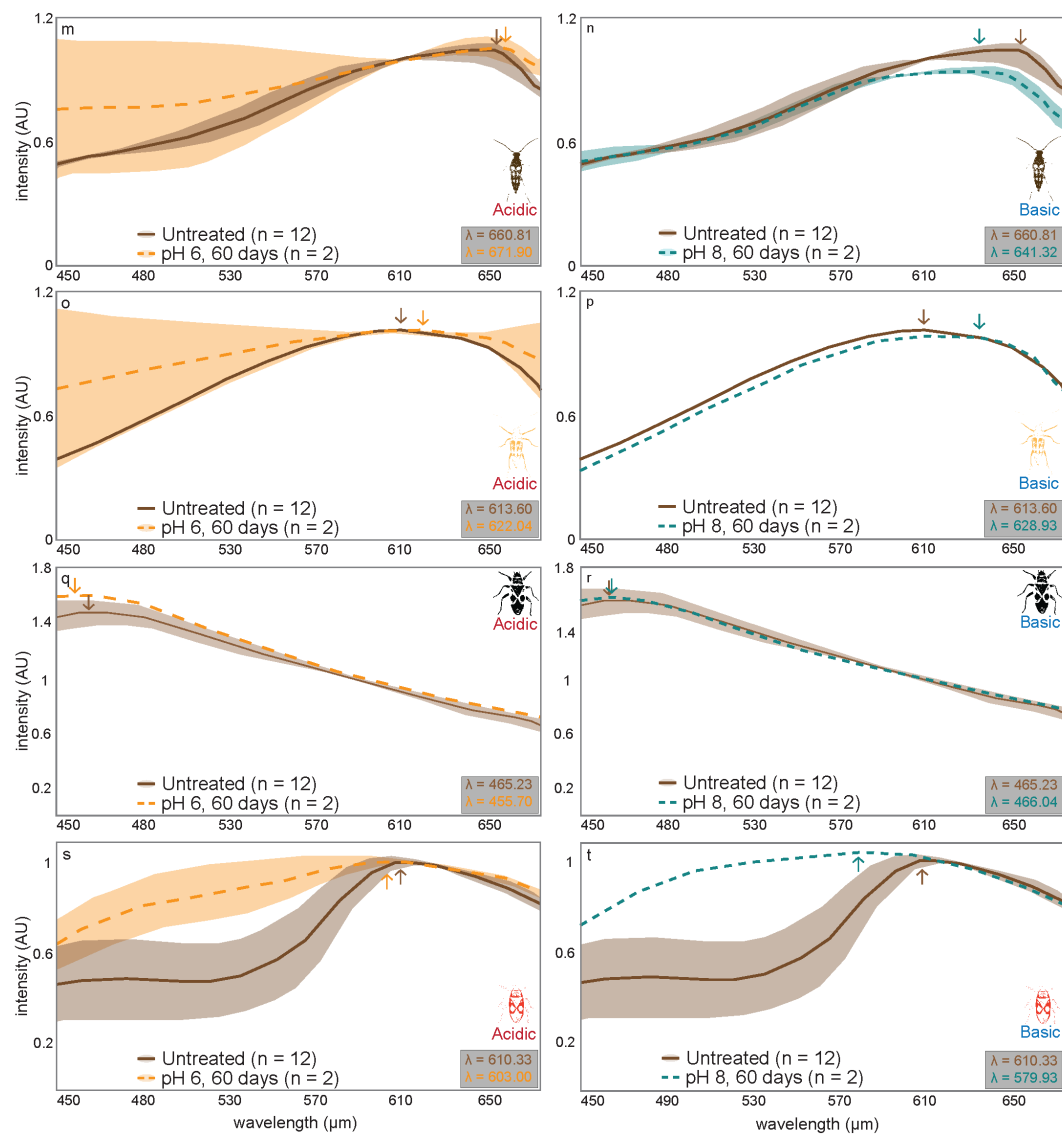
Chromatograms of samples decayed for 180 days are characterized by containing a suite of molecules with low molecular weight, such as lycopene/ $\beta$ -carotene and a suite of unidentified high molecular weight molecules (Supplementary Fig. 3.16g–i and Supplementary Table 3.9).

### **A3.2. Supplementary Figures:**



**Supplementary Figure 3.1.** Light microscopy images showing the high colour plasticity displayed in cuticles of *Harmonia axyridis* before decay commenced. Scale bars: 500  $\mu\text{m}$ .

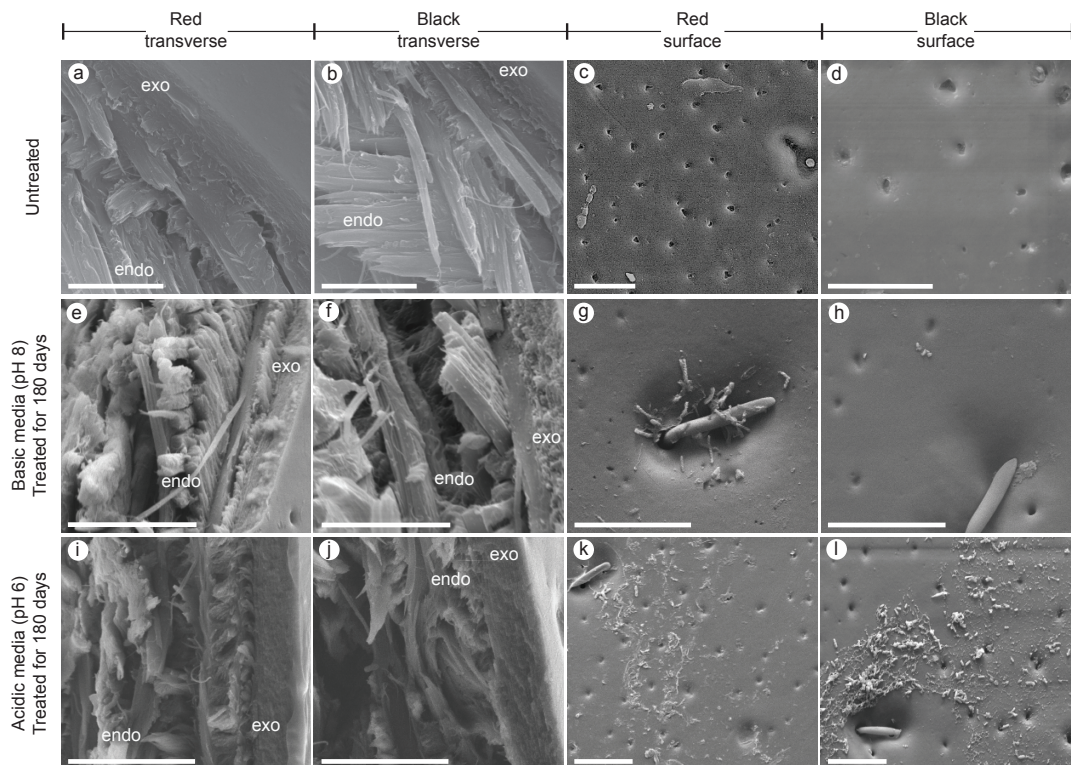




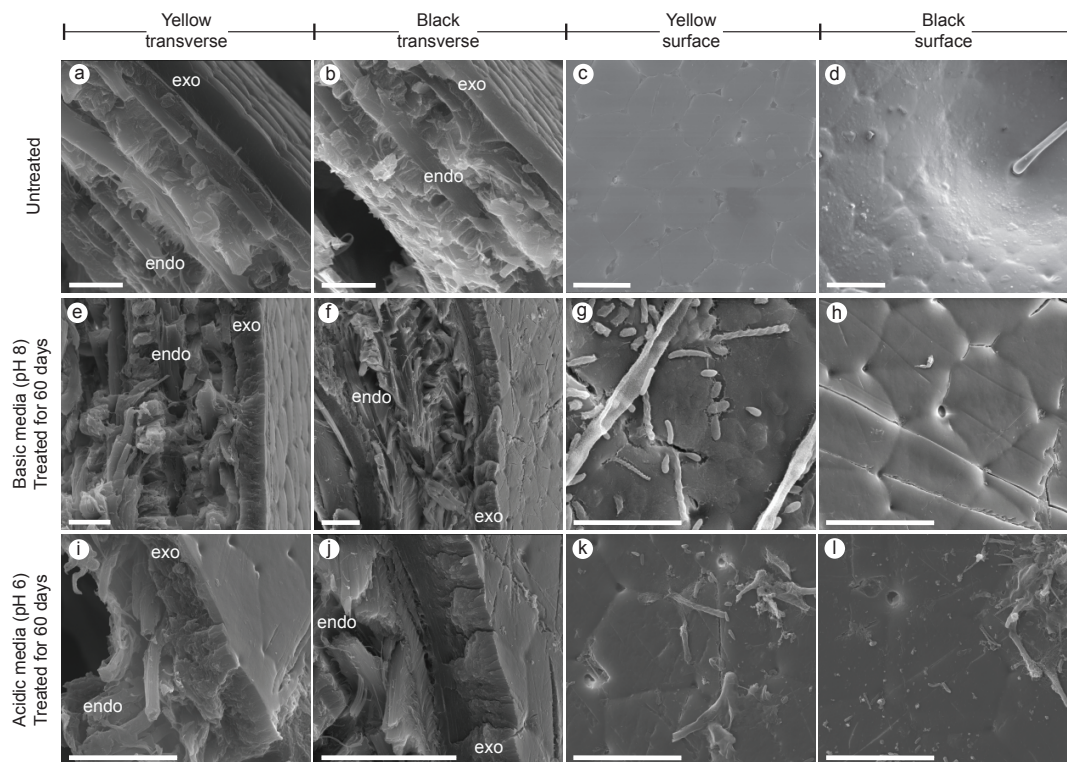
Supplementary Figure 3.2. Caption overleaf.



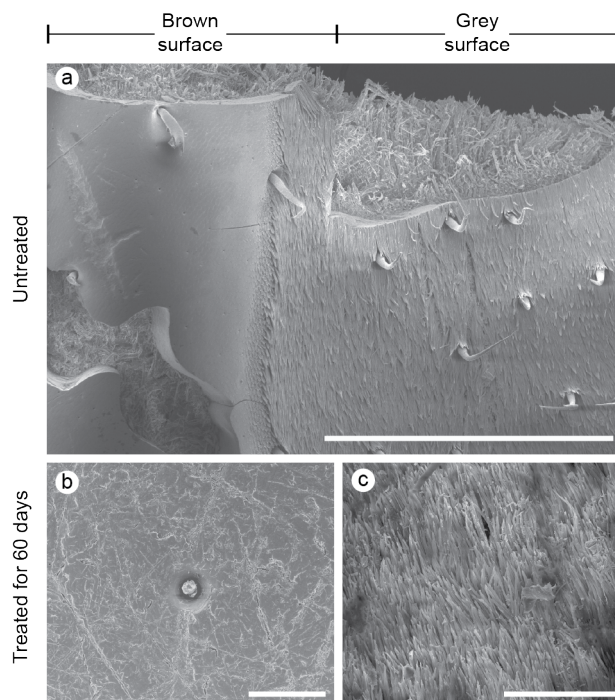
**Supplementary Figure 3.2 (previous page). Changes in colour spectra during decay of *Harmonia axyridis* (a–d), *Leptinotarsa decemlineata* (e–h), *Goliathus goliathus* (i–l), *Typocerus velutinus* (m–p) and *Pyrrhocoris apterus* (q–t). a–d** Cuticle regions from specimens of *H. axyridis* displaying black spots degraded in acidic (a) and basic (b) conditions or red background degraded in acidic (c) and basic (d) conditions. **e–h** Cuticle regions from specimens of *L. decemlineata* displaying black stripes degraded in acidic (e) and basic (f) conditions or yellow stripes degraded in acidic (g) and basic (h) conditions. **i–l** Cuticle regions from specimens of *G. goliathus* displaying dark brown patches degraded in acidic (i) and basic (j) conditions or grey patches degraded in acidic (k) and basic (l) conditions. **m–p** Cuticle regions from specimens of *T. velutinus* displaying orange to brown background degraded in acidic (m) and basic (n) conditions or yellow spots degraded in acidic (o) and basic (p) conditions. **q–t** Cuticle regions from specimens of *P. apterus* displaying black spots degraded in acidic (q) and basic (r) conditions or red background degraded in acidic (s) and basic (t) conditions. Solid and dashed lines denote means and shaded regions denote standard deviation values. Standard deviation for untreated specimens in **a**, **b**, **o** and **p**, specimens treated in acidic media (pH 5) for 60 days in **c**, specimens treated in acidic media (pH 6) for 60 days in **q** and specimens treated in basic media (pH 8) for 60 days in **b**, **h**, **p**, **r** and **t** is minimal and not visible at this scale.



**Supplementary Figure 3.3. Scanning electron micrographs of cuticular ultrastructure of *Harmonia axyridis* during 180 days of decay.** Note prominent endocuticular chitin bundles (e.g., **e**) and exocuticular laminations (e.g., **f**) in treated (**e–f**, **i–j**) relative to untreated (**a–b**) cuticles. Bacteria on red surfaces of decayed specimens (**g**, **k**) are qualitative more abundant and diverse than those observed on black regions of the cuticle (**h–l**). Scale bars 10  $\mu\text{m}$ .

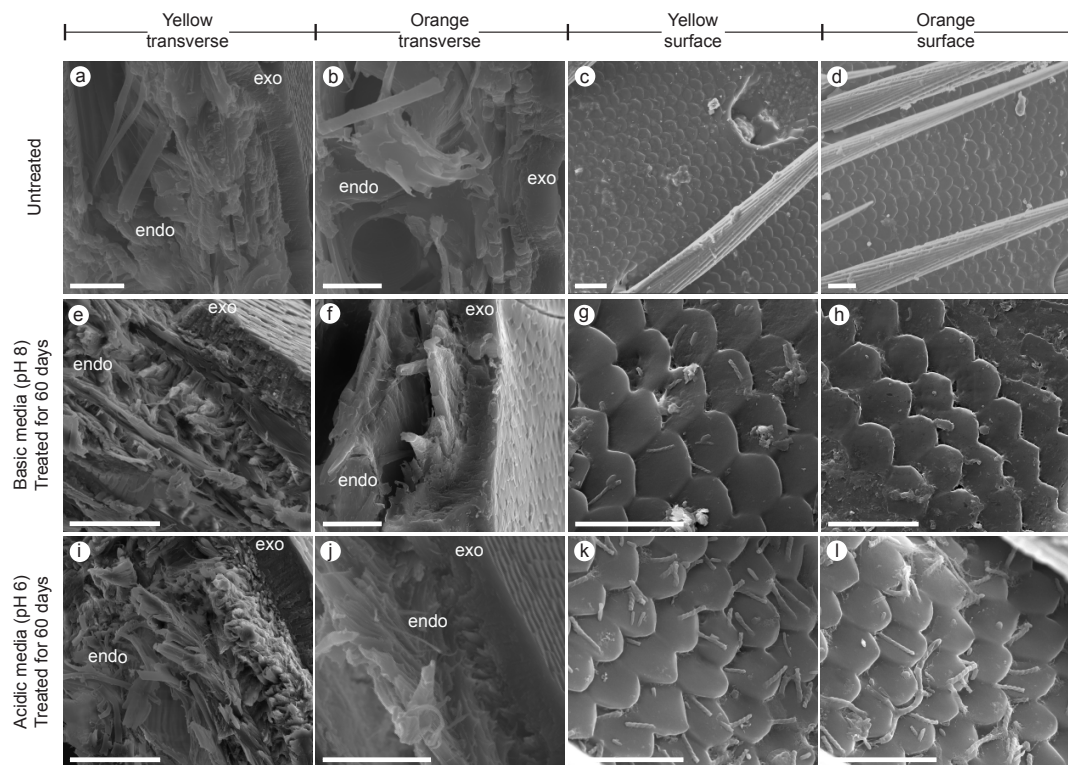


**Supplementary Figure 3.4. Scanning electron micrographs of cuticular ultrastructure of *Leptinotarsa decemlineata* during 60 days of decay.** Note prominent endocuticular chitin bundles (e.g., **e**) and exocuticular laminations (e.g., **i**) in treated (**e–f**, **i–j**) relative to untreated (**a–b**) cuticles. Bacteria on red surfaces of decayed specimens (**g**, **k**) are qualitative more abundant and diverse than those observed on black regions of the cuticle (**h–l**). Scale bars in **a–f**, **h–l** are 10 µm. Scale bar in **g** is 5 µm.

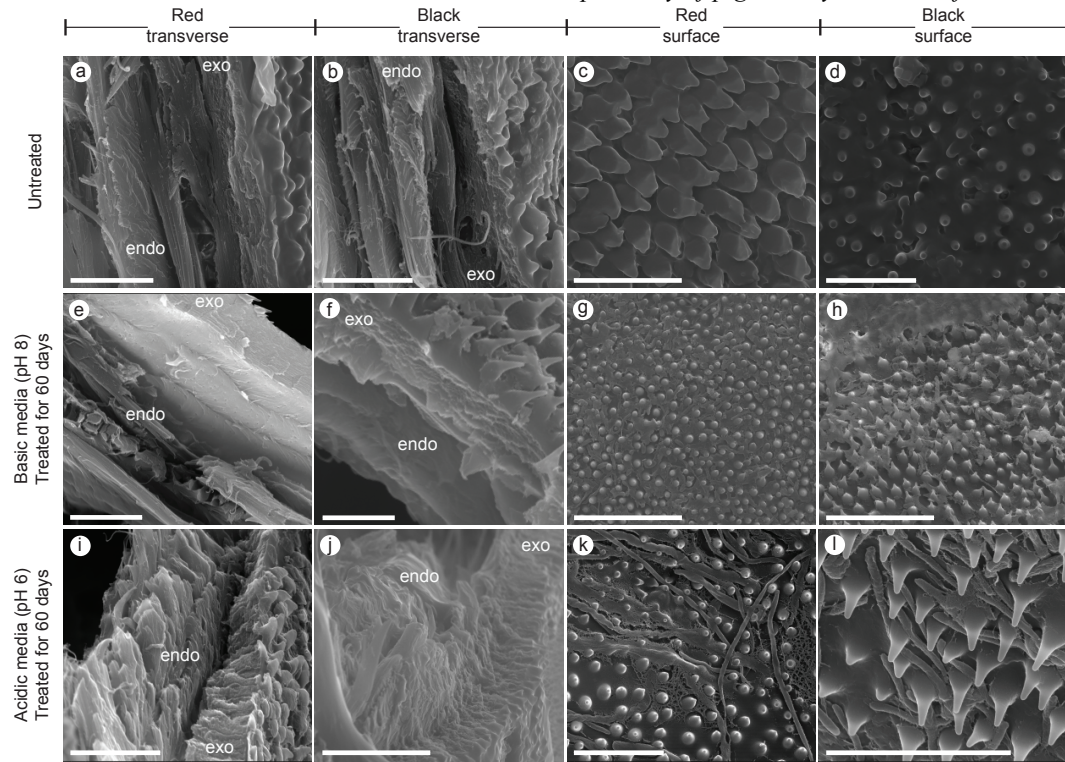


**Supplementary Figure 3.5. Scanning electron micrographs of cuticular ultrastructure of *Goliathus goliathus* during 60 days of decay.** Note distortion of the image caused by charging on the surface of the cuticle (a). After 60 days of decay bacterial growth was not observed in any cuticular region (b–c). Scale bar in a is 500  $\mu\text{m}$ . Scale bars in b–c is 100  $\mu\text{m}$ .



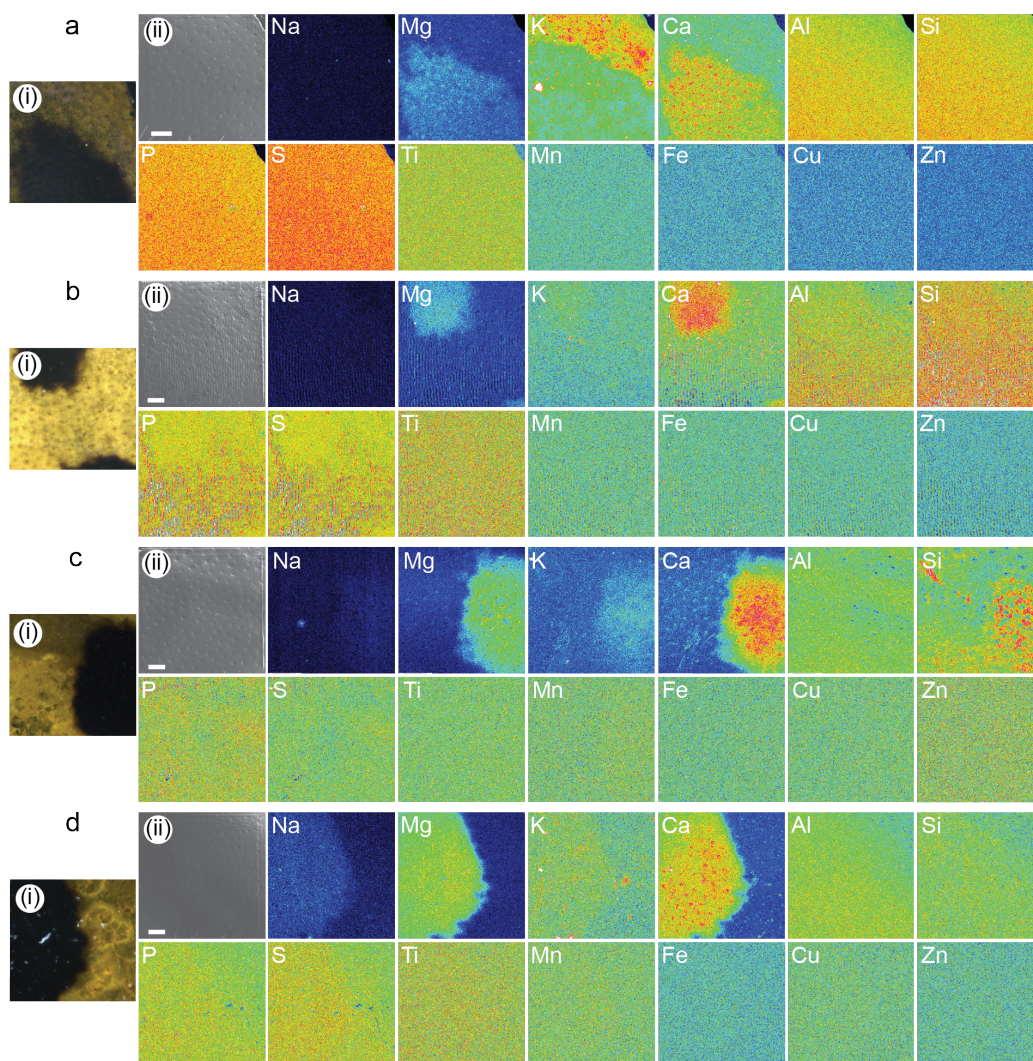


**Supplementary Figure 3.6. Scanning electron micrographs of cuticular ultrastructure of *Typocerus velutinus* during 60 days of decay.** Note prominent endocuticular chitin bundles (e.g., **f**) and exocuticular laminations (e.g., **e**) in treated (**e–f**, **i–j**) relative to untreated (**a–b**) cuticles. Bacteria on yellow surfaces of decayed specimens (**g**, **k**) are qualitative more abundant and diverse than those observed on black regions of the cuticle (**h–l**). Scale bars in **a–b**, **e–f**, **i–j** 20 µm. Scale bars in **c–d**, **g–h**, **k–l** are 10 µm.

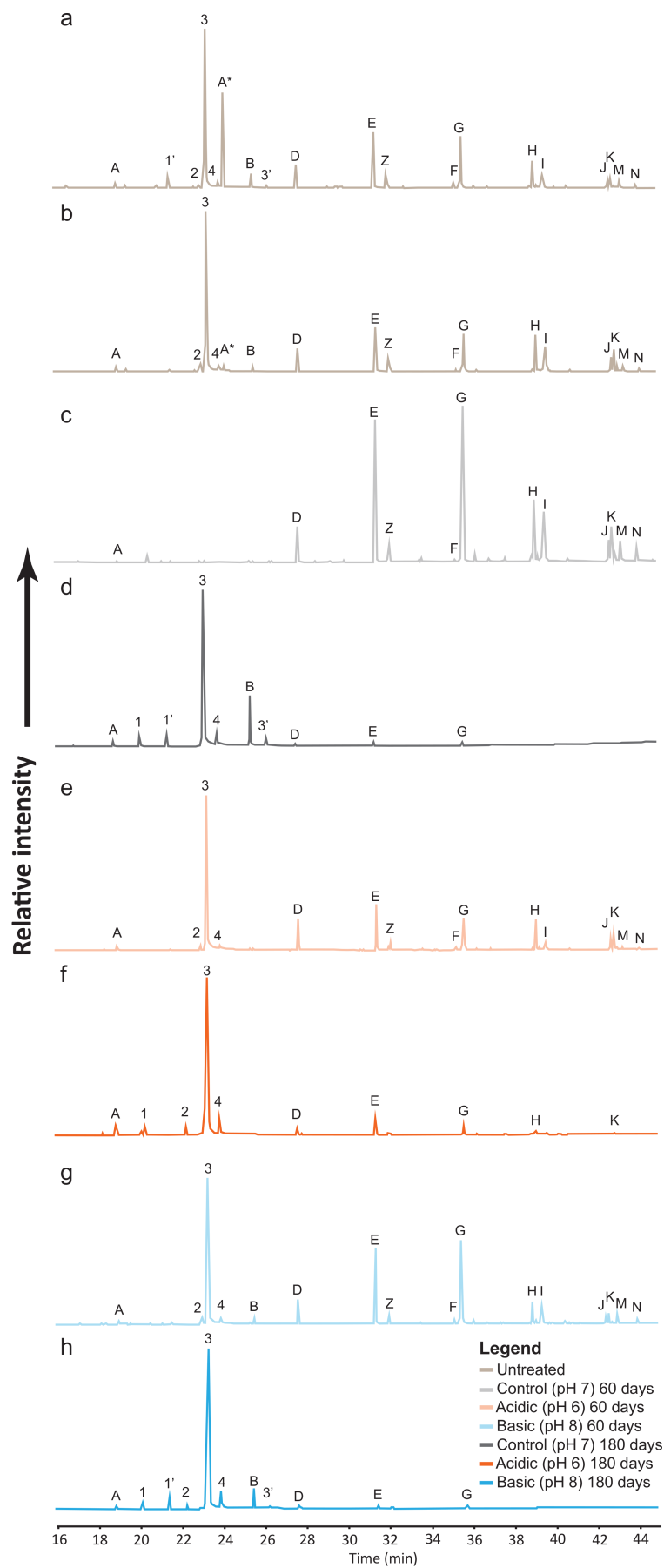


**Supplementary Figure 3.7. Scanning electron micrographs of cuticular ultrastructure of *Pyrrhocoris apterus* during 60 days of decay.** Note prominent endocuticular chitin bundles (e.g., **i**) and exocuticular laminations (e.g., **i**) in treated (**e–f**, **i–j**) relative to untreated (**a–b**) cuticles. Antiglare tubercular structures in the surface of the cuticle (**c–d**, **g–h**, **k–l**). Bacteria are abundant throughout the surface of the cuticle independently of the colour displayed (**g–h**, **k–l**). Scale bars in **a–f**, **i–l** are 5  $\mu\text{m}$ . Scale bars in **g–h** are 10  $\mu\text{m}$ .





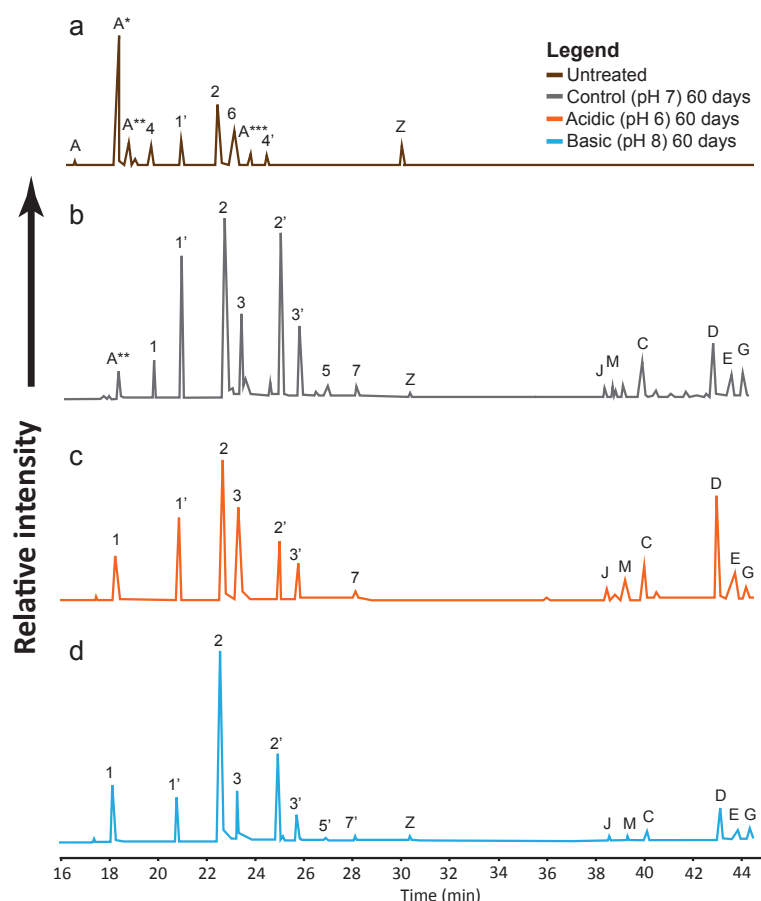
**Supplementary Figure 3.8. Colour patterning and trace element chemistry in cuticles of *Harmonia axyridis* during 60 days of decay. a** Untreated cuticles. **b** Cuticles decayed in neutral media (pH 7), **c** basic media (pH 8) and **d** acidic media (pH 6). **i** Light micrographs of the dorsal surface of the cuticles. **ii** Scanning electron micrographs of regions in **i**. Elemental maps are of regions in **i** and **ii**. Elemental maps for Na, Mg, K and Ca are EPMA maps; maps for all other elements are EDS X-ray maps. Scale bars: 100  $\mu\text{m}$ .



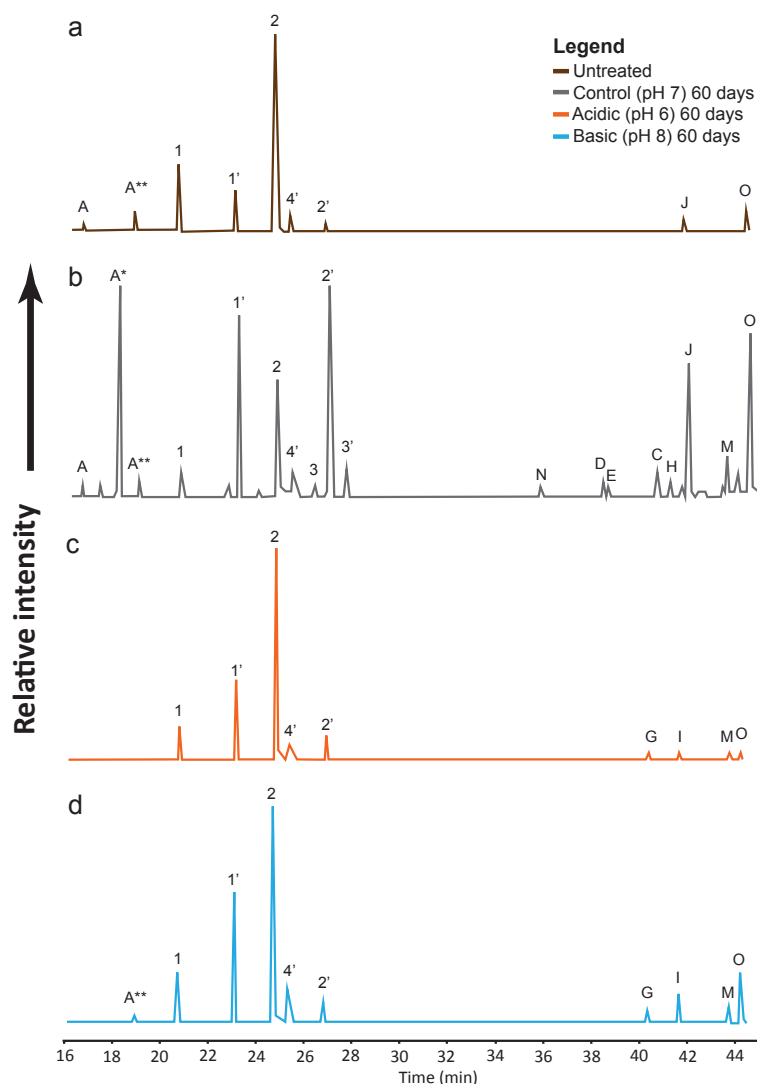
Supplementary Figure 3.9. Caption overleaf.



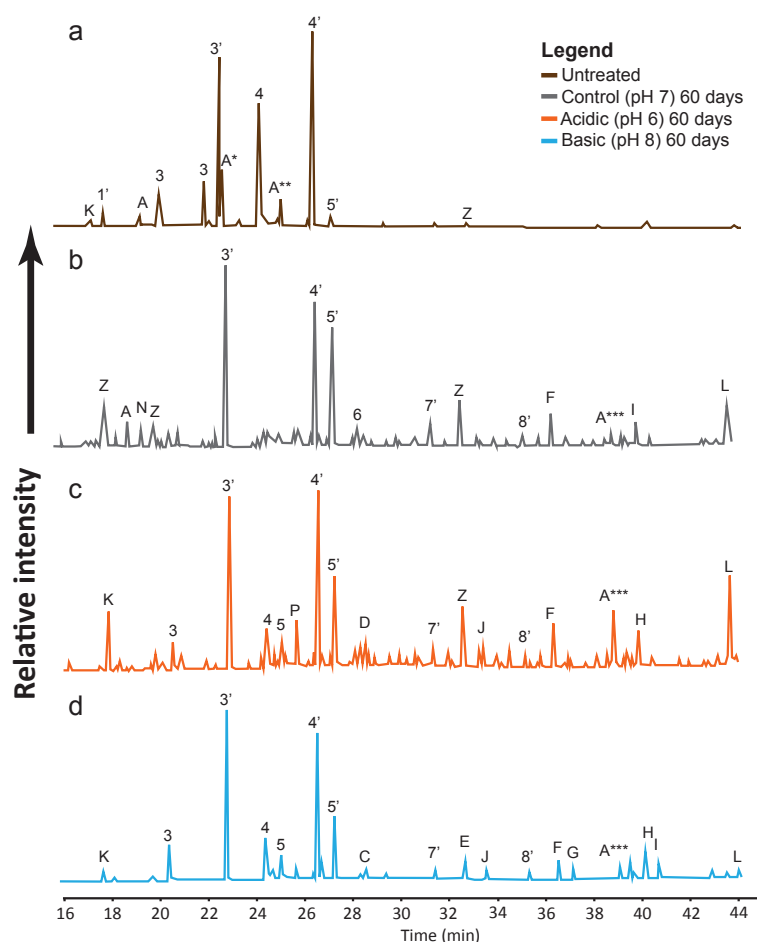
**Supplementary Figure 3.9 (previous page). Gas chromatography-mass spectrometry chromatograms displaying molecular identifications of the largest peaks in *Harmonia axyridis*. a–b** Untreated specimens. **c–d** Specimens decayed in neutral pH conditions for 60 days (**c**) and 180 days (**d**). **e–f** Specimens decayed in acidic pH conditions for 60 days (**e**) and 180 days (**f**). **g–h** Specimens decayed in basic pH conditions for 60 days (**g**) and 180 days (**h**). Peaks represented with numbers were identified as fatty acids, major components of insect cuticle. Peaks represented with letters were identified as sugars, hydrocarbons, sterols and various other taxon-specific compounds. Note that the largest peak in all chromatograms except in **c**, where is non-existent, was identified as C<sub>18</sub> monounsaturated fatty acid. Asterisk following a peak's label indicates recurring occurrence of the compound in the sample. Apostrophe following a peak's label indicates a molecule with a trimethylsilyl (TMS) group. All identifications are listed in Appendix 3, a summary of these identifications are represented in Supplementary Table 3.2.



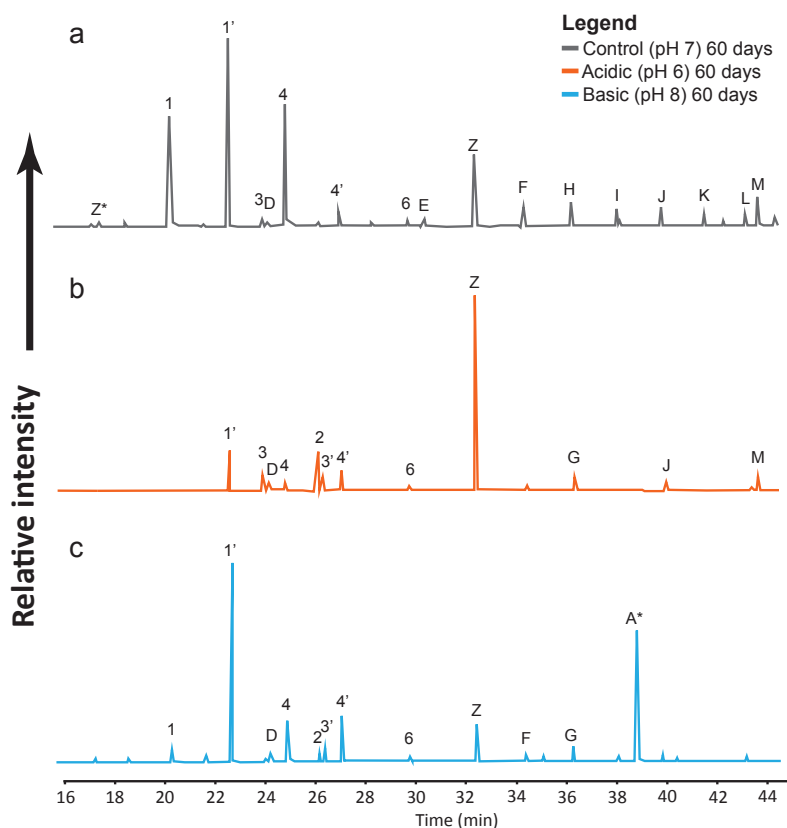
**Supplementary Figure 3.10. Gas chromatography-mass spectrometry chromatograms displaying molecular identifications of the largest peaks in *Leptinotarsa decemlineata*.** **a** Untreated specimen. **b** Specimen decayed in neutral pH conditions for 60 days. **c** Specimen decayed in acidic pH conditions for 60 days. **d** Specimen decayed in basic pH conditions for 60 days. Peaks represented with numbers were identified as fatty acids, major components of insect cuticle. Peaks represented with letters were identified as sugars, hydrocarbons, sterols and various other taxon-specific compounds. Note that the largest peak in all chromatograms except in **a**, where the largest peak is a sugar, was identified as C<sub>18</sub> monounsaturated fatty acid. Asterisk following a peak's label indicates recurring occurrence of the compound in the sample. Apostrophe following a peak's label indicates a molecule with a trimethylsilyl (TMS) group. All identifications are listed in Appendix 3, a summary of these identifications are represented in Supplementary Table 3.3.



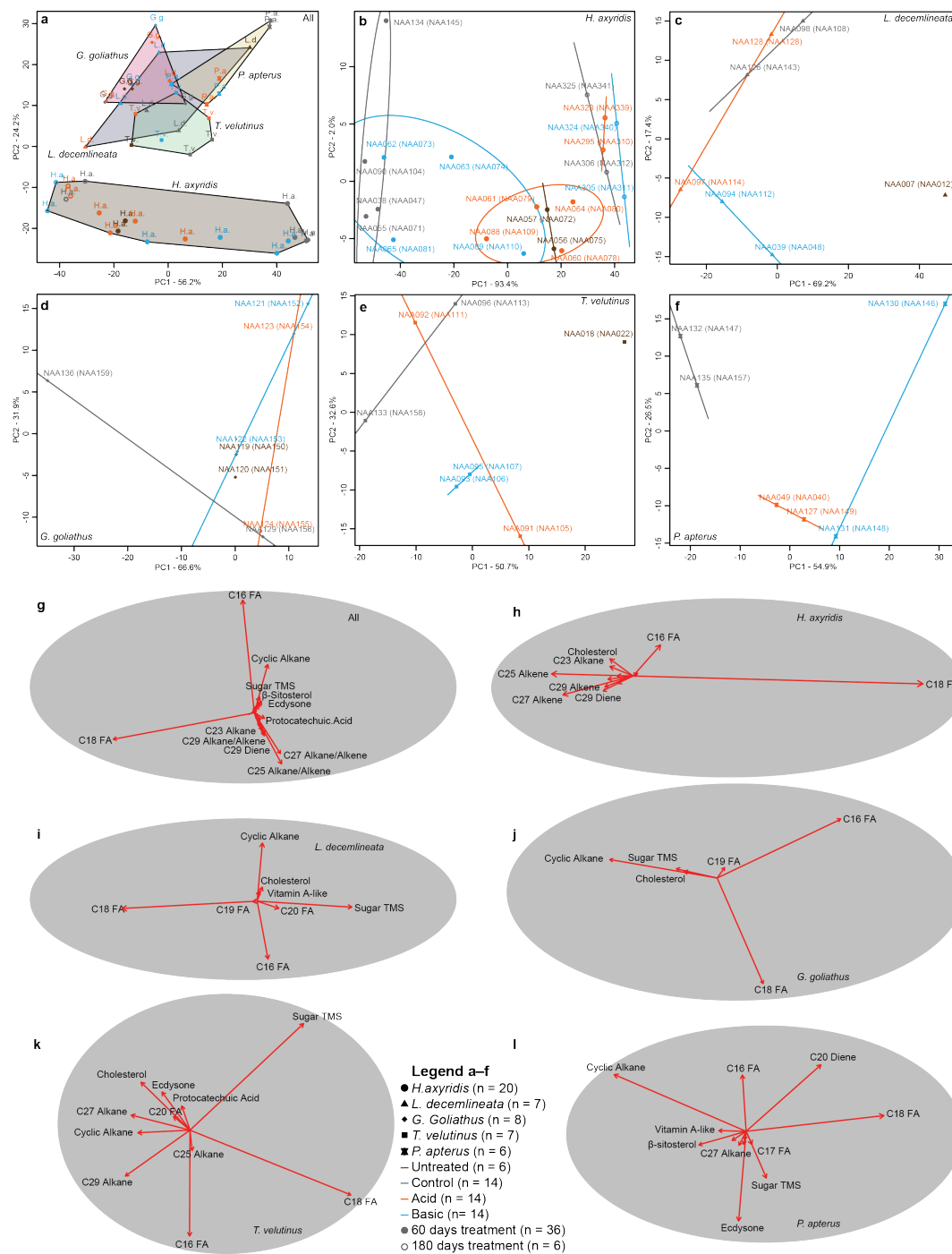
**Supplementary Figure 3.11. Gas chromatography-mass spectrometry chromatograms displaying molecular identifications of the largest peaks in *Goliathus goliathus*.** **a** Untreated specimen. **b** Specimen decayed in neutral pH conditions for 60 days. **c** Specimen decayed in acidic pH conditions for 60 days. **d** Specimen decayed in basic pH conditions for 60 days. Peaks represented with numbers were identified as fatty acids, major components of insect cuticle. Peaks represented with letters were identified as sugars, hydrocarbons, sterols and various other taxon-specific compounds. Note that the largest peak in all chromatograms was identified as C<sub>18</sub> monounsaturated fatty acid. Asterisk following a peak's label indicates recurring occurrence of the compound in the sample. Apostrophe following a peak's label indicates a molecule with a trimethylsilyl (TMS) group. All identifications are listed in Appendix 3, a summary of these identifications are represented in Supplementary Table 3.4.



**Supplementary Figure 3.12. Gas chromatography-mass spectrometry chromatograms displaying molecular identifications of the largest peaks in *Typocerus velutinus*.** **a** Untreated specimen. **b** Specimen decayed in neutral pH conditions for 60 days. **c** Specimen decayed in acidic pH conditions for 60 days. **d** Specimen decayed in basic pH conditions for 60 days. Peaks represented with numbers were identified as fatty acids, major components of insect cuticle. Peaks represented with letters were identified as sugars, hydrocarbons, sterols and various other taxon-specific compounds. Note that the largest peaks in all chromatograms were identified as C<sub>18</sub> monounsaturated fatty acid (**b**, **d**) and C<sub>18</sub> saturated fatty acid (**a**, **c**). Asterisk following a peak's label indicates recurring occurrence of the compound in the sample. Apostrophe following a peak's label indicates a molecule with a trimethylsilyl (TMS) group. All identifications are listed in Appendix 3, a summary of these identifications are represented in Supplementary Table 3.5.

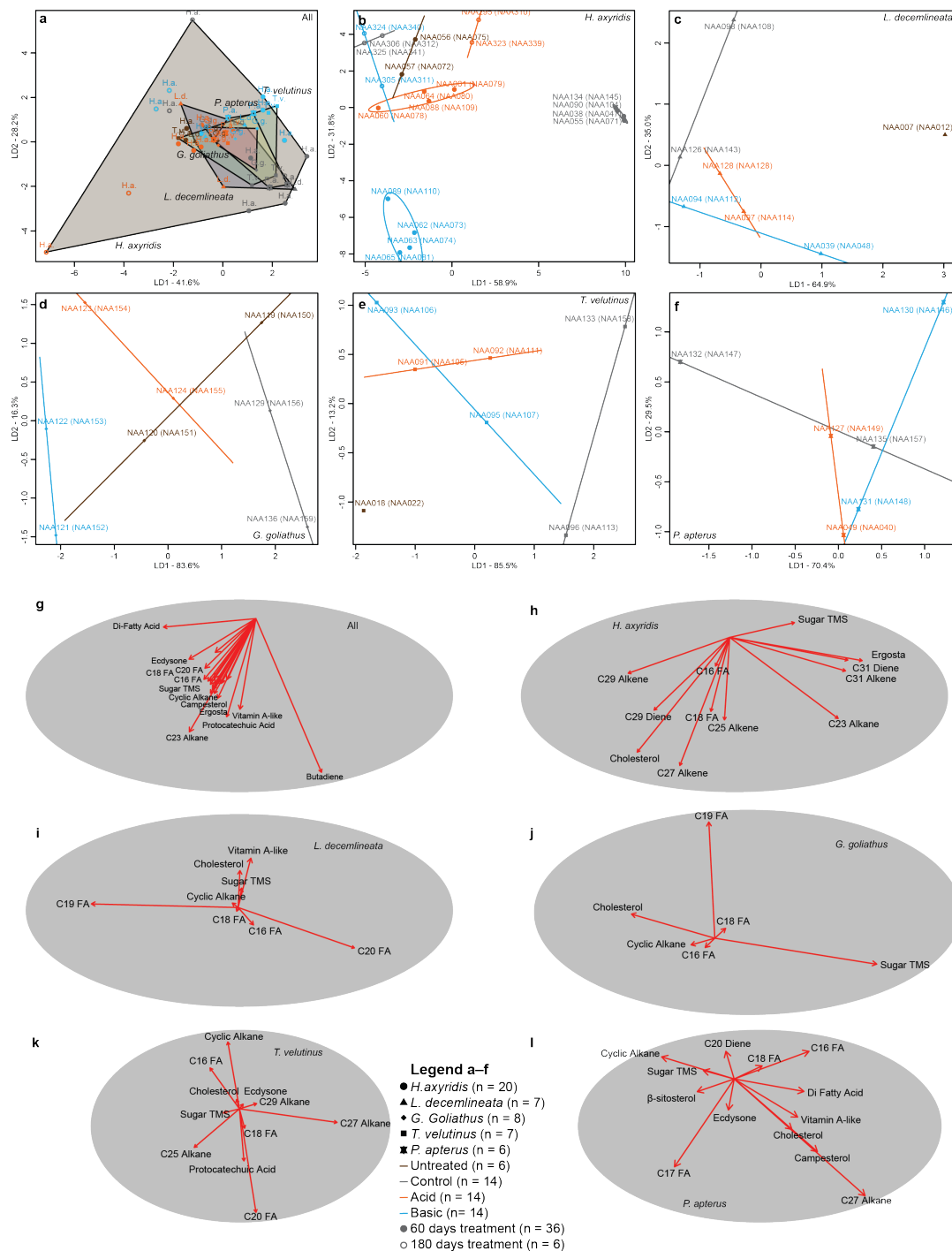


**Supplementary Figure 3.13. Gas chromatography-mass spectrometry chromatograms displaying molecular identifications of the largest peaks in *Pyrrhocoris apterus*.** **a** Specimen decayed in neutral pH conditions for 60 days. **b** Specimen decayed in acidic pH conditions for 60 days. **c** Specimen decayed in basic pH conditions for 60 days. Peaks represented with numbers were identified as fatty acids, major components of insect cuticle. Peaks represented with letters were identified as sugars, hydrocarbons, sterols and various other taxon-specific compounds. Note that the largest peak in all chromatograms was identified as C<sub>14</sub> saturated fatty acid. Largest peak in **b** was identified as phthalate, which is a product of contamination, hence it was ignored in the analysis. Asterisk following a peak's label indicates recurring occurrence of the compound in the sample. Apostrophe following a peak's label indicates a molecule with a trimethylsilyl (TMS) group. All identifications are listed in Appendix 3, a summary of these identifications are represented in Supplementary Table 3.6.



Supplementary Figure 3.14. Caption overleaf.

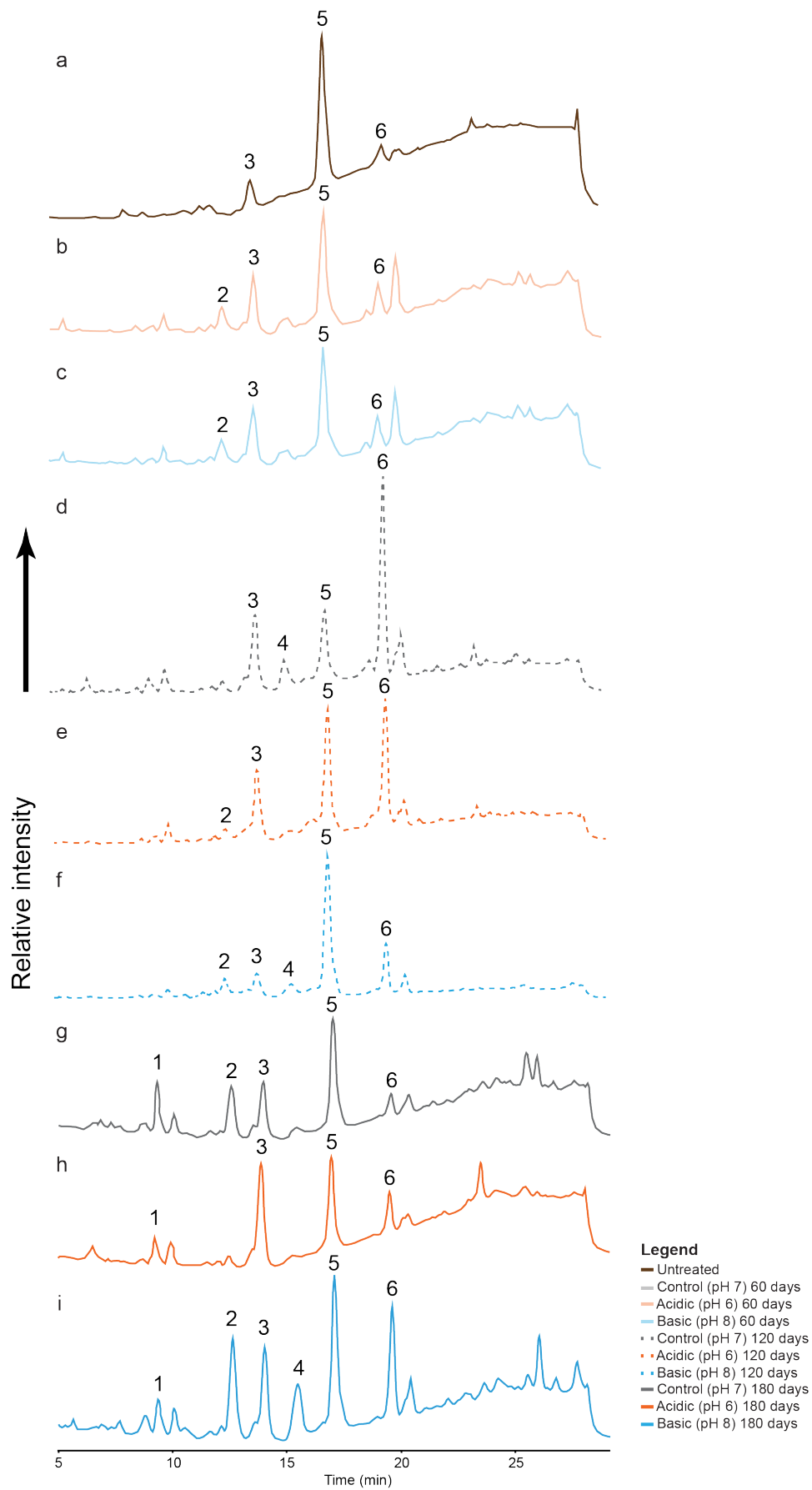
**Supplementary Figure 3.14 (previous page). Principal Component Analysis of relative concentrations of the principal lipid molecules in the cuticle of extant insects prior and after decay. a** PCA plots with data grouped by taxon with 75% confidence ellipses. **b–f** PCA plots of individual taxa with 75% confidence ellipses. **b** *Harmonia axyridis*. **c** *Leptinotarsa decemlineata*. **d** *Goliathus goliathus*. **e** *Typocerus velutinus*. **f** *Pyrrhocoris apterus*. **g–l** Biplot vectors showing the contribution of each molecule to the variation in the dataset. **g** all taxa analyzed. **h** *Harmonia axyridis*. **i** *Leptinotarsa decemlineata*. **j** *Goliathus goliathus*. **k** *Typocerus velutinus*. **l** *Pyrrhocoris apterus*. FA, Fatty Acid; TMS, trimethylsilyl group.



Supplementary Figure 3.15. Cation overleaf.



**Supplementary Figure 3.15 (previous page). Linear Discrimination Analysis of relative concentrations of the principal lipid molecules in the cuticle of extant insects prior and after decay. a** LDA plots with data grouped by taxon with 75% confidence ellipses. **b–f** LDA plots of individual taxa with 75% confidence ellipses. **b** *Harmonia axyridis*. **c** *Leptinotarsa decemlineata*. **d** *Goliathus goliathus*. **e** *Typocerus velutinus*. **f** *Pyrrhocoris apterus*. **g–l** Biplot vectors showing the contribution of each molecule to the variation in the dataset. **g** all taxa analyzed. **h** *Harmonia axyridis*. **i** *Leptinotarsa decemlineata*. **j** *Goliathus goliathus*. **k** *Typocerus velutinus*. **l** *Pyrrhocoris apterus*. FA, Fatty Acid; TMS, trimethylsilyl group.



**Supplementary Figure 3.16. Caption overleaf.**

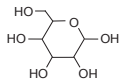
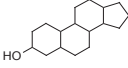
**Supplementary Figure 3.16 (previous page). High performance liquid chromatography-mass spectrometry chromatograms displaying molecular identifications of peaks in *Harmonia axyridis*. a** Untreated specimen. **b–c** Specimens decayed for 60 days in acidic (**b**) and basic (**c**) pH conditions. **d–f** Specimens decayed for 120 days in neutral (**d**), acidic (**e**) and basic (**f**) pH conditions. **g–i** Specimens decayed for 180 days in neutral (**g**), acidic (**h**) and basic pH conditions (**i**). Note that the largest peak in all chromatograms except in **d**, where is the second largest, was identified lutein. All identifications are listed in Appendix 3, a summary of these identifications are represented in Supplementary Table 3.8.

### **A3.3. Supplementary Tables:**

| Harmonia axyridis         |                        |                        |                     |                        |                  |                  |            |                  |            |                  |                  |                  |            |                  |
|---------------------------|------------------------|------------------------|---------------------|------------------------|------------------|------------------|------------|------------------|------------|------------------|------------------|------------------|------------|------------------|
| Duration of experiment    | Colour                 | pH                     | Hue (λmax)          | Intensity (Rmax)       | Purity (S)       | R mean           | St dev     | variance         | F test (F) | F test (p-value) |                  |                  |            |                  |
| Untreated                 | Red                    | untreated              | 591.59              | 1.030933               | 251.413          | 0.840            | 0.15939    | 0.02533          |            |                  |                  |                  |            |                  |
|                           |                        | untreated              | 467.26              | 1.656555               | 356.610          | 1.179            | 0.30624    | 0.02533          |            |                  |                  |                  |            |                  |
|                           | Black                  | total pH 6,8           | 595.27              | 1.026948               | 251.870          | 0.842            | 0.17397    | 0.03016          | 0.83992    | 0.00066          |                  |                  |            |                  |
|                           |                        | total pH 6,7,8         | 591.59              | 1.032789               | 255.303          | 0.853            | 0.17137    | 0.02911          | 0.87019    | 0.00661          |                  |                  |            |                  |
|                           |                        | total pH 5,10          | 563.51              | 1.049316               | 263.793          | 0.881            | 0.15542    | 0.02407          | 1.05220    | 0.32009          |                  |                  |            |                  |
|                           |                        | total pH 5,7,10        | 563.71              | 0.826850               | 263.252          | 0.694            | 0.11986    | 0.02679          | 0.94561    | 0.27451          |                  |                  |            |                  |
|                           |                        | pH 8                   | 606.67              | 1.021132               | 241.867          | 0.810            | 0.19808    | 0.12473          | 0.20370    | 0.00000          |                  |                  |            |                  |
|                           |                        | pH 10                  | 563.51              | 1.065293               | 274.057          | 0.914            | 0.14071    | 0.01974          | 1.28324    | 1.13252 E-6      |                  |                  |            |                  |
|                           |                        | pH 6                   | 591.59              | 1.038406               | 261.448          | 0.873            | 0.15566    | 0.02376          | 1.06616    | 0.21063          |                  |                  |            |                  |
|                           |                        | pH 5                   | 591.59              | 1.043456               | 253.528          | 0.848            | 0.17665    | 0.03110          | 0.81452    | 0.00006          |                  |                  |            |                  |
| 60 days                   | Red                    | pH 7                   | 564.30              | 1.074672               | 262.171          | 0.875            | 0.17296    | 0.02981          | 0.84967    | 0.00146          |                  |                  |            |                  |
|                           |                        | pH 6,8                 | 484.83              | 1.378884               | 324.305          | 1.074            | 0.23274    | 0.05436          | 0.46596    | 0.00000          |                  |                  |            |                  |
|                           |                        | total pH 6,7,8         | 480.59              | 1.393694               | 326.987          | 1.074            | 0.22324    | 0.04833          | 0.52405    | 0.00000          |                  |                  |            |                  |
|                           |                        | pH 5,10                | 465.64              | 1.484410               | 332.407          | 1.101            | 0.21665    | 0.04705          | 0.53838    | 0.00000          |                  |                  |            |                  |
|                           |                        | total pH 5,7,10        | 469.30              | 1.198563               | 332.388          | 0.870            | 0.17701    | 0.04548          | 0.55691    | 0.00000          |                  |                  |            |                  |
|                           | Black                  | pH 8                   | 471.51              | 1.370756               | 322.916          | 1.070            | 0.22612    | 0.05070          | 0.49954    | 0.00000          |                  |                  |            |                  |
|                           |                        | pH 10                  | 458.20              | 1.364861               | 353.463          | 1.168            | 0.28940    | 0.08368          | 0.30269    | 0.00000          |                  |                  |            |                  |
|                           |                        | pH 6                   | 485.63              | 1.402998               | 325.010          | 1.076            | 0.24218    | 0.05818          | 0.43536    | 0.00000          |                  |                  |            |                  |
|                           |                        | pH 5                   | 487.04              | 1.301624               | 311.303          | 1.033            | 0.18558    | 0.03453          | 0.73359    | 1.52181 E-9      |                  |                  |            |                  |
|                           |                        | pH 7                   | 467.70              | 1.499628               | 332.351          | 1.101            | 0.21271    | 0.04534          | 0.55867    | 0.00000          |                  |                  |            |                  |
| 180 days                  | Black                  | pH 6,8                 | 561.55              | 1.065422               | 276.045          | 0.920            | 0.12904    | 0.01233          | 2.05358    | 0.00000          |                  |                  |            |                  |
|                           |                        | total pH 6,7,8         | 561.55              | 0.906283               | 273.818          | 0.783            | 0.11118    | 0.01664          | 1.52187    | 0.00000          |                  |                  |            |                  |
|                           |                        | pH 8                   | 563.71              | 1.077391               | 276.667          | 0.922            | 0.13749    | 0.01885          | 1.34366    | 8.30522 E-9      |                  |                  |            |                  |
|                           |                        | pH 6                   | 561.55              | 1.053673               | 275.423          | 0.918            | 0.12111    | 0.01464          | 1.72995    | 0.00000          |                  |                  |            |                  |
|                           |                        | pH 7                   | 555.86              | 1.052779               | 269.365          | 0.905            | 0.14167    | 0.02187          | 1.15831    | 0.00410          |                  |                  |            |                  |
|                           | Red                    | pH 6,8                 | 463.40              | 2.064199               | 366.118          | 1.210            | 0.32885    | 0.16795          | 0.15081    | 0.00000          |                  |                  |            |                  |
|                           |                        | total pH 6,7,8         | 464.07              | 2.397103               | 379.830          | 1.114            | 0.41104    | 0.23534          | 0.10763    | 0.00000          |                  |                  |            |                  |
|                           |                        | pH 8                   | 456.10              | 1.836110               | 344.657          | 1.141            | 0.23070    | 0.05325          | 0.47569    | 0.00000          |                  |                  |            |                  |
|                           |                        | pH 6                   | 470.70              | 2.554616               | 387.579          | 1.279            | 0.43217    | 0.18608          | 0.13612    | 0.00000          |                  |                  |            |                  |
|                           |                        | pH 7                   | 465.40              | 3.553263               | 407.255          | 1.342            | 0.56497    | 0.31609          | 0.08013    | 0.00000          |                  |                  |            |                  |
| Leptinotarsa decemlineata |                        |                        |                     |                        |                  |                  |            |                  |            |                  |                  |                  |            |                  |
| Duration of experiment    | Colour                 | pH                     | Hue (λmax)          | Intensity (Rmax)       | Purity (S)       | R mean           | St dev     | variance         | F test (F) | F test (p-value) |                  |                  |            |                  |
| Untreated                 | Yellow                 | untreated              | 606.09              | 1.034196               | 264.069          | 0.882            | 0.13963    | 0.11544          |            |                  |                  |                  |            |                  |
|                           |                        | untreated              | 457.73              | 1.420483               | 334.462          | 1.108            | 0.21436    | 0.11544          |            |                  |                  |                  |            |                  |
|                           | Black                  | total pH 6,8           | 617.44              | 1.002520               | 228.955          | 0.769            | 0.22491    | 0.04256          | 2.71282    | 0.00000          |                  |                  |            |                  |
|                           |                        | pH 8                   | 617.44              | 1.003278               | 219.078          | 0.198            | 0.25398    | 0.06433          | 1.79470    | 0.00000          |                  |                  |            |                  |
|                           |                        | pH 6                   | 626.83              | 1.002248               | 238.832          | 0.801            | 0.19629    | 0.03829          | 3.00684    | 0.00000          |                  |                  |            |                  |
|                           |                        | total pH 6,8           | 465.84              | 1.408315               | 332.665          | 1.103            | 0.20595    | 0.04256          | 2.71282    | 0.00000          |                  |                  |            |                  |
|                           |                        | Black                  | pH 8                | 472.72                 | 1.459027         | 338.121          | 1.120      | 0.22157          | 0.04924    | 2.34449          | 0.00000          |                  |            |                  |
|                           |                        |                        | pH 6                | 466.04                 | 1.365960         | 327.208          | 1.085      | 0.19085          | 0.03656    | 3.15801          | 0.00000          |                  |            |                  |
|                           |                        |                        | Goliathus goliathus | Duration of experiment | Colour           | pH               | Hue (λmax) | Intensity (Rmax) | Purity (S) | R mean           | St dev           | variance         | F test (F) | F test (p-value) |
|                           |                        |                        |                     | Untreated              | Grey             | untreated        | 497.49     | 1.377920         | 307.693    | 1.022            | 0.24146          | 0.05833          |            |                  |
| untreated                 | 488.85                 | 1.497135               |                     |                        |                  | 337.715          | 1.121      | 0.21653          | 0.04692    |                  |                  |                  |            |                  |
| 60 days                   | Grey                   | total pH 6,8           |                     |                        | 492.27           | 1.704730         | 362.630    | 1.202            | 0.28798    | 0.08301          | 0.70269          | 6.10193 E-12     |            |                  |
|                           |                        | pH 8                   |                     | 492.27                 | 1.792551         | 374.599          | 1.241      | 0.31079          | 0.09667    | 0.60337          | 0.00000          |                  |            |                  |
|                           | Black                  | pH 6                   |                     | 495.28                 | 1.617162         | 350.661          | 1.163      | 0.27038          | 0.07318    | 0.79710          | 9.56205 E-6      |                  |            |                  |
|                           |                        | total pH 6,8           |                     | 477.37                 | 1.402109         | 332.136          | 1.101      | 0.18476          | 0.03422    | 1.37111          | 7.50757 E-10     |                  |            |                  |
|                           | Brown                  | pH 8                   |                     | 477.37                 | 1.523974         | 348.543          | 1.154      | 0.24138          | 0.0584     | 0.80342          | 0.00002          |                  |            |                  |
|                           |                        | pH 6                   |                     | 482.81                 | 1.282371         | 315.729          | 1.049      | 0.13250          | 0.01759    | 2.66693          | 0.00000          |                  |            |                  |
|                           | Typocerus velutinus    |                        |                     |                        |                  |                  |            |                  |            |                  |                  |                  |            |                  |
|                           | Duration of experiment | Colour                 | pH                  | Hue (λmax)             | Intensity (Rmax) | Purity (S)       | R mean     | St dev           | variance   | F test (F)       | F test (p-value) |                  |            |                  |
| Untreated                 | Yellow                 | untreated              | 613.60              | 1.005976               | 216.533          | 0.728            | 0.25107    | 0.01972          |            |                  |                  |                  |            |                  |
|                           |                        | untreated              | 660.81              | 1.497135               | 221.391          | 0.744            | 0.21937    | 0.03748          |            |                  |                  |                  |            |                  |
|                           | 60 days                | Yellow                 | total pH 6,8        | 628.93                 | 1.002891         | 235.854          | 0.791      | 0.19264          | 0.03702    | 0.53274          | 0.00000          |                  |            |                  |
|                           |                        |                        | pH 8                | 628.93                 | 1.004846         | 209.054          | 0.704      | 0.27058          | 0.07305    | 0.26998          | 0.00000          |                  |            |                  |
|                           |                        | Black                  | pH 6                | 622.04                 | 1.002566         | 262.653          | 0.878      | 0.11501          | 0.01319    | 1.49505          | 0.00000          |                  |            |                  |
|                           |                        |                        | total pH 6,8        | 660.81                 | 1.042009         | 221.467          | 0.744      | 0.21937          | 0.02231    | 1.68006          | 0.00000          |                  |            |                  |
|                           |                        | Brown                  | pH 8                | 641.32                 | 1.004984         | 228.956          | 0.768      | 0.18812          | 0.03531    | 1.06161          | 0.24272          |                  |            |                  |
|                           |                        |                        | pH 6                | 671.90                 | 1.053743         | 260.786          | 0.872      | 0.11483          | 0.01318    | 2.84480          | 0.00000          |                  |            |                  |
|                           |                        | Pyrrhocoris apterus    |                     |                        |                  |                  |            |                  |            |                  |                  |                  |            |                  |
|                           |                        | Duration of experiment | Colour              | pH                     | Hue (λmax)       | Intensity (Rmax) | Purity (S) | R mean           | St dev     | variance         | F test (F)       | F test (p-value) |            |                  |
| Untreated                 | Red                    | untreated              | 610.33              | 1.013554               | 199.233          | 0.669            | 0.21628    | 0.04678          |            |                  |                  |                  |            |                  |
|                           |                        | untreated              | 465.23              | 1.575968               | 351.301          | 1.162            | 0.28404    | 0.08089          |            |                  |                  |                  |            |                  |
|                           | Black                  | total pH 6,8           | 599.72              | 1.027627               | 254.863          | 0.852            | 0.17523    | 0.03059          | 1.15294    | 0.00000          |                  |                  |            |                  |
|                           |                        | pH 8                   | 579.93              | 1.047184               | 260.580          | 0.870            | 0.18325    | 0.03346          | 1.39817    | 6.38802 E-11     |                  |                  |            |                  |
|                           |                        | pH 6                   | 603.00              | 1.017271               | 249.145          | 0.833            | 0.17251    | 0.02965          | 1.57780    | 0.00000          |                  |                  |            |                  |
|                           |                        | total pH 6,8           | 455.70              | 1.681537               | 364.716          | 1.206            | 0.30318    | 0.30348          | 0.87825    | 0.01121          |                  |                  |            |                  |
|                           |                        | Black                  | pH 8                | 466.04                 | 1.636985         | 357.326          | 1.182      | 0.28557          | 0.08175    | 0.98940          | 0.83497          |                  |            |                  |
|                           |                        |                        | pH 6                | 455.70                 | 1.762781         | 372.106          | 1.230      | 0.32197          | 0.10382    | 0.77909          | 1.10535 E-6      |                  |            |                  |

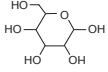
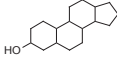
Supplementary Table 3.1. Caption overleaf.

**Supplementary Table 3.1. F-test analysis of changes of hue, intensity and purity of colour during decay of cuticles of *Harmonia axyridis*, *Leptinotarsa decemlineata*, *Goliathus goliathus*, *Typocerus velutinus* and *Pyrrhocoris apterus*.** Values where the null hypothesis was rejected are highlighted in bold. Highlighted in blue are values where  $F$  is larger than the critical value (F Critical value 2-tailed = 1.10552).

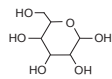
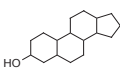
| Peak  | MS characteristics           | Identification             | Common name             | Chemical formula                                           | Possible origin of compound                                                                                       | Percentage of samples | Chemical structure                                                                    |
|-------|------------------------------|----------------------------|-------------------------|------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------|-----------------------|---------------------------------------------------------------------------------------|
| 1, 1' | 256, 129, 73, 60, 57         | C16:0 Fatty Acid           | Palmitic Acid           | C <sub>16</sub> H <sub>32</sub> O <sub>2</sub>             | Component of insect cuticle (Jackson and Baker 1970; Thompson 1973; Lockey 1988; Bozkuş 2003)                     | 70%                   |                                                                                       |
| 2     | 280, 121, 95, 81, 67, 55     | C18:2 Fatty Acid           | Linolenic Acid?         | C <sub>18</sub> H <sub>30</sub> O <sub>2</sub>             | Component of insect cuticle (Thompson 1973; Blomquist <i>et al.</i> 1991; Bozkuş 2003; Womeni <i>et al.</i> 2009) | 60%                   |                                                                                       |
| 3, 3' | 282, 222, 97, 83, 69, 55     | C18:1 Fatty Acid           | Oleic Acid              | C <sub>18</sub> H <sub>34</sub> O <sub>2</sub>             | Component of insect cuticle (Thompson 1973; Hughes <i>et al.</i> 1994; Bozkuş 2003; Womeni <i>et al.</i> 2009)    | 80%                   |    |
| 4     | 284, 241, 129, 73, 60, 55    | C18:0 Fatty Acid           | Stearic Acid            | C <sub>18</sub> H <sub>36</sub> O <sub>2</sub>             | Component of insect cuticle (Thompson 1973; Hughes <i>et al.</i> 1994; Bozkuş 2003; Womeni <i>et al.</i> 2009)    | 65%                   |                                                                                       |
| 5     | 252, 123, 97, 81, 71, 57     | C16:2 Fatty Acid           | (Hexadecadienoic Acid)  | C <sub>16</sub> H <sub>28</sub> O <sub>2</sub>             |                                                                                                                   | 15%                   |                                                                                       |
| 6, 6' | 228, 185, 129, 73, 60        | C14:0 Fatty Acid           | Myristic Acid           | C <sub>14</sub> H <sub>28</sub> O <sub>2</sub>             | Component of insect cuticle (Thompson 1973; Lockey 1988; Bozkuş 2003)                                             | 15%                   |                                                                                       |
| 7     | 312, 143, 87, 74, 55         | C20:0 Fatty Acid           | Arachidic Acid          | C <sub>20</sub> H <sub>40</sub> O <sub>2</sub>             | Component of insect cuticle (Hughes <i>et al.</i> 1994)                                                           | 10%                   |                                                                                       |
| 8     | 340, 143, 96, 87, 74, 55     | C22:0 Fatty Acid           | Behenic Acid            | C <sub>22</sub> H <sub>44</sub> O <sub>2</sub>             |                                                                                                                   | 10%                   |                                                                                       |
| 9     | 410, 147, 129, 73            | C27:0 Fatty Acid           | Carbocerlic Acid        | C <sub>27</sub> H <sub>54</sub> O <sub>2</sub>             |                                                                                                                   | 5%                    |                                                                                       |
| A, A* | 147, 73                      | Sugar TMS                  |                         |                                                            | Blood sugars (Wyatt and Kalf 1957; Thompson 2003) or decay of polysaccharides (Briggs 1999)                       | 55%                   |    |
| B     | 324, 99, 85, 71, 57          | C23 Acyclic Alkane         | Tricosane               | C <sub>23</sub> H <sub>48</sub>                            | Component of insect cuticle (Nelson 1978; Blomquist and Jackson 1979; Hackman 1984)                               | 85%                   |                                                                                       |
| C     | 338, 99, 85, 71, 57          | C24 Acyclic Alkane         | Tetracosane             | C <sub>24</sub> H <sub>50</sub>                            | Component of insect cuticle (Nelson 1978; Blomquist and Jackson 1979; Hackman 1984)                               | 5%                    |                                                                                       |
| D     | 350, 97, 83, 69, 55          | C25 Acyclic Alkene         | Pentacosene             | C <sub>25</sub> H <sub>50</sub>                            | Insect pheromone; Communication (Appelgren <i>et al.</i> 1991; Howard and Blomquist 2005)                         | 95%                   |                                                                                       |
| E     | 352, 99, 85, 71, 57          | C25 Acyclic Alkane         | Pentacosane             | C <sub>25</sub> H <sub>52</sub>                            | Component of insect cuticle; Paraffin wax (Nelson 1978; Blomquist and Jackson 1979; Hackman 1984)                 | 75%                   |                                                                                       |
| F     | 376, 96, 82, 69, 55          | C27 Acyclic Diene          | Heptacosadiene          | C <sub>27</sub> H <sub>52</sub>                            | Insect pheromone; Sex pheromone? (Brown and McDonough 1986; Chertemps 2006)                                       | 100%                  |                                                                                       |
| G     | 378, 97, 83, 69, 55          | C27 Acyclic Alkene         | Heptacosene             | C <sub>27</sub> H <sub>54</sub>                            | Insect pheromone; Communication (Appelgren <i>et al.</i> 1991; Howard and Blomquist 2005)                         | 100%                  |    |
| H     | 404, 96, 82, 69, 55          | C29 Acyclic Diene          | Nonacosadiene           | C <sub>29</sub> H <sub>56</sub>                            | Insect pheromone; Sex pheromone? (Brown and McDonough 1986; Chertemps 2006)                                       | 90%                   |                                                                                       |
| I     | 406, 97, 83, 69, 55          | C29 Acyclic Alkene         | Nonacosene              | C <sub>29</sub> H <sub>58</sub>                            | Insect pheromone; Communication (Appelgren <i>et al.</i> 1991; Howard and Blomquist 2005)                         | 100%                  |                                                                                       |
| J     | 430, 96, 82, 69, 55          | C31 Acyclic Diene          | Hentriacontadiene       | C <sub>31</sub> H <sub>60</sub>                            | Insect pheromone; Sex pheromone? (Brown and McDonough 1986; Chertemps 2006)                                       | 80%                   |                                                                                       |
| K     | 432, 97, 83, 69, 55          | C31 Acyclic Alkene         | Hentriacontene          | C <sub>31</sub> H <sub>62</sub>                            | Insect pheromone; Communication (Appelgren <i>et al.</i> 1991; Howard and Blomquist 2005)                         | 40%                   |                                                                                       |
| L     | 432, 96, 82, 69, 55          | C32 Acyclic Diene          | Dotriacontadiene        | C <sub>32</sub> H <sub>66</sub>                            | Insect pheromone; Sex pheromone? (Brown and McDonough 1986; Chertemps 2006)                                       | 70%                   |                                                                                       |
| M     | 434, 97, 83, 69, 55          | ? Cyclic Alkane            |                         | C <sub>31</sub> H <sub>n</sub>                             | Component of insect cuticle? (Cvačka <i>et al.</i> 2006)                                                          | 25%                   |                                                                                       |
| N     | 458, 368, 105, 75, 73        | C27 Sterol                 | Cholesterol             | C <sub>27</sub> H <sub>46</sub> O                          | Component of insect cuticle (Hutchins and Kaplanis 1969; Hadley 1984)                                             | 75%                   |                                                                                       |
| O     | 472, 207, 129, 73            | C28 Sterol                 | Campesterol             | C <sub>28</sub> H <sub>48</sub> O                          | Component of insect cuticles (Hutchins and Kaplanis 1969; Svoboda and Lusby 1994)                                 | 25%                   |  |
| P     | 386, 341, 105, 75, 73        | C28 Sterol                 | Ergosta-7, 22-dien-3-ol | C <sub>28</sub> H <sub>46</sub> O                          |                                                                                                                   | 15%                   |                                                                                       |
| Q     | 396, 357, 129, 73, 57        | C29 Sterol                 | β-sitosterol            | C <sub>29</sub> H <sub>50</sub> O                          | Phytosterol derived from plants (Hutchins and Kaplanis 1969; Svoboda and Lusby 1994)                              | 25%                   |                                                                                       |
| R     | 211, 99, 85, 71, 57          | ? N-butyloctadec-9-enamide |                         |                                                            |                                                                                                                   | 5%                    |                                                                                       |
| S     | 227, 143, 87, 74             | Unknown compound 6         |                         |                                                            |                                                                                                                   | 5%                    |                                                                                       |
| T     | 205, 175, 109, 99, 57        | Unknown compound 8         |                         |                                                            |                                                                                                                   | 5%                    |                                                                                       |
| U     | 281, 207, 98, 97, 83, 69, 55 | Unknown compound 10        |                         |                                                            |                                                                                                                   | 5%                    |                                                                                       |
| V     | 147, 95, 81, 69              | Unknown compound 20        |                         |                                                            |                                                                                                                   | 5%                    |                                                                                       |
| Z     | 149                          | Benzene-1,2-dicarboxylate  | Phthalate               | C <sub>8</sub> H <sub>4</sub> O <sub>4</sub> <sup>-2</sup> | Contamination                                                                                                     | 50%                   |                                                                                       |

**Supplementary Table 3.2 (previous page). Molecules identified in the chromatograms of *Harmonia axyridis* and percentages of appearance in all samples of the taxon.** Peaks represented with numbers were identified as fatty acids, major components of insect cuticle. Peaks represented with letters were identified as sugars, hydrocarbons, sterols and various other taxon-specific compounds. In the “Peak” column, asterisk following a peak’s label indicates recurring occurrence of the compound in the sample; apostrophe following a peak’s label indicates a molecule with a trimethylsilyl (TMS) group; labels in bold indicate presence of the molecule in the chromatograms represented in Supplementary Fig. 3.9. In the “MS characteristics” column, bold numbers indicate molecular ion and underlined numbers characteristic ion.



| Peak             | MS characteristics              | Identification                  | Common name          | Chemical formula                                           | Possible origin of compound                                                                                                 | Percentage of samples | Chemical structure                                                                  |
|------------------|---------------------------------|---------------------------------|----------------------|------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------|-----------------------|-------------------------------------------------------------------------------------|
| 1, 1'            | 256, 129, 73, 60, 57            | C16:0 Fatty acid                | Palmitic acid        | C <sub>16</sub> H <sub>32</sub> O <sub>2</sub>             | Component of insect cuticle (Jackson and Baker 1970; Thompson 1973; Lockey 1988; Bozkuş 2003)                               | 100%                  |                                                                                     |
| 2, 2'            | 282, 222, 97, 83, 69, 55        | C18:1 Fatty acid                | Oleic acid           | C <sub>18</sub> H <sub>34</sub> O <sub>2</sub>             | Component of insect cuticle (Thompson 1973; Lockey 1988; Hughes <i>et al.</i> 1994; Bozkuş 2003; Womeni <i>et al.</i> 2009) | 100%                  |                                                                                     |
| 3, 3'            | 284, 241, 129, 73, 60, 55       | C18:0 Fatty Acid                | Stearic acid         | C <sub>18</sub> H <sub>36</sub> O <sub>2</sub>             | Component of insect cuticle (Thompson 1973; Lockey 1988; Hughes <i>et al.</i> 1994; Bozkuş 2003; Womeni <i>et al.</i> 2009) | 85.71%                |  |
| 4, 4'            | 157, 101, 88, 55                | Branched Unsaturated Fatty Acid | Nonadecyclic acid    | C <sub>19</sub> H <sub>38</sub> O <sub>2</sub>             |                                                                                                                             | 28.57%                |                                                                                     |
| 5, 5'            | 298, 129, 75                    | C19:0 Fatty Acid                |                      |                                                            |                                                                                                                             | 28.57%                |                                                                                     |
| 6                | 310, 264, 222, 97, 83, 69, 74   | C20:1 Fatty Acid                |                      |                                                            |                                                                                                                             | 14.28%                |                                                                                     |
| 7, 7'            | 312, 129, 97, 73                | C20:0 Fatty Acid                | Arachidic acid       | C <sub>20</sub> H <sub>40</sub> O <sub>2</sub>             | Component of insect cuticle (Hughes <i>et al.</i> 1994)                                                                     | 85.71%                |                                                                                     |
| A, A*, A**, A*** | 147, 73                         | Sugar TMS                       |                      |                                                            | Blood sugars (Wyatt and Kalf 1957; Thompson 2003) or decay of polysaccharides (Briggs 1999)                                 | 57.14%                |  |
| B                | 408, 99, 85, 71, 57             | C29 Acyclic Alkane              | Nonacosane           | C <sub>29</sub> H <sub>60</sub>                            | Component of insect cuticle (Nelson 1978; Blomquist and Jackson 1979; Hackman 1984)                                         | 14.28%                |                                                                                     |
| C                | 281, 99, 85, 71, 57             | ? Cyclic Alkane                 |                      |                                                            | Component of insect cuticle? (Cvačka <i>et al.</i> 2006)                                                                    | 100%                  |                                                                                     |
| D                | 281, 99, 85, 71, 57             | ? Cyclic Alkane                 |                      |                                                            | Component of insect cuticle? (Cvačka <i>et al.</i> 2006)                                                                    | 100%                  |                                                                                     |
| E                | 281, 99, 85, 71, 57             | ? Cyclic Alkane                 |                      |                                                            | Component of insect cuticle? (Cvačka <i>et al.</i> 2006)                                                                    | 100%                  |                                                                                     |
| F                | 194, 154, 73                    | 3,4-Dihydroxybenzoic Acid       | Protocatechuic Acid  | C <sub>7</sub> H <sub>6</sub> O <sub>4</sub>               | Component of insect cuticle (Wigglesworth 1948; Wigglesworth 1957; Atkinson and Gilby 1970; Atkinson <i>et al.</i> 1973)    | 14.28%                |  |
| G                | 458, 368, 105, 75, 73           | C27 Sterol                      | Cholesterol          | C <sub>27</sub> H <sub>46</sub> O                          | Component of insect cuticle (Hutchins and Kaplanis 1969; Hadley 1984)                                                       | 85.71%                |                                                                                     |
| H                | 396, 357, 129, 73, 57           | C29 Sterol                      | β-sitosterol         | C <sub>29</sub> H <sub>50</sub> O                          | Phytosterol derived from plants (Hutchins and Kaplanis 1969; Svoboda and Lusby 1994)                                        | 28.57%                |  |
| I                | 464, 229, 207, 147, 124, 91, 55 | 20-hydroxyecdysone              | Ecdysone             | C <sub>27</sub> H <sub>44</sub> O <sub>6</sub>             | Moulting controlling hormone (Svoboda and Lusby 1994)                                                                       | 14.28%                |                                                                                     |
| J                | 261, 229, 124                   | ? Similar to Vitamin A molecule | Similar to Vitamin A |                                                            | ? Degradation of carotenoids                                                                                                | 71.43%                |  |
| K                | 207, 179, 132, 75, 73           | Unknown compound 18             |                      |                                                            |                                                                                                                             | 14.28%                |                                                                                     |
| L                | 337, 295, 117, 73               | Unknown compound 21             |                      |                                                            |                                                                                                                             | 14.28%                |                                                                                     |
| M                | 137, 81                         | Unknown compound 22             |                      |                                                            |                                                                                                                             | 14.28%                |                                                                                     |
| Z                | 149                             | Benzene-1,2-dicarboxylate       | Phthalate            | C <sub>8</sub> H <sub>6</sub> O <sub>4</sub> <sup>-2</sup> | Contamination                                                                                                               | 57.14%                |                                                                                     |

**Supplementary Table 3.3 (previous page). Molecules identified on the mayor peaks in the chromatograms of *Leptinotarsa decemlineata* and percentages of appearance in all samples of the taxon.** Peaks represented with numbers were identified as fatty acids, major components of insect cuticle. Peaks represented with letters were identified as sugars, hydrocarbons, sterols and various other taxon-specific compounds. In the “Peak” column, asterisk following a peak’s label indicates recurring occurrence of the compound in the sample; apostrophe following a peak’s label indicates a molecule with a trimethylsilyl (TMS) group; labels in bold indicate presence of the molecule in the chromatograms represented in Supplementary Fig. 3.10. In the “MS characteristics” column, bold numbers indicate molecular ion and underlined numbers characteristic ion.

| Peak       | MS characteristics                         | Identification                 | Common name      | Chemical formula                                           | Possible origin of compound                                                                                                 | Percentage of samples | Chemical structure                                                                   |
|------------|--------------------------------------------|--------------------------------|------------------|------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------|-----------------------|--------------------------------------------------------------------------------------|
| 1, 1'      | <b>256</b> , <u>129</u> , 73, 60, 57       | C16:0 Fatty acid               | Palmitic Acid    | C <sub>16</sub> H <sub>32</sub> O <sub>2</sub>             | Component of insect cuticle (Jackson and Baker 1970; Thompson 1973; Lockey 1988; Bozkuş 2003)                               | 100%                  |                                                                                      |
| 2, 2'      | <b>282</b> , <u>222</u> , 97, 83, 69, 55   | C18:1 Fatty acid               | Oleic Acid       | C <sub>18</sub> H <sub>34</sub> O <sub>2</sub>             | Component of insect cuticle (Thompson 1973; Lockey 1988; Hughes <i>et al.</i> 1994; Bozkuş 2003; Womeni <i>et al.</i> 2009) | 100%                  |                                                                                      |
| 3, 3'      | <b>284</b> , <u>241</u> , 129, 73, 60, 55  | C18:0 Fatty Acid               | Stearic Acid     | C <sub>18</sub> H <sub>36</sub> O <sub>2</sub>             | Component of insect cuticle (Thompson 1973; Lockey 1988; Hughes <i>et al.</i> 1994; Bozkuş 2003; Womeni <i>et al.</i> 2009) | 87.50%                |                                                                                      |
| 4'         | <b>355</b> , 185, <u>129</u> , 97, 83, 73  | C19:0 Fatty Acid               | Nonadecylic Acid | C <sub>19</sub> H <sub>38</sub> O <sub>2</sub>             | Component of insect cuticle (Thompson 1973; Bozkuş 2003)                                                                    | 50%                   |                                                                                      |
| 5'         | <b>311</b> , 145, <u>129</u> , 117, 75, 73 | C16:1 Fatty Acid               | Palmitoleic Acid | C <sub>16</sub> H <sub>32</sub> O <sub>2</sub>             | Component of insect cuticle (Thompson 1973; Bozkuş 2003)                                                                    | 25%                   |   |
| 6          | <b>270</b> , <u>143</u> , 87, 74           | C16:0 Fatty Acid Methyl Ester  | Palmitic Acid    | C <sub>16</sub> H <sub>32</sub> O <sub>2</sub>             | Component of insect cuticle (Jackson and Baker 1970; Thompson 1973; Lockey 1988; Bozkuş 2003)                               | 100%                  |                                                                                      |
| 7, 7'      | <b>280</b> , <u>121</u> , 95, 81, 67, 55   | C18:2 Fatty Acid               | Linolenic Acid?  | C <sub>18</sub> H <sub>30</sub> O <sub>2</sub>             | Component of insect cuticle (Thompson 1973; Blomquist <i>et al.</i> 1991; Bozkuş 2003; Womeni <i>et al.</i> 2009)           | 62.50%                |                                                                                      |
| 8          | <b>310</b> , <u>222</u> , 97, 83, 69, 74   | C20:1 Fatty Acid               | Arachidic Acid   | C <sub>20</sub> H <sub>40</sub> O <sub>2</sub>             | Component of insect cuticle (Hughes <i>et al.</i> 1994)                                                                     | 25%                   |                                                                                      |
| 9'         | <b>325</b> , 147, <u>129</u> , 73          | C22:0 Fatty Acid               | Behenic Acid     | C <sub>22</sub> H <sub>44</sub> O <sub>2</sub>             |                                                                                                                             | 12.50%                |                                                                                      |
| 10         | <b>368</b> , <u>143</u> , 87, 74           | C23:0 Fatty Acid Methyl Ester  | Tricosylic Acid  | C <sub>23</sub> H <sub>46</sub> O <sub>2</sub>             |                                                                                                                             | 12.50%                |                                                                                      |
| 11         | <u>101</u> , 88                            | Branched Saturated Fatty Acid  |                  |                                                            |                                                                                                                             | 25%                   |                                                                                      |
| A, A*, A** | <u>147</u> , 73                            | Sugar TMS                      |                  |                                                            | Blood sugars (Wyatt and Kalf 1957; Thompson 2003) or decay of polysaccharides (Briggs 1999)                                 | 62.50%                |   |
| B          | <b>366</b> , <u>99</u> , 85, 71, 57        | C26 Acyclic Alkane             | Hexacosane       | C <sub>26</sub> H <sub>54</sub>                            | Component of insect cuticle (Nelson 1978; Blomquist and Jackson 1979; Hackman 1984)                                         | 12.50%                |                                                                                      |
| C          | <b>394</b> , <u>99</u> , 85, 71, 57        | C28 Acyclic Alkane             | Octacosane       | C <sub>28</sub> H <sub>58</sub>                            | Component of insect cuticle (Nelson 1978; Blomquist and Jackson 1979; Hackman 1984)                                         | 12.50%                |                                                                                      |
| D          | <u>281</u> , 99, 85, 71, 57                | ? Cyclic Alkane                |                  |                                                            | Component of insect cuticle? (Cvačka <i>et al.</i> 2006)                                                                    | 100%                  |                                                                                      |
| E          | <u>281</u> , 99, 85, 71, 57                | ? Cyclic Alkane                |                  |                                                            | Component of insect cuticle? (Cvačka <i>et al.</i> 2006)                                                                    | 100%                  |                                                                                      |
| F          | <b>343</b> , <u>97</u> , 83, 69, 55        | C31 Acyclic Alkene             | Hentriacontene   | C <sub>31</sub> H <sub>62</sub>                            | Insect pheromone; Communication (Appelgren <i>et al.</i> 1991; Howard and Blomquist 2005)                                   | 37.50%                |                                                                                      |
| G          | <b>464</b> , <u>99</u> , 85, 71, 57        | C33 Acyclic Alkane             | Tritriacontane   | C <sub>33</sub> H <sub>68</sub>                            | Component of insect cuticle (Nelson 1978; Blomquist and Jackson 1979; Hackman 1984)                                         | 12.50%                |   |
| H          | <u>281</u> , 99, 85, 71, 57                | ? Cyclic Alkane                |                  |                                                            | Component of insect cuticle? (Cvačka <i>et al.</i> 2006)                                                                    | 100%                  |                                                                                      |
| I          | <b>492</b> , <u>99</u> , 85, 71, 57        | C35 Acyclic Alkane             | Pentatriacontane | C <sub>35</sub> H <sub>76</sub>                            | Component of insect cuticle (Nelson 1978; Blomquist and Jackson 1979; Hackman 1984)                                         | 12.50%                |                                                                                      |
| J          | <u>281</u> , 99, 85, 71, 57                | ? Cyclic Alkane                |                  |                                                            | Component of insect cuticle? (Cvačka <i>et al.</i> 2006)                                                                    | 100%                  |                                                                                      |
| K          | <b>506</b> , <u>99</u> , 85, 71, 57        | C36 Acyclic Alkane             | Hexatriacontane  | C <sub>36</sub> H <sub>74</sub>                            | Component of insect cuticle (Nelson 1978; Blomquist and Jackson 1979; Hackman 1984)                                         | 12.50%                |                                                                                      |
| L          | <b>534</b> , <u>99</u> , 85, 71, 57        | C38 Acyclic Alkane             | Octatriacontane  | C <sub>38</sub> H <sub>78</sub>                            | Component of insect cuticle (Nelson 1978; Blomquist and Jackson 1979; Hackman 1984)                                         | 12.50%                |                                                                                      |
| M          | <u>281</u> , 99, 85, 71, 58                | ? Cyclic Alkane                |                  |                                                            | Component of insect cuticle? (Cvačka <i>et al.</i> 2006)                                                                    | 100%                  |                                                                                      |
| N          | 99, 85, 71, 57                             | Branching Alkaloid             |                  |                                                            |                                                                                                                             | 12.50%                |                                                                                      |
| O          | <b>458</b> , <u>368</u> , 105, 75, 73      | C27 Sterol                     | Cholesterol      | C <sub>27</sub> H <sub>46</sub> O                          | Component of insect cuticle (Hutchins and Kaplanis 1969; Hadley 1984)                                                       | 100%                  |  |
| P          | 281, 252, 208, 111, 99, 85, 71, 57         | Molecule with ? Glycerol group |                  |                                                            |                                                                                                                             | 12.50%                |                                                                                      |
| Q          | 147, 105, 91, 79                           | Unknown compound 6             |                  |                                                            |                                                                                                                             | 12.50%                |                                                                                      |
| R          | 72, 59                                     | Unknown compound 19            |                  |                                                            |                                                                                                                             | 12.50%                |                                                                                      |
| S          | 95, 81, 69                                 | Unknown compound 20            |                  |                                                            |                                                                                                                             | 25%                   |                                                                                      |
| T          | <u>191</u>                                 | ? Bacterial lipids             |                  |                                                            | Archaeal lipids? Product of decay                                                                                           | 12.50%                |                                                                                      |
| Z          | <u>149</u>                                 | Benzene-1,2-dicarboxylate      | Phthalate        | C <sub>8</sub> H <sub>6</sub> O <sub>4</sub> <sup>-2</sup> | Contamination                                                                                                               | 12.50%                |                                                                                      |

**Supplementary Table 3.4 (previous page). Molecules identified on the mayor peaks in the chromatograms of *Goliathus goliathus* and percentages of appearance in all samples of the taxon.** Peaks represented with numbers were identified as fatty acids, major components of insect cuticle. Peaks represented with letters were identified as sugars, hydrocarbons, sterols and various other taxon-specific compounds. In the “Peak” column, asterisk following a peak’s label indicates recurring occurrence of the compound in the sample; apostrophe following a peak’s label indicates a molecule with a trimethylsilyl (TMS) group; labels in bold indicate presence of the molecule in the chromatograms represented in Supplementary Fig. 3.11. In the “MS characteristics” column, bold numbers indicate molecular ion and underlined numbers characteristic ion.

| Peak             | MS characteristics              | Identification                | Common name         | Chemical formula                                           | Possible origin of compound                                                                                              | Percentage of samples | Chemical structure                                                                   |
|------------------|---------------------------------|-------------------------------|---------------------|------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------|-----------------------|--------------------------------------------------------------------------------------|
| 1'               | 285, 129, 117, 75, 73           | C14:0 Fatty Acid              | Myristic acid       | C <sub>14</sub> H <sub>28</sub> O <sub>2</sub>             | Component of insect cuticle (Thompson 1973; Lockey 1988; Bozkuş 2003)                                                    | 14.29%                |                                                                                      |
| 2'               | 311, 145, 129, 117, 75, 73      | C16:1 Fatty acid              | Palmitoleic acid    | C <sub>16</sub> H <sub>30</sub> O <sub>2</sub>             | Component of insect cuticle (Thompson 1973; Bozkuş 2003)                                                                 | 28.57%                |                                                                                      |
| 3, 3'            | 256, 129, 73, 60, 57            | C16:0 Fatty acid              | Palmitic acid       | C <sub>16</sub> H <sub>32</sub> O <sub>2</sub>             | Component of insect cuticle (Jackson and Baker 1970; Thompson 1973; Bozkuş 2003)                                         | 100%                  |                                                                                      |
| 4, 4'            | 282, 222, 97, 83, 69, 55        | C18:1 Fatty acid              | Oleic acid          | C <sub>18</sub> H <sub>34</sub> O <sub>2</sub>             | Component of insect cuticle (Thompson 1973; Hughes <i>et al.</i> 1994; Bozkuş 2003; Womeni <i>et al.</i> 2009)           | 100%                  |   |
| 5, 5'            | 284, 241, 129, 73, 60, 55       | C18:0 Fatty Acid              | Stearic acid        | C <sub>18</sub> H <sub>36</sub> O <sub>2</sub>             | Component of insect cuticle (Thompson 1973; Hughes <i>et al.</i> 1994; Bozkuş 2003; Womeni <i>et al.</i> 2009)           | 100%                  |                                                                                      |
| 6, 6'            | 298, 129, 117, 95, 81, 75, 67   | C19:0 Fatty Acid              | Nonadecylic acid    | C <sub>19</sub> H <sub>38</sub> O <sub>2</sub>             | Component of insect cuticle (Thompson 1973; Blomquist <i>et al.</i> 1991; Bozkuş 2003; Womeni <i>et al.</i> 2009)        | 28.57%                |                                                                                      |
| 7, 7'            | 382, 227, 129, 75, 57           | C20:0 Fatty Acid              | Arachidic acid      | C <sub>20</sub> H <sub>40</sub> O <sub>2</sub>             | Component of insect cuticle (Hughes <i>et al.</i> 1994)                                                                  | 50%                   |                                                                                      |
| 8'               | 325, 147, 129, 73               | C22:0 Fatty Acid              | Behenic acid        | C <sub>22</sub> H <sub>44</sub> O <sub>2</sub>             | Component of insect cuticle (Major and Blomquist 1978; Womeni <i>et al.</i> 2009; Gasco <i>et al.</i> 2018)              | 14.29%                |                                                                                      |
| 9'               | 257, 145, 131, 129, 117, 75, 73 | C12:0 Fatty Acid              | Lauric acid         | C <sub>12</sub> H <sub>24</sub> O <sub>2</sub>             | Component of insect cuticle (Thompson 1973; Blomquist <i>et al.</i> 1991; Bozkuş 2003; Womeni <i>et al.</i> 2009)        | 14.29%                |                                                                                      |
| 10'              | 337, 129, 117, 81, 75           | C18:2 Fatty Acid              | Linoleic acid       | C <sub>18</sub> H <sub>32</sub> O <sub>2</sub>             |                                                                                                                          | 28.57%                |                                                                                      |
| A, A*, A**, A*** | 147, 73                         | Sugar TMS                     |                     |                                                            | Blood sugars (Wyatt and Kalf 1957; Thompson 2003) or decay of polysaccharides (Briggs 1999)                              | 28.57%                |   |
| B                | 236, 97, 83, 69, 55             | C17 Acyclic Diene             | Heptadecadiene      | C <sub>17</sub> H <sub>32</sub>                            | Insect pheromone; Sex pheromone? (Brown and McDonough 1986; Chertemps <i>et al.</i> 2006)                                | 14.29%                |                                                                                      |
| C                | 281, 99, 85, 71, 57             | ? Cyclic Alkane               |                     |                                                            | Component of insect cuticle? (Cvačka <i>et al.</i> 2006)                                                                 | 51.14%                |                                                                                      |
| D                | 324, 99, 85, 71, 57             | C23 Acyclic Alkane            | Tricosane           | C <sub>23</sub> H <sub>46</sub>                            | Component of insect cuticle (Nelson 1978; Blomquist and Jackson 1979; Hackman 1984)                                      | 28.57%                |                                                                                      |
| E                | 352, 99, 85, 71, 57             | C25 Acyclic Alkane            | Pentacosane         | C <sub>25</sub> H <sub>50</sub>                            | Component of insect cuticle (Nelson 1978; Blomquist and Jackson 1979; Hackman 1984)                                      | 50%                   |   |
| F                | 352, 99, 85, 71, 57             | C27 Acyclic Alkane            | Heptacosane         | C <sub>27</sub> H <sub>54</sub>                            | Component of insect cuticle; Paraffin wax (Nelson 1978; Blomquist and Jackson 1979; Hackman 1984)                        | 85.71%                |                                                                                      |
| G                | 281, 99, 85, 71, 57             | ? Cyclic Alkane               |                     |                                                            | Component of insect cuticle? (Cvačka <i>et al.</i> 2006)                                                                 | 71.43%                |                                                                                      |
| H                | 408, 99, 85, 71, 57             | C29 Acyclic Alkane            | Nonacosane          | C <sub>29</sub> H <sub>60</sub>                            | Component of insect cuticle (Nelson 1978; Blomquist and Jackson 1979; Hackman 1984)                                      | 71.43%                |                                                                                      |
| I                | 281, 99, 85, 71, 57             | ? Cyclic Alkane               |                     |                                                            | Component of insect cuticle? (Cvačka <i>et al.</i> 2006)                                                                 | 42.86%                |                                                                                      |
| J                | 341, 239, 179, 75, 73           | C19 Alcohol                   |                     |                                                            | Trace component of insect cuticle (Lockey 1988)                                                                          | 28.57%                |   |
| K                | 154, 73                         | 3,4-Dihydroxybenzoic Acid     | Protocatechuic Acid | C <sub>7</sub> H <sub>6</sub> O <sub>4</sub>               | Component of insect cuticle (Wigglesworth 1948; Wigglesworth 1957; Atkinson and Gilby 1970; Atkinson <i>et al.</i> 1973) | 42.86%                |   |
| L                | 458, 368, 105, 75, 73           | C27 Sterol                    | Cholesterol         | C <sub>27</sub> H <sub>46</sub> O                          | Component of insect cuticle (Hutchins and Kaplanis 1969; Hadley 1984)                                                    | 57.14%                |   |
| M                | 464, 229, 207, 147, 124, 91, 55 | 20-hydroxyecdysone            | Ecdysone            | C <sub>27</sub> H <sub>44</sub> O <sub>6</sub>             | Moulting controlling hormone (Svoboda and Lusby 1994)                                                                    | 42.86%                |                                                                                      |
| N                | 263, 247, 218, 129, 177, 75, 73 | ? Terpenoid                   |                     |                                                            | Defensive substance in some Lepidoptera (Nishida 2002) or break down product of β-carotene (Payzant <i>et al.</i> 1986)  | 14.29%                |  |
| O                | 111, 71                         | Compound with amino group     |                     |                                                            | Derivative of amonia?                                                                                                    | 28.57%                |                                                                                      |
| P                | 240, 165, 152                   | Unknown compound 13 (Cyclic?) |                     |                                                            |                                                                                                                          | 14.29%                |                                                                                      |
| Q                | 335, 320, 247, 218, 117, 73     | Unknown compound 14           |                     |                                                            |                                                                                                                          | 14.29%                |                                                                                      |
| R                | 218, 192, 177, 75, 73           | Unknown compound 15           |                     |                                                            |                                                                                                                          | 14.29%                |                                                                                      |
| S                | 265, 208, 207, 206, 179, 75, 73 | Unknown compound 16           |                     |                                                            |                                                                                                                          | 14.29%                |                                                                                      |
| Z                | 149                             | Benzene-1,2-dicarboxylate     | Phthalate           | C <sub>8</sub> H <sub>4</sub> O <sub>4</sub> <sup>-2</sup> | Contamination                                                                                                            | 57.14%                |                                                                                      |

**Supplementary Table 3.5 (previous page). Molecules identified on the mayor peaks in the chromatograms of *Typocerus velutinus* and percentages of appearance in all samples of the taxon.** Peaks represented with numbers were identified as fatty acids, major components of insect cuticle. Peaks represented with letters were identified as sugars, hydrocarbons, sterols and various other taxon-specific compounds. In the “Peak” column, asterisk following a peak’s label indicates recurring occurrence of the compound in the sample; apostrophe following a peak’s label indicates a molecule with a trimethylsilyl (TMS) group; labels in bold indicate presence of the molecule in the chromatograms represented in Supplementary Fig. 3.12. In the “MS characteristics” column, bold numbers indicate molecular ion and underlined numbers characteristic ion.

| Peak  | MS characteristics              | Identification                  | Common name          | Chemical formula                                           | Possible origin of compound                                                                                       | Percentage of samples | Chemical structure |
|-------|---------------------------------|---------------------------------|----------------------|------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------|-----------------------|--------------------|
| 1, 1' | 256, 129, 73, 60, 57            | C16:0 Fatty acid                | Palmitic acid        | C <sub>16</sub> H <sub>32</sub> O <sub>2</sub>             | Component of insect cuticle (Jackson and Baker 1970; Thompson 1973; Bozkuş 2003)                                  | 100%                  |                    |
| 2, 2' | 280, 121, 95, 81, 67, 55        | C18:2 Fatty Acid                | Linoleic acid        | C <sub>18</sub> H <sub>30</sub> O <sub>2</sub>             | Component of insect cuticle (Thompson 1973; Blomquist <i>et al.</i> 1991; Bozkuş 2003; Womeni <i>et al.</i> 2009) | 50%                   |                    |
| 3, 3' | 282, 222, 97, 83, 69, 55        | C18:1 Fatty acid                | Oleic acid           | C <sub>18</sub> H <sub>34</sub> O <sub>2</sub>             | Component of insect cuticle (Thompson 1973; Hughes <i>et al.</i> 1994; Bozkuş 2003; Womeni <i>et al.</i> 2009)    | 16.67%                |                    |
| 4, 4' | 284, 241, 129, 73, 60, 55       | C18:0 Fatty Acid                | Stearic acid         | C <sub>18</sub> H <sub>36</sub> O <sub>2</sub>             | Component of insect cuticle (Thompson 1973; Hughes <i>et al.</i> 1994; Bozkuş 2003; Womeni <i>et al.</i> 2009)    | 100%                  |                    |
| 5, 5' | 200, 157, 129, 73, 60, 50       | C12:0 Fatty Acid                | Lauric acid          | C <sub>12</sub> H <sub>24</sub> O <sub>2</sub>             | Component of insect cuticle (Womeni <i>et al.</i> 2009; Major and Blomquist 1978; Gasco <i>et al.</i> 2018)       | 50%                   |                    |
| 6     | 129, 111, 83, 71, 70, 57        | Di-Fatty Acid                   |                      |                                                            |                                                                                                                   | 50%                   |                    |
| A, A* | 147, 73                         | Sugar TMS                       |                      |                                                            | Blood sugars (Wyatt and Kalf 1957; Thompson 2003) or decay of polysaccharides (Briggs 1999)                       | 83.33%                |                    |
| B     | 264, 97, 83, 69, 55             | C19 Acyclic Alkene              | Nonadecene           | C <sub>19</sub> H <sub>38</sub>                            | Insect pheromone; Communication (Appelgren <i>et al.</i> 1991; Howard and Blomquist 2005)                         | 16.67%                |                    |
| C     | 268, 95, 81, 67, 55             | C19 Acyclic Alkane              | Nonadecane           | C <sub>19</sub> H <sub>40</sub>                            | Component of insect cuticle (Nelson 1978; Blomquist and Jackson 1979; Hackman 1984)                               | 16.67%                |                    |
| D     | 280, 95, 81, 67, 55             | C20 Acyclic Diene               | Eicosadiene          | C <sub>20</sub> H <sub>40</sub>                            | Insect pheromone; Sex pheromone? (Brown and McDonough 1986; Chertemps <i>et al.</i> 2006)                         | 50%                   |                    |
| E     | 281, 99, 85, 71, 57             | ? Cyclic Alkane                 |                      |                                                            | Component of insect cuticle? (Cvačka <i>et al.</i> 2006)                                                          | 16.67%                |                    |
| F     | 281, 99, 85, 71, 57             | ? Cyclic Alkane                 |                      |                                                            | Component of insect cuticle? (Cvačka <i>et al.</i> 2006)                                                          | 66.67%                |                    |
| G     | 352, 99, 85, 71, 57             | C27 Acyclic Alkane              | Pentacosane          | C <sub>25</sub> H <sub>50</sub>                            | Component of insect cuticle; Paraffin wax (Nelson 1978; Blomquist and Jackson 1979; Hackman 1984)                 | 50%                   |                    |
| H     | 281, 99, 85, 71, 57             | ? Cyclic Alkane                 |                      |                                                            | Component of insect cuticle? (Cvačka <i>et al.</i> 2006)                                                          | 50%                   |                    |
| I     | 281, 99, 85, 71, 57             | ? Cyclic Alkane                 |                      |                                                            | Component of insect cuticle? (Cvačka <i>et al.</i> 2006)                                                          | 50%                   |                    |
| J     | 281, 99, 85, 71, 57             | ? Cyclic Alkane                 |                      |                                                            | Component of insect cuticle? (Cvačka <i>et al.</i> 2006)                                                          | 66.67%                |                    |
| K     | 281, 99, 85, 71, 57             | ? Cyclic Alkane                 |                      |                                                            | Component of insect cuticle? (Cvačka <i>et al.</i> 2006)                                                          | 33.33%                |                    |
| L     | 281, 99, 85, 71, 57             | ? Cyclic Alkane                 |                      |                                                            | Component of insect cuticle? (Cvačka <i>et al.</i> 2006)                                                          | 33.33%                |                    |
| M     | 458, 368, 105, 75, 73           | C27 Sterol                      | Cholesterol          | C <sub>27</sub> H <sub>46</sub> O                          | Component of insect cuticle (Hutchins and Kaplanis 1969; Hadley 1984)                                             | 33.33%                |                    |
| N     | 472, 207, 129, 73               | C28 Sterol                      | Campesterol          | C <sub>28</sub> H <sub>48</sub> O                          | Component of insect cuticles (Hutchins and Kaplanis 1969; Svoboda and Lusby 1994)                                 | 50%                   |                    |
| O     | 486, 396, 357, 129, 73, 57      | C29 Sterol                      | β-sitosterol         | C <sub>29</sub> H <sub>50</sub> O                          | Phytosterol derived from plants (Hutchins and Kaplanis 1969; Svoboda and Lusby 1994)                              | 50%                   |                    |
| P     | 464, 229, 207, 147, 124, 91, 55 | 20-hydroxyecdysone              | Ecdysone             | C <sub>27</sub> H <sub>44</sub> O <sub>6</sub>             | Moulting controlling hormone (Svoboda and Lusby 1994)                                                             | 83.33%                |                    |
| Q     | 194, 166, 149, 121              | ? Benzoic acid                  | Benzoic Acid         | C <sub>7</sub> H <sub>6</sub> COOH                         | Biprodukt of amino acid biosynthesis (Atkinson <i>et al.</i> 1973)                                                | 50%                   |                    |
| R     | 71                              | Buta-1,3-diene                  | Butadiene            | C <sub>4</sub> H <sub>6</sub>                              | Contamination                                                                                                     | 50%                   |                    |
| S     | 261, 229, 124                   | ? Similar to Vitamin A molecule | Similar to Vitamin A |                                                            | ? Degradation of carotenoids                                                                                      | 50%                   |                    |
| T     | 111, 71                         | Molecule with an amyl group     |                      |                                                            |                                                                                                                   | 16.67%                |                    |
| U     | 217, 205, 174, 91, 57           | Unknown compound 8              |                      |                                                            |                                                                                                                   | 33.33%                |                    |
| Z     | 149                             | Benzene-1,2-dicarboxylate       | Phthalate            | C <sub>8</sub> H <sub>6</sub> O <sub>4</sub> <sup>-2</sup> | Contamination                                                                                                     | 100%                  |                    |

**Supplementary Table 3.6 (previous page). Molecules identified on the mayor peaks in the chromatograms of *Pyrrhocoris apterus* and percentages of appearance in all samples of the taxon.** Peaks represented with numbers were identified as fatty acids, major components of insect cuticle. Peaks represented with letters were identified as sugars, hydrocarbons, sterols and various other taxon-specific compounds. In the “Peak” column, asterisk following a peak’s label indicates recurring occurrence of the compound in the sample; apostrophe following a peak’s label indicates a molecule with a trimethylsilyl (TMS) group; labels in bold indicate presence of the molecule in the chromatograms represented in Supplementary Fig. 3.13. In the “MS characteristics” column, bold numbers indicate molecular ion and underlined numbers characteristic ion.



| Media Sample        | Basic 1<br>(NAA039<br>(NAA045) | Basic 1<br>(NAA094<br>(NAA112) | Basic 1<br>(NAA131<br>(NAA146) | Basic 1<br>(NAA130<br>(NAA146) | Basic 1<br>(NAA095<br>(NAA107) | Basic 1<br>(NAA093<br>(NAA106) | Acid 1<br>(NAA060<br>(NAA076) | Acid 1<br>(NAA064<br>(NAA076) | Acid 1<br>(NAA061<br>(NAA077) | Acid 1<br>(NAA088<br>(NAA109) | Acid 1<br>(NAA123<br>(NAA164) | Acid 1<br>(NAA124<br>(NAA157) |
|---------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
| Species             | <i>L. decemlineata</i>         | <i>L. decemlineata</i>         | <i>P. apterus</i>              | <i>P. apterus</i>              | <i>T. velutinus</i>            | <i>T. velutinus</i>            | <i>H. axyridis</i>            | <i>H. axyridis</i>            | <i>H. axyridis</i>            | <i>H. axyridis</i>            | <i>G. goliathus</i>           | <i>G. goliathus</i>           |
| Sugar TMS           | 0                              | 0                              | 11.04                          | 1.65                           | 0                              | 2.2                            | 0                             | 0                             | 0                             | 0                             | 0.65                          | 0                             |
| Butadiene           | 0                              | 0                              | 1.97                           | 0                              | 0                              | 0                              | 0                             | 0                             | 0                             | 0                             | 0                             | 0                             |
| Protocatechuic Acid | 0                              | 0                              | 0                              | 0                              | 2.48                           | 1.61                           | 0                             | 0                             | 0                             | 0                             | 0                             | 0                             |
| C16 Fatty Acid      | 40.43                          | 28.19                          | 21.95                          | 31.7                           | 18.69                          | 29.96                          | 0                             | 5.45                          | 4.03                          | 0                             | 48.15                         | 33.32                         |
| C18 Fatty Acid      | 43.77                          | 59.65                          | 23.55                          | 39.05                          | 46.67                          | 39.54                          | 68.36                         | 71.3                          | 59.15                         | 42.6                          | 44.85                         | 63.96                         |
| C19 Fatty Acid      | 0                              | 1.7                            | 0                              | 0                              | 0                              | 0                              | 0                             | 0                             | 0                             | 0                             | 5.07                          | 0                             |
| C20 Fatty Acid      | 3.25                           | 0                              | 0                              | 0                              | 2.75                           | 2.13                           | 0                             | 0                             | 0                             | 0                             | 0                             | 0                             |
| Di-Fatty Acid       | 0                              | 0                              | 0                              | 0                              | 0                              | 0                              | 0                             | 0                             | 0                             | 0                             | 0                             | 0                             |
| C20 Hydrocarbon     | 0                              | 0                              | 0                              | 25.33                          | 0                              | 0                              | 0                             | 0                             | 0                             | 0                             | 0                             | 0                             |
| C23 Hydrocarbon     | 0                              | 0                              | 0                              | 0                              | 0                              | 0                              | 1.85                          | 2.49                          | 4.36                          | 5.75                          | 0                             | 0                             |
| C25 Hydrocarbon     | 0                              | 0                              | 0                              | 0                              | 2.74                           | 2.85                           | 4.35                          | 4.11                          | 8.13                          | 11.78                         | 0                             | 0                             |
| C27 Hydrocarbon     | 0                              | 0                              | 2.9                            | 0                              | 5.45                           | 3.57                           | 6.53                          | 3.95                          | 8.19                          | 13.74                         | 0                             | 0                             |
| C29 Hydrocarbon     | 0                              | 0                              | 0                              | 0                              | 9.84                           | 4.76                           | 10.19                         | 0                             | 9.19                          | 15.86                         | 0                             | 0                             |
| C31 Hydrocarbon     | 0                              | 0                              | 0                              | 0                              | 0                              | 0                              | 7.22                          | 4.96                          | 6.96                          | 10.26                         | 0                             | 0                             |
| Cyclic Alkane       | 10.45                          | 9.55                           | 2.78                           | 0.72                           | 8.8                            | 9.96                           | 0                             | 0                             | 0                             | 0                             | 0.56                          | 1.84                          |
| Cholesterol         | 0.38                           | 0.91                           | 0                              | 0                              | 0.89                           | 1.5                            | 1.52                          | 0                             | 0                             | 0                             | 0.72                          | 0.9                           |
| Ergosta             | 0                              | 0                              | 0                              | 0                              | 0                              | 0                              | 0                             | 0                             | 0                             | 0                             | 0                             | 0                             |
| Campesterol         | 0                              | 0                              | 0                              | 0                              | 0                              | 0                              | 0                             | 0                             | 0                             | 0                             | 0                             | 0                             |
| β-Sitosterol        | 0                              | 0                              | 11.39                          | 0                              | 0                              | 0                              | 0                             | 0                             | 0                             | 0                             | 0                             | 0                             |
| Ecdysone            | 0                              | 0                              | 19.19                          | 0                              | 0                              | 0                              | 0                             | 0                             | 0                             | 0                             | 0                             | 0                             |
| Vitamin A-like      | 0.6                            | 0                              | 5.23                           | 1.55                           | 0                              | 0                              | 0                             | 0                             | 0                             | 0                             | 0                             | 0                             |

| Media Sample        | Acid 1<br>(NAA129)<br>(NAA114) | Acid 1<br>(NAA112)<br>(NAA149) | Acid 1<br>(NAA112)<br>(NAA149) | Acid 1<br>(NAA112)<br>(NAA149) | Acid 1<br>(NAA112)<br>(NAA149) | Acid 1<br>(NAA112)<br>(NAA149) | Control 2<br>(NAA112)<br>(NAA149) | Control 2<br>(NAA112)<br>(NAA149) | Basic 2<br>(NAA112)<br>(NAA149) | Basic 2<br>(NAA112)<br>(NAA149) | Acid 2<br>(NAA112)<br>(NAA149) | Acid 2<br>(NAA112)<br>(NAA149) |
|---------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|-----------------------------------|-----------------------------------|---------------------------------|---------------------------------|--------------------------------|--------------------------------|
| Species             | <i>L. decemlineata</i>         | <i>L. decemlineata</i>         | <i>P. apterus</i>              | <i>P. apterus</i>              | <i>T. velutinus</i>            | <i>T. velutinus</i>            | <i>H. axyridis</i>                | <i>H. axyridis</i>                | <i>H. axyridis</i>              | <i>H. axyridis</i>              | <i>G. goliathus</i>            | <i>G. goliathus</i>            |
| Sugar TMS           | 0                              | 0                              | 13.38                          | 0                              | 8.51                           | 0                              | 1.24                              | 0                                 | 0                               | 0                               | 10.08                          | 0.83                           |
| Butadiene           | 0                              | 0                              | 2.03                           | 1.46                           | 0                              | 0                              | 0                                 | 0                                 | 0                               | 0                               | 0                              | 0                              |
| Protocatechuic Acid | 0                              | 0                              | 0                              | 0                              | 4.25                           | 0                              | 0                                 | 0                                 | 0                               | 0                               | 0                              | 0                              |
| C16 Fatty Acid      | 21.92                          | 19.26                          | 23.7                           | 12.26                          | 17.61                          | 29.82                          | 8.25                              | 14.99                             | 5.61                            | 13.26                           | 8.55                           | 11.72                          |
| C18 Fatty Acid      | 43.14                          | 73.62                          | 23.13                          | 30.92                          | 29.04                          | 54.65                          | 82.36                             | 75.4                              | 89.45                           | 85.29                           | 81.24                          | 80.99                          |
| C19 Fatty Acid      | 0                              | 0.04                           | 0                              | 0                              | 0                              | 0                              | 0                                 | 0                                 | 0                               | 0                               | 0                              | 0                              |
| C20 Fatty Acid      | 1.49                           | 2.14                           | 0                              | 0                              | 2.34                           | 0                              | 0                                 | 0                                 | 0                               | 0                               | 0                              | 0                              |
| Di-Fatty Acid       | 0                              | 0                              | 0                              | 2.34                           | 0                              | 0                              | 0                                 | 0                                 | 0                               | 0                               | 0                              | 0                              |
| C20 Hydrocarbon     | 0                              | 0                              | 0                              | 0                              | 0                              | 0                              | 0                                 | 0                                 | 0                               | 0                               | 0                              | 0                              |
| C23 Hydrocarbon     | 0                              | 0                              | 0                              | 0                              | 0                              | 0                              | 0.95                              | 0                                 | 1.42                            | 0.45                            | 1.3                            | 5.52                           |
| C25 Hydrocarbon     | 0                              | 0                              | 0                              | 0                              | 0                              | 2.44                           | 0.8                               | 8.1                               | 0.79                            | 1.01                            | 3.9                            | 0.35                           |
| C27 Hydrocarbon     | 0                              | 0                              | 0                              | 4.45                           | 8.17                           | 4.54                           | 1.18                              | 1.52                              | 0.5                             | 0                               | 1.24                           | 0.58                           |
| C29 Hydrocarbon     | 0                              | 0                              | 0                              | 0                              | 0                              | 3.69                           | 3.36                              | 0                                 | 0                               | 0                               | 0.87                           | 0                              |
| C31 Hydrocarbon     | 0                              | 0                              | 0                              | 0                              | 0                              | 0                              | 1.87                              | 0                                 | 0.88                            | 0                               | 0                              | 0                              |
| Cyclic Alkane       | 32.57                          | 4.95                           | 8.33                           | 30                             | 9.48                           | 3.29                           | 0                                 | 0                                 | 0                               | 0                               | 0                              | 0                              |
| Cholesterol         | 0                              | 0                              | 0                              | 0                              | 11.17                          | 1.58                           | 0                                 | 0                                 | 0                               | 0                               | 0                              | 0                              |
| Ergosta             | 0                              | 0                              | 0                              | 0                              | 0                              | 0                              | 0                                 | 0                                 | 0                               | 0                               | 0                              | 0                              |
| Campesterol         | 0                              | 0                              | 0                              | 0                              | 0                              | 0                              | 0                                 | 0                                 | 0                               | 0                               | 0                              | 0                              |
| β-Sitosterol        | 0                              | 0                              | 5.99                           | 10.75                          | 0                              | 0                              | 0                                 | 0                                 | 0                               | 0                               | 0                              | 0                              |
| Ecdysone            | 0                              | 0                              | 17.74                          | 7.81                           | 9.44                           | 0                              | 0                                 | 0                                 | 0                               | 0                               | 0                              | 0                              |
| Vitamin A-like      | 0.88                           | 0                              | 5.7                            | 0                              | 0                              | 0                              | 0                                 | 0                                 | 0                               | 0                               | 0                              | 0                              |

| Media Sample        | Untreated<br>(NAA057)<br>(NAA072) | Untreated<br>(NAA057)<br>(NAA072) | Untreated<br>(NAA112)<br>(NAA150) | Untreated<br>(NAA112)<br>(NAA150) | Untreated<br>(NAA112)<br>(NAA150) | Untreated<br>(NAA112)<br>(NAA150) | Control 1<br>(NAA112)<br>(NAA150) | Control 1<br>(NAA112)<br>(NAA150) | Control 1<br>(NAA112)<br>(NAA150) | Control 1<br>(NAA112)<br>(NAA150) | Control 1<br>(NAA112)<br>(NAA150) | Control 1<br>(NAA112)<br>(NAA150) |
|---------------------|-----------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|
| Species             | <i>H. axyridis</i>                | <i>H. axyridis</i>                | <i>G. goliathus</i>               | <i>G. goliathus</i>               | <i>G. goliathus</i>               | <i>L. decemlineata</i>            | <i>T. velutinus</i>               | <i>H. axyridis</i>                | <i>H. axyridis</i>                | <i>H. axyridis</i>                | <i>H. axyridis</i>                | <i>G. goliathus</i>               |
| Sugar TMS           | 0                                 | 3.76                              | 2.99                              | 0.94                              | 38.45                             | 28.06                             | 0                                 | 0                                 | 2.4                               | 0                                 | 10.58                             | 1.03                              |
| Butadiene           | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 |
| Protocatechuic Acid | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0.81                              | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 |
| C16 Fatty Acid      | 0                                 | 3.24                              | 33.55                             | 33.62                             | 29.02                             | 14.22                             | 7.81                              | 0                                 | 0                                 | 0                                 | 14.26                             | 33.24                             |
| C18 Fatty Acid      | 64.96                             | 62.77                             | 54.29                             | 57.43                             | 10.79                             | 56.65                             | 4.51                              | 4.06                              | 0                                 | 0                                 | 38.14                             | 64.51                             |
| C19 Fatty Acid      | 0                                 | 0                                 | 3.16                              | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 |
| C20 Fatty Acid      | 0                                 | 0                                 | 0                                 | 0                                 | 9.92                              | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 |
| Di-Fatty Acid       | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 |
| C20 Hydrocarbon     | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 |
| C23 Hydrocarbon     | 2.77                              | 3.68                              | 0                                 | 0                                 | 0                                 | 0                                 | 14.18                             | 4.57                              | 7.72                              | 6.34                              | 0                                 | 0                                 |
| C25 Hydrocarbon     | 5.47                              | 7.36                              | 0                                 | 0                                 | 0                                 | 0                                 | 21.53                             | 19.93                             | 27.63                             | 21.94                             | 0                                 | 0                                 |
| C27 Hydrocarbon     | 1.33                              | 7.49                              | 0                                 | 0                                 | 0                                 | 0                                 | 9.68                              | 22.72                             | 21.51                             | 20.88                             | 0                                 | 0                                 |
| C29 Hydrocarbon     | 16.7                              | 9.08                              | 0                                 | 0                                 | 0                                 | 0                                 | 15.46                             | 19.48                             | 15.25                             | 23.17                             | 0                                 | 0                                 |
| C31 Hydrocarbon     | 8.78                              | 2.62                              | 0                                 | 0                                 | 0                                 | 0                                 | 14.37                             | 10.28                             | 5.65                              | 23.68                             | 0                                 | 0                                 |
| Cyclic Alkane       | 0                                 | 0                                 | 4.23                              | 6.59                              | 10.58                             | 0.25                              | 0                                 | 0                                 | 0                                 | 0                                 | 28.17                             | 1.03                              |
| Cholesterol         | 0                                 | 0                                 | 1.79                              | 1.43                              | 1.24                              | 0                                 | 12.46                             | 3.86                              | 3.86                              | 3.99                              | 8.84                              | 0.19                              |
| Ergosta             | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 11.79                             | 8.42                              | 0                                 | 0                                 | 0                                 |
| Campesterol         | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 3.32                              | 7.56                              | 0                                 | 0                                 | 0                                 |
| β-Sitosterol        | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 |
| Ecdysone            | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 |
| Vitamin A-like      | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 |

| Media Sample        | Control 1<br>(NAA088)<br>(NAA128) | Control 1<br>(NAA126)<br>(NAA143) | Control 1<br>(NAA132)<br>(NAA147) | Control 1<br>(NAA132)<br>(NAA147) | Control 1<br>(NAA132)<br>(NAA147) | Control 1<br>(NAA132)<br>(NAA147) | Basic 1<br>(NAA062)<br>(NAA073) | Basic 1<br>(NAA062)<br>(NAA073) | Basic 1<br>(NAA062)<br>(NAA073) | Basic 1<br>(NAA062)<br>(NAA073) | Basic 1<br>(NAA062)<br>(NAA073) | Basic 1<br>(NAA062)<br>(NAA073) |
|---------------------|-----------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| Species             | <i>L. decemlineata</i>            | <i>L. decemlineata</i>            | <i>P. apterus</i>                 | <i>P. apterus</i>                 | <i>T. velutinus</i>               | <i>T. velutinus</i>               | <i>H. axyridis</i>              | <i>H. axyridis</i>              | <i>H. axyridis</i>              | <i>H. axyridis</i>              | <i>G. goliathus</i>             | <i>G. goliathus</i>             |
| Sugar TMS           | 13.77                             | 1.04                              | 0                                 | 0                                 | 10.31                             | 0                                 | 0                               | 0                               | 0                               | 0                               | 0.39                            | 0.81                            |
| Butadiene           | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                               | 0                               | 0                               | 0                               | 0                               | 0                               |
| Protocatechuic Acid | 0                                 | 0                                 | 0                                 | 0                                 | 6.25                              | 0                                 | 0                               | 0                               | 0                               | 0                               | 0                               | 0                               |
| C16 Fatty Acid      | 8.64                              | 18.49                             | 29.22                             | 32.8                              | 9.17                              | 17.88                             | 0                               | 0                               | 0                               | 0                               | 53.19                           | 34.97                           |
| C18 Fatty Acid      | 40.81                             | 51.6                              | 4.42                              | 4.46                              | 36.93                             | 29.04                             | 7.06                            | 30.58                           | 11.97                           | 56.46                           | 43.11                           | 54.6                            |
| C19 Fatty Acid      | 0                                 | 1.41                              | 0                                 | 0                                 | 0                                 | 0                                 | 0                               | 0                               | 0                               | 0                               | 0.42                            | 0                               |
| C20 Fatty Acid      | 2.31                              | 1.14                              | 0                                 | 0                                 | 5.7                               | 2.32                              | 0                               | 0                               | 0                               | 0                               | 0                               | 0                               |
| Di-Fatty Acid       | 0                                 | 0                                 | 1.61                              | 5.33                              | 0                                 | 0                                 | 0                               | 0                               | 0                               | 0                               | 0                               | 0                               |
| C20 Hydrocarbon     | 0                                 | 0                                 | 6.55                              | 0                                 | 0                                 | 0                                 | 0                               | 0                               | 0                               | 0                               | 0                               | 0                               |
| C23 Hydrocarbon     | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 6.55                            | 4.99                            | 6.63                            | 4.01                            | 0                               | 0                               |
| C25 Hydrocarbon     | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 24.41                           | 18.1                            | 28.23                           | 11.53                           | 0                               | 0                               |
| C27 Hydrocarbon     | 0                                 | 0                                 | 0                                 | 4.35                              | 9.74                              | 12.59                             | 21.68                           | 14.24                           | 22.41                           | 13.74                           | 0                               | 0                               |
| C29 Hydrocarbon     | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 19.58                             | 14.28                           | 10.37                           | 21.7                            | 9.22                            | 0                               | 0                               |
| C31 Hydrocarbon     | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 7.07                            | 7.93                            | 9.08                            | 5.03                            | 0                               | 0                               |
| Cyclic Alkane       | 20.02                             | 22.17                             | 33.58                             | 24.28                             | 7.48                              | 8.94                              | 0                               | 0                               | 0                               | 0                               | 1.15                            | 5.63                            |
| Cholesterol         | 9.02                              | 2.11                              | 0                                 | 0                                 | 9.78                              | 7.15                              | 9.39                            | 10.98                           | 0                               | 0                               | 0.48                            | 3.99                            |
| Ergosta             | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 3.58                            | 2.81                            | 0                               | 0                               | 0                               | 0                               |
| Campesterol         | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 0                                 | 6                               | 0                               | 0                               | 0                               | 0                               | 0                               |
| β-Sitosterol        | 0                                 | 0                                 | 14.7                              | 7.77                              | 0                                 | 0                                 | 0                               | 0                               | 0                               | 0                               | 0                               | 0                               |
| Ecdysone            | 0                                 | 0                                 | 4.86                              | 7.35                              | 4.64                              | 2.5                               | 0                               | 0                               | 0                               | 0                               | 0                               | 0                               |
| Vitamin A-like      | 5.45                              | 0.85                              | 5.05                              | 13.68                             | 0                                 | 0                                 | 0                               | 0                               | 0                               | 0                               | 0                               | 0                               |

Supplementary Table 3.7. Caption overleaf.

**Supplementary Table 3.7 (previous page). Presence/absence of carotenoid molecules identified in gas chromatography-mass spectrometry analysis of cuticles of *Harmonia axyridis*, *Leptinotarsa decemlineata*, *Goliathus goliathus*, *Typocerus velutinus* and *Pyrrhocoris apterus*. When present, compounds are symbolized with the percentage of relative abundance in the sample.**

| Peak | MS characteristics                            | Common name                 | Chemical formula                               | Percentage of samples |
|------|-----------------------------------------------|-----------------------------|------------------------------------------------|-----------------------|
| 1    | 422, 433.5, <u>445.15</u> , 469, <b>537.4</b> | lycopene/ $\beta$ -carotene | C <sub>40</sub> H <sub>56</sub>                | 24%                   |
| 2    | 433.5, 464.5, 488.9, <b>537.4</b>             | $\alpha$ -carotene          | C <sub>40</sub> H <sub>56</sub>                | 6%                    |
| 3    | 445.5, 470, 488, <b>537.4</b>                 | lycopene/ $\beta$ -carotene | C <sub>40</sub> H <sub>56</sub>                | 88%                   |
| 4    | <u>441</u> , 469.25, <b>553.4</b>             | $\beta$ -cryptoxanthin      | C <sub>40</sub> H <sub>56</sub> O              | 24%                   |
| 5    | <u>450</u> , 476, <b>569.4</b>                | lutein                      | C <sub>40</sub> H <sub>56</sub> O <sub>2</sub> | 81%                   |
| 6    | 453.50, 481, <u>563</u> , <b>569.4</b>        | zeaxanthin                  | C <sub>40</sub> H <sub>56</sub> O <sub>2</sub> | 65%                   |

**Supplementary Table 3.8. Molecules identified on the mayor peaks in high performance liquid chromatography – mass spectrometry chromatograms of *Harmonia axyridis* and percentages of appearance in all samples of the taxon.**

Peak numbers correspond to those represented in Supplementary Fig. 3.16. In the “MS characteristics” column, bold numbers indicate molecular ion and underlined numbers characteristic ion. All identifications are listed in Appendix 3.

| Media Sample                | Untreated<br>H S0 R | Untreated<br>H | Basic 1<br>H47 B 60 | Basic 1<br>H48 B 60 | Acid 1<br>H45 A 60 | Acid 1<br>H46 A 60 |
|-----------------------------|---------------------|----------------|---------------------|---------------------|--------------------|--------------------|
| Lycopene/ $\beta$ -carotene | 0                   | 0              | 0                   | 22.677              | 6.343              | 0                  |
| $\alpha$ -carotene          | 0                   | 0              | 0                   | 0                   | 5.487              | 0                  |
| Lycopene/ $\beta$ -carotene | 0                   | 0              | 23.458              | 23.848              | 30.955             | 4.293              |
| $\beta$ -cryptoxanthin      | 13.579              | 0              | 0                   | 0                   | 0                  | 0                  |
| Lutein                      | 77.982              | 100            | 54.313              | 53.475              | 40.132             | 70.034             |
| Unknown                     | 0                   | 0              | 0                   | 0                   | 0                  | 0                  |
| Zeaxanthin                  | 8.439               | 0              | 22.229              | 0                   | 17.082             | 25.674             |

| Media Sample                | Control 2<br>H21 C 120 | Control 2<br>H22 C 120 | Basic 2<br>H6 B 120 | Basic 2<br>H7 B 120 | Acid 2<br>H1 A 120 | Acid 2<br>H2 A 120 |
|-----------------------------|------------------------|------------------------|---------------------|---------------------|--------------------|--------------------|
| Lycopene/ $\beta$ -carotene | 0                      | 0                      | 0                   | 0                   | 0                  | 0                  |
| $\alpha$ -carotene          | 0                      | 0                      | 5.7                 | 49.172              | 0                  | 0                  |
| Lycopene/ $\beta$ -carotene | 17.783                 | 46.121                 | 7.847               | 0                   | 0                  | 100                |
| $\beta$ -cryptoxanthin      | 8.995                  | 0                      | 6.484               | 0                   | 0                  | 0                  |
| Lutein                      | 20.247                 | 53.879                 | 58.348              | 50.828              | 37.776             | 0                  |
| Unknown                     | 41.621                 | 0                      | 15.651              | 0                   | 34.025             | 0                  |
| Zeaxanthin                  | 11.327                 | 0                      | 6.07                | 0                   | 8.291              | 0                  |

| Media Sample                | Control 3<br>H25 C 180 | Control 3<br>H26 C 180 | Basic 3<br>H16 B 180 | Basic 3<br>H17 B 180 | Acid 3<br>H11 A 180 | Acid 3<br>H12 A 180 |
|-----------------------------|------------------------|------------------------|----------------------|----------------------|---------------------|---------------------|
| Lycopene/ $\beta$ -carotene | 0                      | 15.467                 | 0                    | 0                    | 0                   | 0                   |
| $\alpha$ -carotene          | 4.784                  | 20.17                  | 19.325               | 6.436                | 0                   | 0                   |
| Lycopene/ $\beta$ -carotene | 0                      | 17.841                 | 14.881               | 9.911                | 47.499              | 0                   |
| $\beta$ -cryptoxanthin      | 0                      | 0                      | 13.89                | 0                    | 0                   | 0                   |
| Lutein                      | 76.697                 | 46.522                 | 28.429               | 74.627               | 52.501              | 54.467              |
| Unknown                     | 11.258                 | 0                      | 17.109               | 9.027                | 0                   | 45.533              |
| Zeaxanthin                  | 7.26                   | 0                      | 6.366                | 0                    | 0                   | 0                   |

**Supplementary Table 3.9. Presence/absence of carotenoid molecules identified in high performance liquid chromatography-mass spectrometry analysis of cuticles of *Harmonia axyridis*.** When present, compounds are symbolized with the percentage of relative abundance in the sample.

| Order      | taxon                            | Treatment | PTCA in<br>native tissue<br>(ng/mg) | 4-AHP<br>(ng/mg) | EM<br>(ug/mg) | PM<br>(ug/mg) | TM<br>(ug/mg) |
|------------|----------------------------------|-----------|-------------------------------------|------------------|---------------|---------------|---------------|
| Coleoptera | <i>Harmonia axyridis</i>         | pH 7      | 8.90                                | 35.90            | 0.22          | 0.25          | 0.47          |
|            |                                  | pH 6      | 4.40                                | 7.70             | 0.11          | 0.05          | 0.16          |
|            |                                  | pH 8      | 4.40                                | 1.30             | 0.11          | 0.01          | 0.12          |
|            | <i>Leptinotarsa decemlineata</i> | pH 7      | 105.00                              | 175.00           | 2.63          | 1.23          | 3.86          |
|            |                                  | pH 6      | 20.00                               | 237.00           | 0.50          | 1.66          | 2.16          |
|            |                                  | pH 8      | 17.80                               | 25.60            | 0.45          | 0.18          | 0.63          |
|            | <i>Typocerus velutinus</i>       | pH 7      | 8.90                                | 48.70            | 0.22          | 0.34          | 0.56          |
|            |                                  | pH 6      | 8.90                                | 5.10             | 0.22          | 0.04          | 0.26          |
|            |                                  | pH 8      | 4.40                                | 16.40            | 0.11          | 0.11          | 0.22          |

**Supplementary Table 3.10. Alkaline hydrogen peroxide oxidation and hydroiodic acid hydrolysis from dark regions from the cuticle of untreated *Harmonia axyridis*, and treated *Harmonia axyridis*, *Leptinotarsa decemlineata* and *Typocerus velutinus* for 60 days in acidic (pH 6), neutral (pH 7) and basic (pH 8) media.** PTCA, pyrrole-2,3,5-tricarboxylic acid; 4-AHP, 4-amino-3-hydroxyphenylalanine ; EM, eumelanin; PM, pheomelanin; TM, total melanin (EM+PM).

**Digital file: Colour\_spectra\_dataset.xlsx**

[https://www.dropbox.com/s/6uxi6w7em7gkr0t/Colour\\_spectra\\_dataset.xlsx?dl=0](https://www.dropbox.com/s/6uxi6w7em7gkr0t/Colour_spectra_dataset.xlsx?dl=0)



**A3.3. R Script**

```

library(MASS)
library(nlme)
library(rpart)
library(spatstat.data)
library(spatstat)
library(ggplot2)
library(magrittr)
library(ggpubr)
library(ellipse)
library(permute)
library(lattice)
library(vegan)

# Major peaks identified

setwd("/Users/aidin_13_/Desktop/thesis(laptop)/compounds/peaks/CSV/")
all <- read.csv("all_2.csv")
attach(all)
names(all)

# Not logged:
pca <- prcomp(all[,2:24])
attach(pca)
prop.pca = pca$sdev^2 / sum(pca$sdev^2) #proportions
print (prop.pca) # If we are going to be using proportions for LDA as we can't use
percentages then THIS is the one we use for PCA

# Plot scores
pcascores <- pca$x
plot(pcascores[,1:2],pch=19, col=c("red", "grey", "green", "orange", "yellow",
  "black", "blue", "pink", "brown", "salmon", "gold", "purple"), xlab="PC1 - 56.2%",
  ylab="PC2 - 24.2%")

```

```
#Plot loadings
```

```
plot(pca, col=c("wheat", "slategrey", "yellowgreen", "steelblue2", "seagreen2",  
"palevioletred1", "coral1", "lightpink", "dodgerblue", "purple1"), xlab="Principal  
component")
```

```
pcaloadings <- pca$rotation
```

```
plot(pcaloadings[,1:2], pch=19, col=c("red", "grey", "mediumaquamarine", "orange",  
"yellow", "black", "blue", "pink", "brown", "salmon", "deepskyblue", "purple",  
"chartreuse", "cornflowerblue", "darkgreen", "firebrick", "darkturquoise",  
"deeppink", "indianred1", "khaki", "navy", "thistle", "tan"), xlab="PC1 - 56.2%",  
ylab="PC2 - 24.2%")
```

```
legend(locator(1), c("Sugar TMS", "Butadiene", "Protocatechuic A.", "C16 Fatty A.",  
"C18 Fatty A.", "C19 Fatty A.", "C20 Fatty A.", "Di-Fatty A.", "C20 Diene", "C23  
Alkaloid", "C25 Alkaloid", "C27 Alkaloid", "C29 Alkaloid", "C29 Diene", "C31  
Alkaloid", "C31 Diene", "Cyclic Alkane", "Cholesterol", "Ergosta", "Campesterol",  
"B-Sitosterol", "Ecdysone", "Vitamin A-like"), pch=19, col=c("red", "grey",  
"mediumaquamarine", "orange", "yellow", "black", "blue", "pink", "brown",  
"salmon", "deepskyblue", "purple", "chartreuse", "cornflowerblue", "darkgreen",  
"firebrick", "darkturquoise", "deeppink", "indianred1", "khaki", "navy", "thistle",  
"tan"), cex=2/3, bty = "n")
```

```
plot(pcaloadings[,3:4], pch=19, col=c("red", "grey", "mediumaquamarine", "orange",  
"yellow", "black", "blue", "pink", "brown", "salmon", "deepskyblue", "purple",  
"chartreuse", "cornflowerblue", "darkgreen", "firebrick", "darkturquoise",  
"deeppink", "indianred1", "khaki", "navy", "thistle", "tan"), xlab="PC1 - 7.2%",  
ylab="PC2 - 4.9%")
```

```
legend(locator(1), c("Sugar TMS", "Butadiene", "Protocatechuic A.", "C16 Fatty A.",  
"C18 Fatty A.", "C19 Fatty A.", "C20 Fatty A.", "Di-Fatty A.", "C20 Diene", "C23  
Alkaloid", "C25 Alkaloid", "C27 Alkaloid", "C29 Alkaloid", "C29 Diene", "C31  
Alkaloid", "C31 Diene", "Cyclic Alkane", "Cholesterol", "Ergosta", "Campesterol",  
"B-Sitosterol", "Ecdysone", "Vitamin A-like"), pch=19, col=c("red", "grey",
```



```
"mediumaquamarine", "orange", "yellow", "black", "blue", "pink", "brown",
"salmon", "deepskyblue", "purple", "chartreuse", "cornflowerblue", "darkgreen",
"firebrick", "darkturquoise", "deeppink", "indianred1", "khaki", "navy", "thistle",
"tan"),cex=2/3, bty = "n")
```

```
plot(pcaloadings[,5:6], pch=19, col=c("red", "grey", "mediumaquamarine", "orange",
"yellow", "black", "blue", "pink", "brown", "salmon", "deepskyblue", "purple",
"chartreuse", "cornflowerblue", "darkgreen", "firebrick", "darkturquoise",
"deeppink", "indianred1", "khaki", "navy", "thistle", "tan"), xlab="PC1 - 1.9%",
ylab="PC2 - 1.3%")
```

```
legend(locator(1), c("Sugar TMS", "Butadiene", "Protocatechuic A.", "C16 Fatty A.",
"C18 Fatty A.", "C19 Fatty A.", "C20 Fatty A.", "Di-Fatty A.", "C20 Diene", "C23
Alkaloid", "C25 Alkaloid", "C27 Alkaloid", "C29 Alkaloid", "C29 Diene", "C31
Alkaloid", "C31 Diene", "Cyclic Alkane", "Cholesterol", "Ergosta", "Campesterol",
"B-Sitosterol", "Ecdysone", "Vitamin A-like"), pch=19, col=c("red", "grey",
"mediumaquamarine", "orange", "yellow", "black", "blue", "pink", "brown",
"salmon", "deepskyblue", "purple", "chartreuse", "cornflowerblue", "darkgreen",
"firebrick", "darkturquoise", "deeppink", "indianred1", "khaki", "navy", "thistle",
"tan"),cex=2/3, bty = "n")
```

```
plot(pcaloadings[,7:8], pch=19, col=c("red", "grey", "mediumaquamarine", "orange",
"yellow", "black", "blue", "pink", "brown", "salmon", "deepskyblue", "purple",
"chartreuse", "cornflowerblue", "darkgreen", "firebrick", "darkturquoise",
"deeppink", "indianred1", "khaki", "navy", "thistle", "tan"), xlab="PC1 - 1.1%",
ylab="PC2 - 0.9%")
```

```
legend(locator(1), c("Sugar TMS", "Butadiene", "Protocatechuic A.", "C16 Fatty A.",
"C18 Fatty A.", "C19 Fatty A.", "C20 Fatty A.", "Di-Fatty A.", "C20 Diene", "C23
Alkaloid", "C25 Alkaloid", "C27 Alkaloid", "C29 Alkaloid", "C29 Diene", "C31
Alkaloid", "C31 Diene", "Cyclic Alkane", "Cholesterol", "Ergosta", "Campesterol",
"B-Sitosterol", "Ecdysone", "Vitamin A-like"), pch=19, col=c("red", "grey",
"mediumaquamarine", "orange", "yellow", "black", "blue", "pink", "brown",
"salmon", "deepskyblue", "purple", "chartreuse", "cornflowerblue", "darkgreen",
"firebrick", "darkturquoise", "deeppink", "indianred1", "khaki", "navy", "thistle",
"tan"),cex=2/3, bty = "n")
```

```
plot(pcaloadings[,9:10], pch=19, col=c("red", "grey", "mediumaquamarine",
"orange", "yellow", "black", "blue", "pink", "brown", "salmon", "deepskyblue",
"purple", "chartreuse", "cornflowerblue", "darkgreen", "firebrick", "darkturquoise",
"deeppink", "indianred1", "khaki", "navy", "thistle", "tan"), xlab="PC1 - 0.7%",
ylab="PC2 - 0.4%")
legend(locator(1), c("Sugar TMS", "Butadiene", "Protocatechuic A.", "C16 Fatty A.",
"C18 Fatty A.", "C19 Fatty A.", "C20 Fatty A.", "Di-Fatty A.", "C20 Diene", "C23
Alkaloid", "C25 Alkaloid", "C27 Alkaloid", "C29 Alkaloid", "C29 Diene", "C31
Alkaloid", "C31 Diene", "Cyclic Alkane", "Cholesterol", "Ergosta", "Campesterol",
"B-Sitosterol", "Ecdysone", "Vitamin A-like"), pch=19, col=c("red", "grey",
"mediumaquamarine", "orange", "yellow", "black", "blue", "pink", "brown",
"salmon", "deepskyblue", "purple", "chartreuse", "cornflowerblue", "darkgreen",
"firebrick", "darkturquoise", "deeppink", "indianred1", "khaki", "navy", "thistle",
"tan"), cex=2/3, bty = "n")
```

```
plot(pcaloadings[,11:12], pch=19, col=c("red", "grey", "mediumaquamarine",
"orange", "yellow", "black", "blue", "pink", "brown", "salmon", "deepskyblue",
"purple", "chartreuse", "cornflowerblue", "darkgreen", "firebrick", "darkturquoise",
"deeppink", "indianred1", "khaki", "navy", "thistle", "tan"), xlab="PC1 - 0.3%",
ylab="PC2 - 0.2%")
legend(locator(1), c("Sugar TMS", "Butadiene", "Protocatechuic A.", "C16 Fatty A.",
"C18 Fatty A.", "C19 Fatty A.", "C20 Fatty A.", "Di-Fatty A.", "C20 Diene", "C23
Alkaloid", "C25 Alkaloid", "C27 Alkaloid", "C29 Alkaloid", "C29 Diene", "C31
Alkaloid", "C31 Diene", "Cyclic Alkane", "Cholesterol", "Ergosta", "Campesterol",
"B-Sitosterol", "Ecdysone", "Vitamin A-like"), pch=19, col=c("red", "grey",
"mediumaquamarine", "orange", "yellow", "black", "blue", "pink", "brown",
"salmon", "deepskyblue", "purple", "chartreuse", "cornflowerblue", "darkgreen",
"firebrick", "darkturquoise", "deeppink", "indianred1", "khaki", "navy", "thistle",
"tan"), cex=2/3, bty = "n")
```

```
plot(pcaloadings[,13:14], pch=19, col=c("red", "grey", "mediumaquamarine",
"orange", "yellow", "black", "blue", "pink", "brown", "salmon", "deepskyblue",
"purple", "chartreuse", "cornflowerblue", "darkgreen", "firebrick", "darkturquoise",
```

```

"deeppink", "indianred1", "khaki", "navy", "thistle", "tan"), xlab="PC1 - 0.2%",
ylab="PC2 - 0.1%")
legend(locator(1), c("Sugar TMS", "Butadiene", "Protocatechuic A.", "C16 Fatty A.",
"C18 Fatty A.", "C19 Fatty A.", "C20 Fatty A.", "Di-Fatty A.", "C20 Diene", "C23
Alkaloid", "C25 Alkaloid", "C27 Alkaloid", "C29 Alkaloid", "C29 Diene", "C31
Alkaloid", "C31 Diene", "Cyclic Alkane", "Cholesterol", "Ergosta", "Campesterol",
"B-Sitosterol", "Ecdysone", "Vitamin A-like"), pch=19, col=c("red", "grey",
"mediumaquamarine", "orange", "yellow", "black", "blue", "pink", "brown",
"salmon", "deepskyblue", "purple", "chartreuse", "cornflowerblue", "darkgreen",
"firebrick", "darkturquoise", "deeppink", "indianred1", "khaki", "navy", "thistle",
"tan"),cex=2/3, bty = "n")

hex <- all$hex
hex <- as.vector(hex)

med <- all$Compounds
med <- as.vector(med)

nm <- all$Name
nm <- as.vector(nm)

sm <- all$Sample
sm <- as.vector(sm)

sp <- all$Species
sp <- as.vector(sp)

sp[which(sp == "G. goliathus")] <- "G.g."
sp[which(sp == "H. axyridis")] <- "H.a."
sp[which(sp == "L. decemlineata")] <- "L.d."
sp[which(sp == "P. apterus")] <- "P.a."
sp[which(sp == "T. velutinus")] <- "T.v."

```

```

plot(pca$x[,1:2], col = hex, pch=
c(19,19,18,18,17,15,19,19,19,19,18,18,17,17,11,11,15,15,19,19,19,19,18,18,17,17,1
1,11,15,15,19,19,19,19,18,18,17,17,11,11,15,15,21,21,21,21,21,21), xlab="PC1 -
56.2%", ylab="PC2 - 24.2%")
text(x[,1],x[,2], cex=0.5, labels = sp, col = hex , pos=3)

# Confidence Ellipses Media

ordiellipse(pca, all$Compounds, conf= 0.99, col = hex)

# Plot media

levels(Compounds)

# Plot Biplot for PCA Media

biplot(pca, scale = 0)

# LDA: Media
lda.all <- lda(Compounds ~ Sugar.TMS + Butadiene + Protocatechuic.Acid +
C16.F..A. + C18.F..A. + C19.F..A. + C20.F..A. + Di.fatty.Acid + C20.Diene +
C23.Alk + C25.Alk + C27.Alk + C29.Alk + C29.Die + C31.Alk + C31.Die +
Cyclic.Alkane + Cholesterol + Ergosta + Campesterol + X_.Sitosterol + Ecdysone +
Vitamin.A.like, all)
attach(lda.all)
prop.lda.all = lda.all$svd^2/sum(lda.all$svd^2) # proportions
print(prop.lda.all)

# predict LDA. It returns the classification and the posterior probabilities of the new
data based on the LD model:
plda.all <- predict (object = lda.all, newdata = all)# plot

plot(plda.all$x[,1], plda.all$x[,2], pch=
c(19,19,18,18,17,15,19,19,19,19,18,18,17,17,11,11,15,15,19,19,19,19,18,18,17,17,1

```

```

1,11,15,15,19,19,19,19,18,18,17,17,11,11,15,15,21,21,21,21,21,21), col =hex,
xlab="LD1 - 41.6%", ylab="LD2 - 28.2%") # make scatterplot
text(plda.all$x[,1],plda.all$x[,2], labels=sp, col=hex, cex=0.5, pos=3)

# Confidence Ellipses Media

ordiellipse(pca, all$Compounds, conf= 0.99, col = hex)

# Plot Biplot for LDA Media

heads.all <- coef(lda.all)
heads.all
biplot.all <- arrows(x0 = 0, y0 = 0, x1 = heads.all [,1], y1 = heads.all [,2], length =
1/10, col = "red") #Make font smalller
text(heads.all[,1:2], labels = row.names(heads.all))

```

## APPENDIX 4

# Supplementary Information on Chapter 4: Colouration bias in the fossil record of insects

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#### **A4.1. Supplementary Text:**

**A.4.1.1. Tumbling.** The first fissured elytron as a result of physical breakage was first detected at 136.75 hours (RBS4). The first fractured elytron (BB2) was observed at 86 hours (Tables 4.2 and 4.3). The first fragmented elytra (RBS4, BB1, BB2 and BBS3) were detected at 329 hours (Tables 4.2 and 4.3).

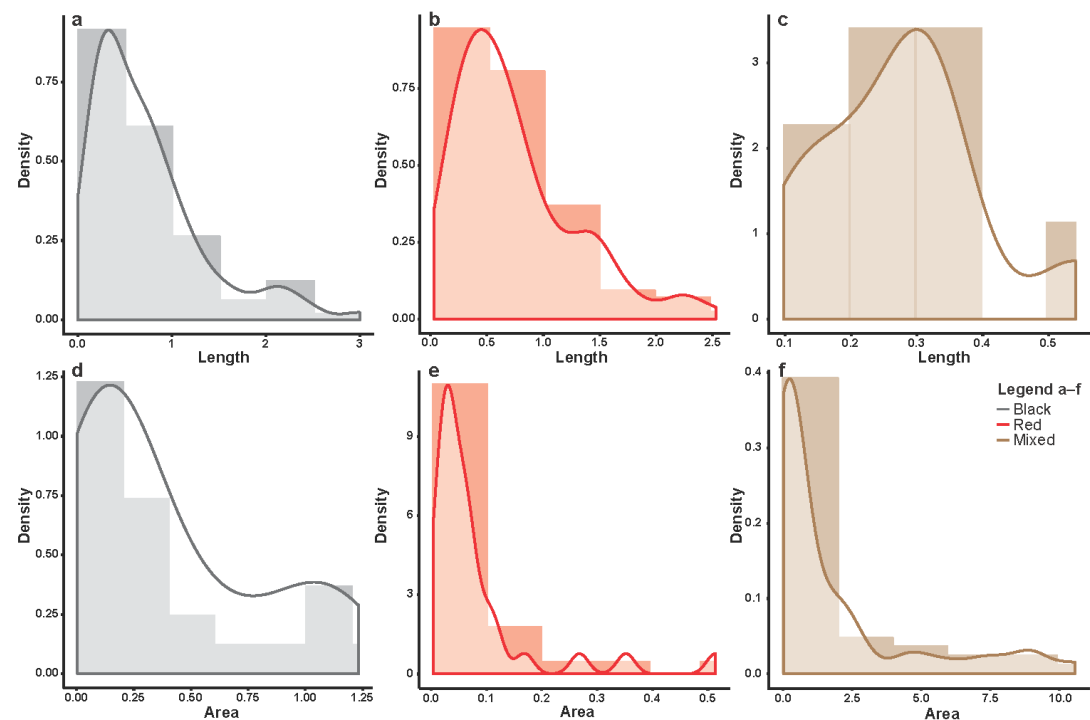
Due to physical efforts undergone by vials during tumbling experiments, four vials were found to slightly leak liquid and were refilled with 1–2 ml of double distilled water at 86 hours (RBS1 and BB3) and 537.95 hours (RB1 and RB2) in order to regain the 14 ml initially introduced at the start of the experiment (Tables 4.2 and 4.3). Another three vials were substituted for new vials and refilled with 3–4 ml of double distilled water after leakage was observed at 109.5 hours (BBS1 and BBS3) and 136.75 hours (BB4) (Tables 4.2 and 4.3). One of the vials previously refilled at 537.95 hours (RB2) was found to leak liquid, sediment and fragments of the sample at 588.35 hours, this sample was discarded from the analysis.

**A.4.1.2. Statistical analysis.** Density graphs and quantile-quantile plots show all datasets skewed normality towards smaller measurements (Supplementary Figs. 4.1a–b, d–f and 4.2a–b, d–f), with the exception of mixed visual colour length of fractures which is a normal distributed dataset (Supplementary Figs. 4.1c and 4.2c). In line with previous visual inspection, Shapiro-Wilk tests indicate that length of fracture for black and orange regions are not normal distributed (Table 4.4;  $p < 0.05$ ), whereas length of fractures in regions with mixed visual colours are not different from a normal distribution (Table 4.4;  $p > 0.05$ ). Shapiro-Wilk tests also show that areas of fragmented cuticle in all coloured regions are not normal distributed (Table 4.4;  $p < 0.05$ ). Kruskal-Wallis Rank tests conclude that medians of length of fracture

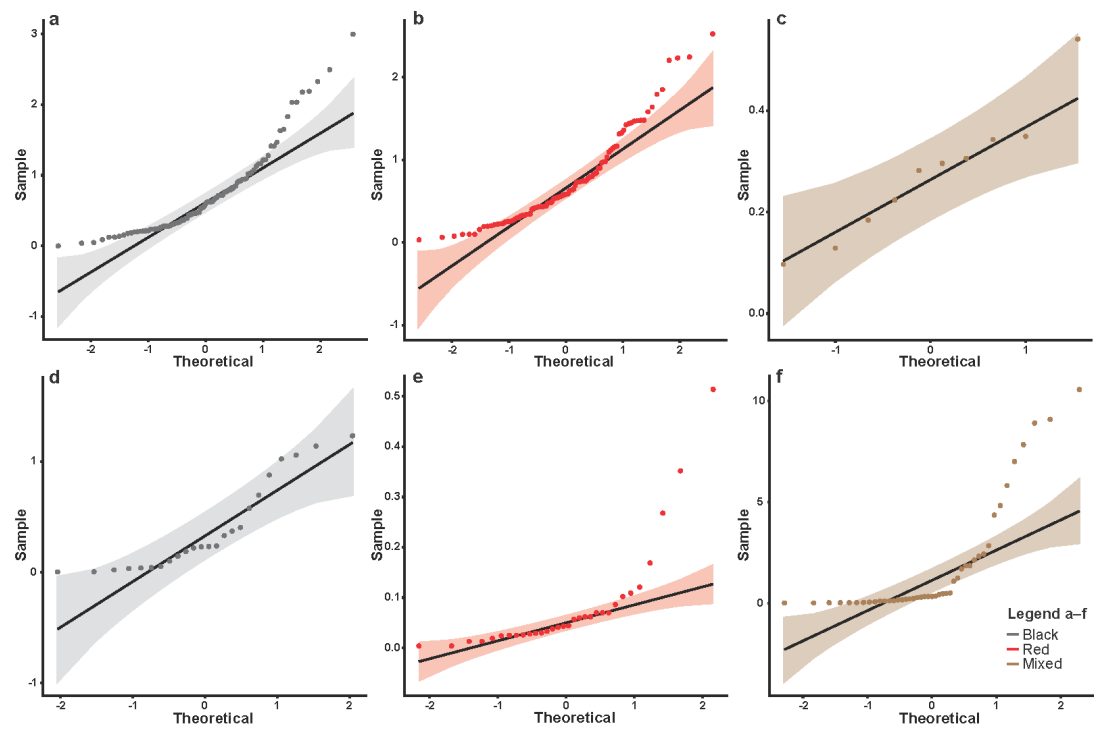


(Table 4.4;  $p < 0.05$ ) and area of fragments (Table 4.4;  $p < 0.05$ ) are not equal, consequently the null hypothesis is rejected, and the differences among groups are statistically significant.

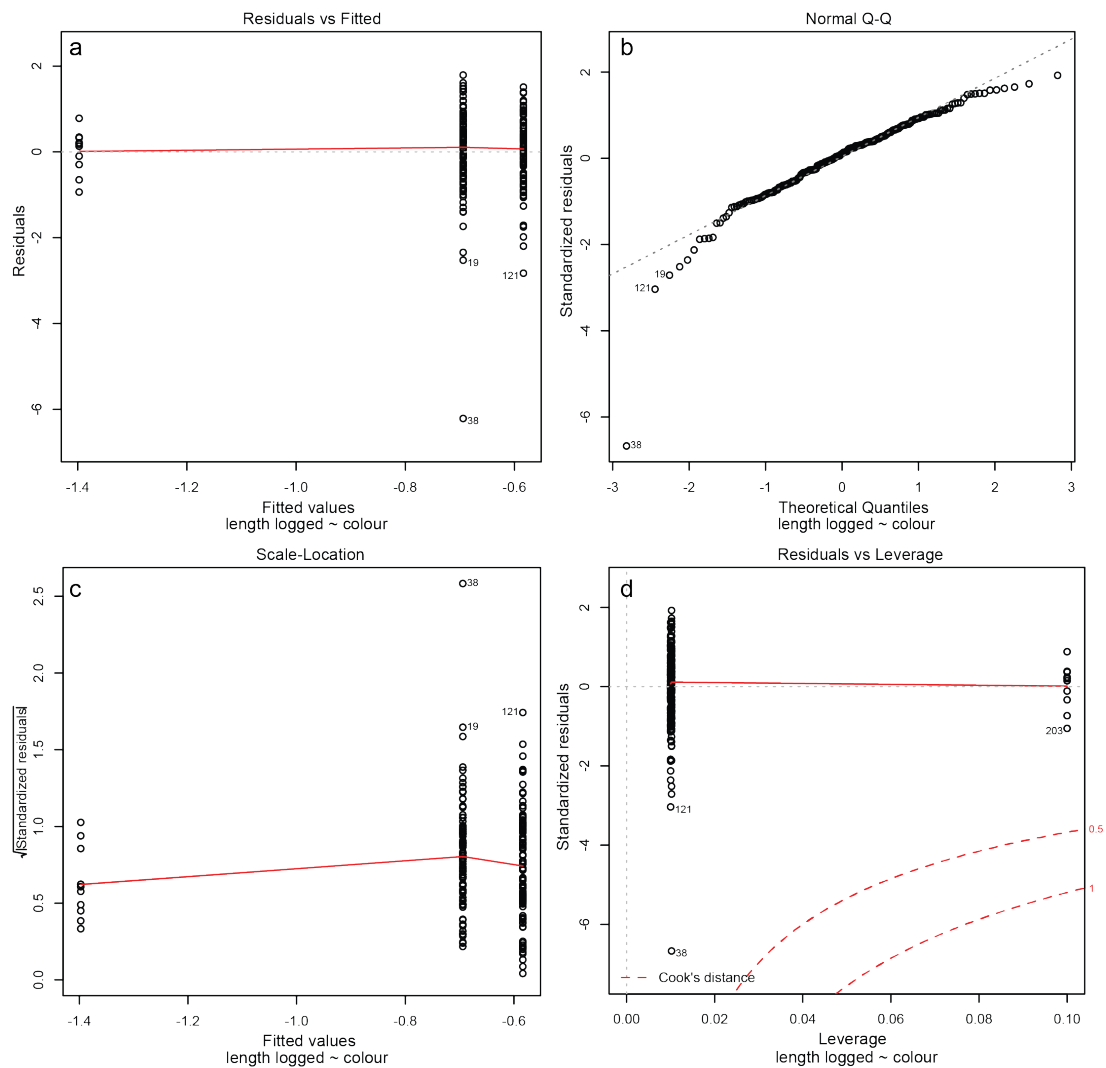
## A4.2. Supplementary Figures:



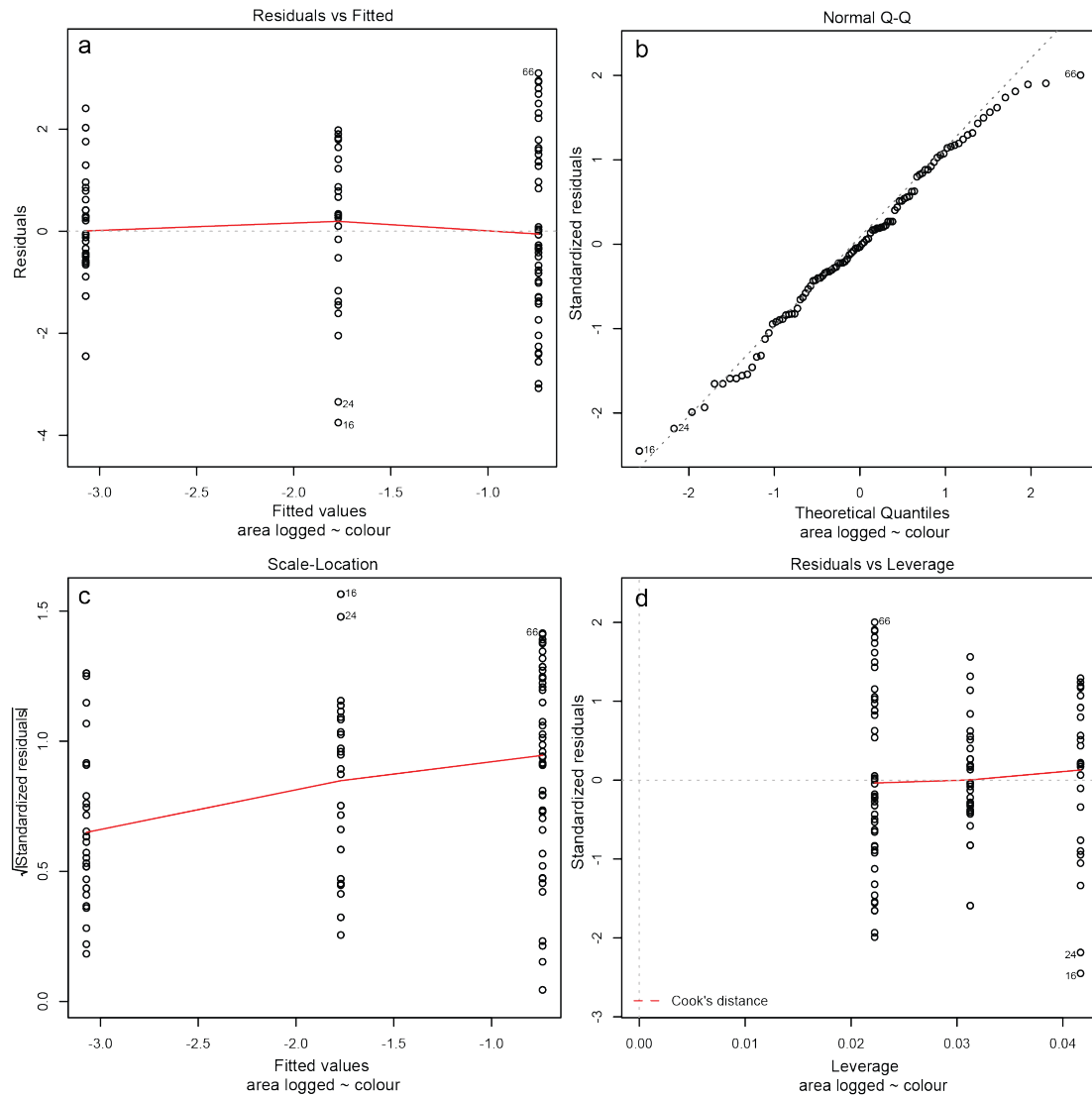
**Supplementary Figure 4.1. Density plots and histograms of datasets from length of fracture (a–c) and area of individual fragments (d–f).** a, d Predominantly black regions of the cuticle. b, e Predominantly orange regions of the cuticle. c, f Mixed (containing black and orange visual colours) regions of the cuticle. a–e; please see f for key.



**Supplementary Figure 4.2. Quantile-quantile plots of length of fracture (a–c) and area of individual fragments (d–f). a, d** Predominantly black regions of the cuticle. **b, e** Predominantly orange regions of the cuticle. **c, f** Mixed (containing black and orange visual colours) regions of the cuticle. **a–e**; please see **f** for key.



**Supplementary Figure 4.3.** ANOVA residual plots of length of fracture. Residuals vs fitted (a), quantile–quantile (b), scale location (c) and residuals vs leverage (d).



**Supplementary Figure 4.4. ANOVA residual plots of area of fracture. Residuals vs fitted (a), quantile–quantile (b), scale location (c) and residuals vs leverage (d).**

### **A4.3. Supplementary Tables:**

|         | Area    |         |         | Length |       |       |
|---------|---------|---------|---------|--------|-------|-------|
|         | Black   | Red     | Mixed   | Black  | Red   | Mixed |
|         | 1.058   | 0.026   | 4.364   | 0.472  | 0.282 | 0.224 |
|         | 1.025   | 0.029   | 1.865   | 0.432  | 1.326 | 0.343 |
|         | 0.332   | 0.060   | 1.259   | 0.329  | 0.483 | 0.184 |
|         | 0.221   | 0.044   | 7.013   | 0.899  | 0.328 | 0.541 |
|         | 0.188   | 0.514   | 2.852   | 1.076  | 0.34  | 0.097 |
|         | 0.697   | 0.062   | 0.445   | 0.654  | 0.977 | 0.129 |
|         | 0.231   | 0.033   | 0.043   | 0.266  | 0.69  | 0.306 |
|         | 0.040   | 0.025   | 2.333   | 0.239  | 1.034 | 0.282 |
|         | 0.145   | 0.057   | 0.024   | 2.033  | 0.077 | 0.296 |
|         | 0.022   | 0.086   | 10.567  | 0.922  | 0.733 | 0.349 |
|         | 0.043   | 0.043   | 4.836   | 1.018  | 1.476 |       |
|         | 0.101   | 0.268   | 2.440   | 0.945  | 0.229 |       |
|         | 0.405   | 0.070   | 0.130   | 0.235  | 0.226 |       |
|         | 0.879   | 0.025   | 5.824   | 0.814  | 0.413 |       |
|         | 0.373   | 0.121   | 1.871   | 1.217  | 0.435 |       |
|         | 0.004   | 0.041   | 7.844   | 2.327  | 0.799 |       |
|         | 0.034   | 0.062   | 1.101   | 0.088  | 0.203 |       |
|         | 1.233   | 0.169   | 9.094   | 0.123  | 0.248 |       |
|         | 0.053   | 0.352   | 8.910   | 0.04   | 0.526 |       |
|         | 0.233   | 0.109   | 1.708   | 0.365  | 0.965 |       |
|         | 0.578   | 0.102   | 2.147   | 0.176  | 0.342 |       |
|         | 0.239   | 0.004   | 0.121   | 0.576  | 0.222 |       |
|         | 1.140   | 0.028   | 0.519   | 1.414  | 0.033 |       |
|         | 0.006   | 0.038   | 0.290   | 0.198  | 0.844 |       |
|         |         | 0.030   | 0.244   | 0.72   | 0.825 |       |
|         |         | 0.013   | 0.210   | 0.201  | 2.246 |       |
|         |         | 0.070   | 0.314   | 0.627  | 0.902 |       |
|         |         | 0.004   | 0.337   | 2.177  | 1.143 |       |
|         |         | 0.013   | 0.495   | 0.776  | 0.432 |       |
|         |         | 0.019   | 0.022   | 0.332  | 0.101 |       |
|         |         | 0.070   | 0.347   | 0.463  | 2.206 |       |
|         |         | 0.024   | 0.363   | 1.632  | 1.478 |       |
|         |         |         | 0.180   | 0.917  | 0.265 |       |
|         |         |         | 0.050   | 0.549  | 1.359 |       |
|         |         |         | 0.132   | 1.166  | 1.096 |       |
|         |         |         | 0.479   | 2.997  | 0.444 |       |
|         |         |         | 0.037   | 0.048  | 0.423 |       |
|         |         |         | 0.037   | 0      | 0.196 |       |
|         |         |         | 0.062   | 0.287  | 0.099 |       |
|         |         |         | 0.173   | 0.543  | 0.647 |       |
|         |         |         | 0.044   | 0.85   | 0.743 |       |
|         |         |         | 0.338   | 0.833  | 0.257 |       |
|         |         |         |         | 0.24   | 0.743 |       |
| TOTAL   | 9.280   | 2.368   | 77.600  | 0.71   | 0.158 |       |
| Mean    | 0.387   | 0.099   | 3.233   | 0.388  | 0.794 |       |
| Std Dev | 0.40085 | 0.11981 | 3.27921 | 0.478  | 0.479 |       |
|         |         |         |         | 1.162  | 0.579 |       |
|         |         |         |         | 0.625  | 1.64  |       |
|         |         |         |         | 0.763  | 0.193 |       |
|         |         |         |         | 0.137  | 0.636 |       |
|         |         |         |         | 0.623  | 0.322 |       |
|         |         |         |         | 0.154  | 1.481 |       |
|         |         |         |         | 1.026  | 0.557 |       |
|         |         |         |         | 1.414  | 1.315 |       |
|         |         |         |         | 0.673  | 1.582 |       |
|         |         |         |         | 0.445  | 0.436 |       |
|         |         |         |         | 2.496  | 0.062 |       |
|         |         |         |         | 0.322  | 0.097 |       |
|         |         |         |         | 0.278  | 0.979 |       |
|         |         |         |         | 1.652  | 0.899 |       |
|         |         |         |         | 1.22   | 0.3   |       |
|         |         |         |         | 0.213  | 1.119 |       |
|         |         |         |         | 2.188  | 0.328 |       |
|         |         |         |         | 1.831  | 0.567 |       |
|         |         |         |         | 1.28   | 1.469 |       |
|         |         |         |         | 1.467  | 0.474 |       |
|         |         |         |         | 0.954  | 0.419 |       |
|         |         |         |         | 0.689  | 0.255 |       |
|         |         |         |         | 0.185  | 0.739 |       |
|         |         |         |         | 0.944  | 0.784 |       |
|         |         |         |         | 0.46   | 2.234 |       |
|         |         |         |         | 2.034  | 1.44  |       |
|         |         |         |         | 0.124  | 1.426 |       |
|         |         |         |         | 1.049  | 0.761 |       |
|         |         |         |         | 1.13   | 0.585 |       |
|         |         |         |         | 0.527  | 0.744 |       |
|         |         |         |         | 0.368  | 0.634 |       |
|         |         |         |         | 0.209  | 0.499 |       |
|         |         |         |         | 0.21   | 0.623 |       |
|         |         |         |         | 0.626  | 1.795 |       |
|         |         |         |         | 0.442  | 0.44  |       |
|         |         |         |         | 0.249  | 0.582 |       |
|         |         |         |         | 0.391  | 1.852 |       |
|         |         |         |         | 0.237  | 0.303 |       |
|         |         |         |         | 0.718  | 0.217 |       |
|         |         |         |         | 0.216  | 0.835 |       |
|         |         |         |         | 0.46   | 0.543 |       |
|         |         |         |         | 0.797  | 2.527 |       |
|         |         |         |         | 0.616  | 1.449 |       |
|         |         |         |         | 0.793  | 0.535 |       |
|         |         |         |         | 0.278  | 0.589 |       |
|         |         |         |         | 0.304  | 1.16  |       |
|         |         |         |         | 0.76   | 0.732 |       |
|         |         |         |         | 0.281  | 0.342 |       |
|         |         |         |         | 0.311  | 1.167 |       |
|         |         |         |         | 0.714  | 0.541 |       |
|         |         |         |         | 0.282  | 0.728 |       |
|         |         |         |         | 0.352  | 0.554 |       |
|         |         |         |         |        | 0.4   |       |
|         |         |         |         |        | 0.432 |       |

**Supplementary Table 4.1 (previous page). Measurements of length of fracture and area of fragments in tumbled cuticles of *Harmonia axyridis*.**



| Length of fracture |     |       |      |         |               |                 |        |       |         |               |
|--------------------|-----|-------|------|---------|---------------|-----------------|--------|-------|---------|---------------|
| raw data           |     |       |      |         |               | log-transformed |        |       |         |               |
| ANOVA              | Df  | Sum   | Mean | F value | p-value       | Df              | Sum    | Mean  | F value | p-value       |
| colour             | 2   | 2.03  | 1.01 | 3.225   | <b>0.0406</b> | 2               | 6.090  | 3.046 | 3.475   | <b>0.0328</b> |
| Residuals          | 205 | 63.79 | 0.31 |         |               | 205             | 179.65 | 0.876 |         |               |

| Area of fragment |    |        |       |         |                |                 |        |       |         |                |
|------------------|----|--------|-------|---------|----------------|-----------------|--------|-------|---------|----------------|
| raw data         |    |        |       |         |                | log-transformed |        |       |         |                |
| ANOVA            | Df | Sum    | Mean  | F value | p-value        | Df              | Sum    | Mean  | F value | p-value        |
| colour           | 2  | 65.70  | 32.87 | 9.084   | <b>2.4E-04</b> | 2               | 101.80 | 50.92 | 20.820  | <b>2.9E-08</b> |
| Residuals        | 98 | 354.60 | 3.62  |         |                | 98              | 239.80 | 2.450 |         |                |

| Length of fracture |               |               |                 |               |               |           |
|--------------------|---------------|---------------|-----------------|---------------|---------------|-----------|
| raw data           |               |               | log-transformed |               |               |           |
|                    | Black/Mixed   | Red/Mixed     | Black/Red       | Black/Mixed   | Red/Mixed     | Black/Red |
| Holm-Ponferronni   | <b>0.0370</b> | <b>0.0370</b> | 0.8760          | <b>0.0490</b> | <b>0.0280</b> | 0.4070    |
| Tukey 95%          | <b>0.0397</b> | <b>0.0332</b> | 0.9867          | 0.0633        | <b>0.0254</b> | 0.6847    |

| Area of fragment |               |               |                 |               |                 |               |
|------------------|---------------|---------------|-----------------|---------------|-----------------|---------------|
| raw data         |               |               | log-transformed |               |                 |               |
|                  | Black/Mixed   | Red/Mixed     | Black/Red       | Black/Mixed   | Red/Mixed       | Black/Red     |
| Holm-Ponferronni | <b>0.0073</b> | <b>0.0004</b> | 0.5539          | <b>0.0105</b> | <b>1.30E-08</b> | <b>0.0054</b> |
| Tukey 95%        | <b>0.0101</b> | <b>0.0004</b> | 0.8237          | <b>0.0280</b> | <b>0.0000</b>   | <b>0.0075</b> |

**Supplementary Table 4.2. Results of parametric statistical analysis of length of fracture and area of fragments following tumbling experiments in *Harmonia axyridis*.** Statistically significant results are indicated in bold font.

**A4.4. R Script:**

```
library(stats)

# Length

setwd("/Users/aidin_13_/Desktop/thesis(laptop)/tumblingfinal/CSV/")

alll <- read.csv("alll_try.csv")

attach(alll)

names(alll)

# Density is the first check

ggdensity(alll$Black, ylab = "Density of length", xlab = "Length")

ggdensity(alll$Red, ylab = "Density of length", xlab = "Length")

ggdensity(alll$Mixed, ylab = "Density of length", xlab = "Length")

hist(alll$Black)

hist(alll$Red)

hist(alll$Mixed)

# Quantile - Quantile plot correlation between a given sample and the normal
distribution.

ggqqplot(alll$Black)

ggqqplot(alll$Red)

ggqqplot(alll$Mixed)
```

```

# Shapiro Normality test if p-value >0.05 the distribution of the data are not
significantly different from normal distribution, we can assume normality.

shapiro.test(all$Black)

shapiro.test(all$Red)

shapiro.test(all$Mixed)


# Anderson-Darling Normality test

ad.test(all)


# Kruskal-Wallis test (alternative to ANOVA for non normal distributions)

Summarize(length~colour) #FSA

kruskal.test(length~colour) #stats


dunnTest(length~colour) #FSA - Zar (2010) Appropriate for groups with unequal
number of observations.

dT <- dunnTest(length~colour, method = "bh") #FSA

cldList(comparison = dT)


NT <- NemenyiTest(length~colour) #Tukey - Zar (2010) Not appropriate for groups
with unequal number of observations.

cldList(comparison = NT)


pairwise.wilcox.test(length,colour, p.adjust.method = "none")

PW <- pairwise.wilcox.test(length,colour, p.adjust.method = "none")

PW<- PT$p.value

```

```
PW
```

```
PW1 <- fullPTable(PT)
```

```
PW1
```

```
multcompLetters(PT1) # Values sharing the same letter are not significantly different.
```

```
# Brunner-Munzel test (for the stochastic equality of two samples)
```

```
brunnermunzel.test(length, colour, alternative = c("two.sided", "greater", "less"),  
  alpha = 0.05, perm = FALSE)
```

```
# plot
```

```
plot(alll)
```

```
plot(alll$Black)
```

```
plot(alll$Red)
```

```
plot(alll$Mixed)
```

```
# Boxplot (To investigate the distribution of the data)
```

```
boxplot(alll)
```

```
#New organisation
```

```
setwd("/Users/aidin_13_/Desktop/thesis(laptop)/tumblingfinal/CSV/")
```

```
alll <- read.csv("alll.csv")
```

```
attach(alll)
```

```
names(alll)
```

```
col <- all$colour
col <- as.vector(col)
levels(colour)

# ANOVA
aovl <- aov (length~colour, data = all)

#Adjust p-values
pairwise.t.test(length, colour, p.adj = "none")
pairwise.t.test(length, colour, p.adj = "bonf") # Bonferroni
pairwise.t.test(length, colour, p.adj = "holm") # Holm
TukeyHSD(aovl) # Tukey comparisons of means 95% family-wise confidence level

# Normalise - Log transform
all.l <- log(all[,2])
all$B <- all.l

# ANOVA logged
aovB <- aov (B~colour, data = all)

B <- all$B
B <- as.vector(B)

#Adjust p-values
pairwise.t.test(B, colour, p.adj = "none")
```

```
pairwise.t.test(B, colour, p.adj = "bonf") # Bonferroni
pairwise.t.test(B, colour, p.adj = "holm") # Holm
TukeyHSD(aovB) # Tukey comparisons of means 95% family-wise confidence level

# Area
setwd("/Users/aidin_13_/Desktop/thesis(laptop)/tumblingfinal/CSV/")
alla <- read.csv("alla_try.csv")
attach(alla)
names(alla)

# Density is the first check
ggdensity(alla$Black, ylab = "Density of length", xlab = "Length")
ggdensity(alla$Red, ylab = "Density of length", xlab = "Length")
ggdensity(alla$Mixed, ylab = "Density of length", xlab = "Length")

hist(alla$Black)
hist(alla$Red)
hist(alla$Mixed)

# Quantile - Quantile plot correlation between a given sample and the normal
distribution.
ggqqplot(alla$Black)
ggqqplot(alla$Red)
ggqqplot(alla$Mixed)
```

# Shapiro Normality test if p-value >0.05 the distribution of the data are not significantly different from normal distribution, we can assume normality.

```
shapiro.test(alla$Black)
```

```
shapiro.test(alla$Red)
```

```
shapiro.test(alla$Mixed)
```

# Anderson-Darling Normality test

```
ad.test(alla)
```

# Kruskal-Wallis test (alternative to ANOVA for non normal distributions)

```
Summarize(area~colour) #FSA
```

```
kruskal.test(area~colour) # FSA
```

dunnTest(area~colour) #FSA - Zar (2010) Appropriate for groups with unequal number of observations.

```
dT <- dunnTest(area~colour, method = "bh") #FSA
```

```
dT
```

```
cldList(comparison = dT) # rcompanion
```

NT <- NemenyiTest(area~colour) #Tukey - Zar (2010) Not appropriate for groups with unequal number of observations. DescTools

```
NT
```

```
cldList(comparison = NT)
```

```
pairwise.wilcox.test(area,colour, p.adjust.method = "none")
```

```
PW <- pairwise.wilcox.test(area,colour, p.adjust.method = "none")

PW<- PW$p.value

PW

PW1 <- fullPTable(PW)

PW1

multcompLetters(PW1) # Values sharing the same letter are not significantly
different. multcompView

# Brunner-Munzel test (for the stochastic equality of two samples)

brunnersmunzel.test(area, colour, alternative = c("two.sided", "greater", "less"), alpha
= 0.05, perm = FALSE)

# plot

plot(alla)

plot(alla$Black)

plot(alla$Red)

plot(alla$Mixed)

# Boxplot (To investigate the distribution of the data)

boxplot(alla)
```



```
#New organisation

setwd("/Users/aidin_13_/Desktop/thesis(laptop)/tumblingfinal/CSV/")

alla <- read.csv("alla.csv")

attach(alla)

names(alla)

col <- alla$colour

col <- as.vector(col)

levels(colour)

# ANOVA

aova <- aov (area~colour, data = alla)

#Adjust p-values

pairwise.t.test(area, colour, p.adj = "none")

pairwise.t.test(area, colour, p.adj = "bonf") # Bonferroni

pairwise.t.test(area, colour, p.adj = "holm") # Holm

TukeyHSD(aova) # Tukey comparisons of means 95% family-wise confidence level

# Normalise - Log transform

alla.l <- log(alla[,1])

alla$B <- alla.l

# ANOVA logged

aovB <- aov (B~colour, data = alla)
```

```
B <- alla$B
```

```
B <- as.vector(B)
```

```
#Adjust p-values
```

```
pairwise.t.test(B, colour, p.adj = "none")
```

```
pairwise.t.test(B, colour, p.adj = "bonf") # Bonferroni
```

```
pairwise.t.test(B, colour, p.adj = "holm") # Holm
```

```
TukeyHSD(aovB) # Tukey comparisons of means 95% family-wise confidence level
```

## APPENDIX 5

# Supplementary Information on Chapter 5: Synchrotron-X-ray fluorescence as a tool for interpreting the original colouration of insects

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### A.5.1 Supplementary Text:

#### A.5.1.1 Linear Discriminant Analysis of elemental chemistry associated with pigment molecules in extant insect cuticle.

For instance, in ladybirds (Coccinellidae: Coleoptera), melanin-rich regions are relatively enriched in Mn, Fe and especially Ca and carotenoid-rich regions, in Cl, Ni and P (Fig. 5.3c, d, Table 5.4, Supplementary Fig. 5.5k, v and Supplementary Table 5.2). In Panorpididae (Mecoptera), melanin-rich regions are enriched in Mn and K and pterin-rich regions, in Ca and S (Fig. 5.3b, d, Table 5.4, Supplementary Fig. 5.5j, v and Supplementary Table 5.2). In Geometridae and Pieridae (Lepidoptera), melanin-rich regions are enriched in Cl, P and S and melanin-poor regions, in K, Mn and Ca (Fig. 5.3a, b, d, Table 5.4, Supplementary Fig. 5.5i, m, v and Supplementary Table 5.2). Melanin-rich regions in Zygaenidae are enriched in S and P while in Sphingidae in Ca and S. Carotenoid-rich regions are enriched in Ca, S and P in Zygaenidae and Cl and P in Sphingidae (Lepidoptera); flavonoid-rich regions are enriched in K, Cl and Ni in Zygaenidae and K in Sphingidae (Fig. 5.3a-d, Table 5.4, Supplementary Fig. 5.5i, m, v and Supplementary Table 5.2). In Hemiptera, melanin-rich regions are enriched in Ca and K in Pentatomidae (Fig. 5.3a,d, Table 5.4, Supplementary Fig. 5.5l,v and Supplementary Table 5.2) and Ca and Mn in Cicadellidae (Fig. 5.3b,d, Table 5.4, Supplementary Fig. 5.5j,v and Supplementary Table 5.2); melanin-poor regions, in Mn, S and Cl in Pentatomidae (Fig. 5.3a,d, Table 5.4, Supplementary Fig. 5.5l,v and Supplementary Table 5.2) and S, Cl and K in Cicadellidae (Fig. 5.3b,d, Table 5.4, Supplementary Fig. 5.5j,v and Supplementary Table 5.2). In Cercopidae (Hemiptera), dark (presumably melanin-rich) regions are enriched in Cl, P and K and pterin-rich regions (red) in Ca and Fe (Table 5.4, Supplementary Fig. 5.5g, v and Supplementary Table 5.6). In

Chrysomelidae (Coleoptera), melanin-rich regions (black) are enriched in K, Mn and Fe and carotenoid-rich regions (yellow) in Cl, Ca and S (Table 5.4, Supplementary Fig. 5.5g, v and Supplementary Table 5.6). In Asilidae (Diptera), melanin-rich regions (black) are enriched in Ca and S and non-melanin-rich regions (colourless) in K, Cl and Ca (Table 5.4, Supplementary Fig. 5.5g, v and Supplementary Table 5.6). In Hesperidae (Lepidoptera), flavonoid-rich regions (brown) are enriched in Ca and S and non-flavonoid-rich regions (orange) in Cl and P (Table 5.4, Supplementary Fig. 5.5g, v and Supplementary Table 5.6). In Calopterygidae (Odonata), melanin-rich regions (black) are enriched in Ca, Mn and Fe and regions lacking of pigments (colourless) in Cl and P (Table 5.4, Supplementary Fig. 5.5h, v and Supplementary Table 5.6). In Papilionidae (Lepidoptera), melanin-rich regions (black and orange) are enriched in S, Mn and P, ommochrome-rich regions (yellow and blue/green) are enriched in K and Mn and carotenoid-rich regions (orange) in Ca and Cl (Table 5.4, Supplementary Fig. 5.5h, v and Supplementary Table 5.6). In Scarabaeidae (Coleoptera), melanin-rich regions (black and brown) are enriched in Cl and P (Table 5.4, Supplementary Figs. 5.3a, d, 5.5h, v, and Supplementary Table 5.6). In Cerambycidae (Coleoptera), melanin-rich regions (black) are enriched in Cl and P, carotenoid-rich regions (orange) are enriched in Fe and S (Table 5.4, Supplementary Figs. 5.3a, d, 5.5i, v and Supplementary Table 5.6). In Corydiidae (Blattodea), dark (presumably melanin-rich) regions are enriched in Ca and S while regions lacking dark colouration in K, Cl and Ni (Table 5.4, Supplementary Fig. 5.5j, v and Supplementary Table 5.6). In Nymphalidae (Lepidoptera), melanin-rich regions (black and brown) are enriched in Ca and Fe, carotenoid-rich (yellow and orange) regions in Ca and S, and ommochrome-rich regions (red) in S (Table 5.4,

Supplementary Figs. 5.3a, d, 5.5k, v and Supplementary Table 5.6). In Libellulidae (Odonata), melanin-rich regions (black) are enriched in Ca and Cu while regions lacking pigment (colourless) are enriched in Cl (Table 5.4, Supplementary Figs. 5.3a, d, 5.5l, v and Supplementary Table 5.6). In Lycaenidae (Lepidoptera), flavonoid-rich regions (brown) are enriched in Mn and carotenoid-rich regions in Ca and P (Table 5.4, Supplementary Fig. 5.5l, v and Supplementary Table 5.6). In Silphidae (Coleoptera), regions containing adipokinetic/red pigment-concentrating hormone (AKH; brown) are enriched in Cl and S (Table 5.4, Supplementary Fig. 5.5m, v and Supplementary Table 5.6). In Vespidae (Hymenoptera), presumably melanin-rich regions (black) are enriched in S, Fe and Cu while regions enriched in pterins (colourless) are enriched in Ca and Ti (Table 5.4, Supplementary Fig. 5.3a, d and Supplementary Table 5.6). In Andrenidae (Hymenoptera), melanin-rich regions (black) are enriched in K and Zn while regions lacking pigment (colourless) are enriched in Cu and S (Table 5.4, Supplementary Fig. 5.3a, d and Supplementary Table 5.6). In Carabidae (Coleoptera), melanin-rich regions (black) are enriched in P and Cl while regions lacking melanin are enriched in S and Fe (Table 5.4, Supplementary Fig. 5.3a, d and Supplementary Table 5.6). In Cordulidae (Odonata), melanin-rich regions (black) are enriched in S and Fe while regions lacking pigment (colourless) are enriched in P, Cl and Ca (Table 5.4, Supplementary Fig. 5.3a, d and Supplementary Table 5.6). In Aeshnidae (Odonata), melanin-rich regions (black) are enriched in Ca and Cu while regions lacking pigment (colourless) are enriched in Fe and Ti (Table 5.4, Supplementary Fig. 5.3a, d and Supplementary Table 5.6). In Coenagrionidae (Odonata), melanin-rich regions (black) are enriched in Cl, Ca and Ni while regions lacking pigment

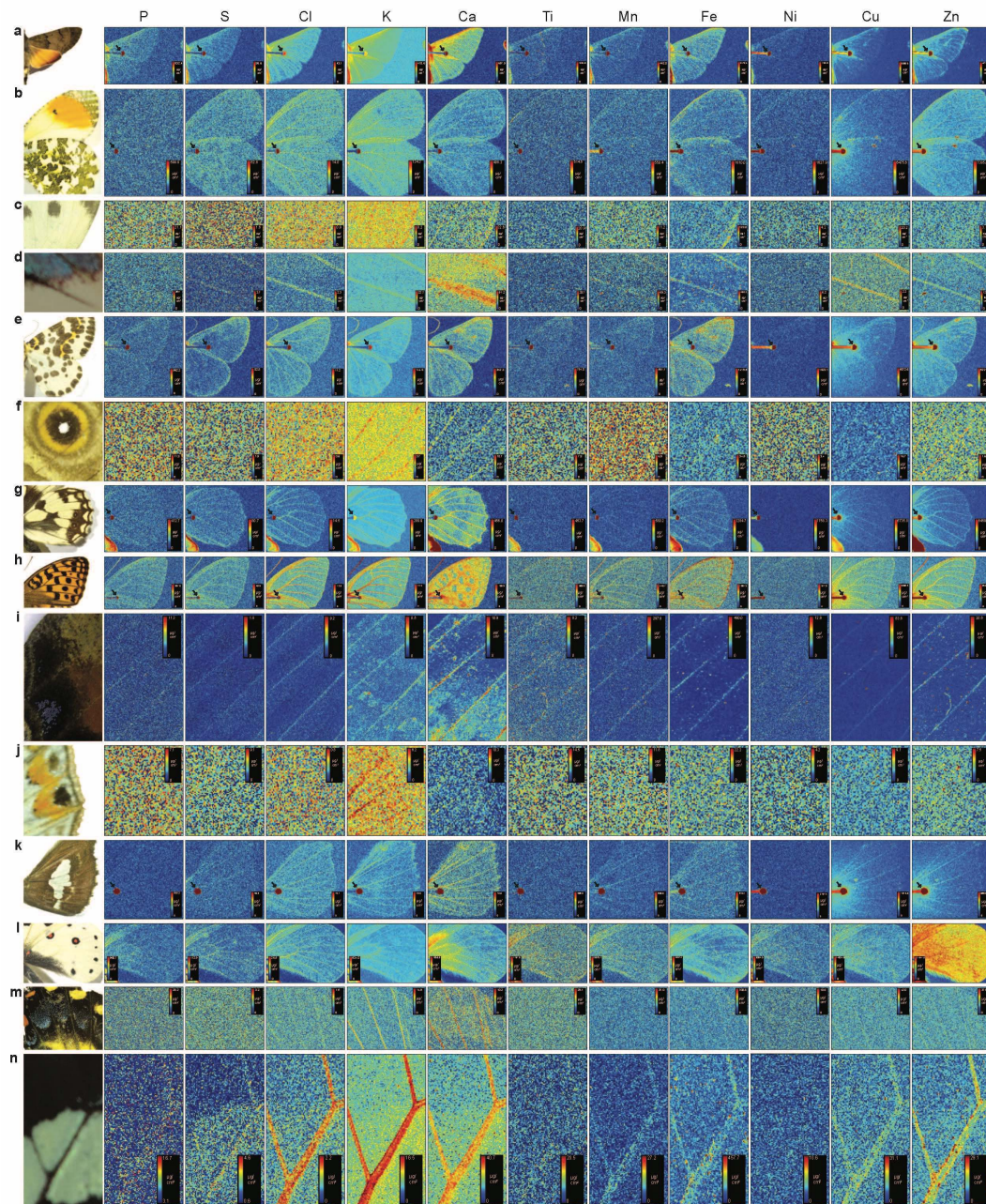
(colourless) are enriched in Fe and S (Table 5.4, Supplementary Fig. 5.3a, d v and Supplementary Table 5.6).

#### **A5.1.2 Elemental chemistry associated to pigment molecules in fossil insect**

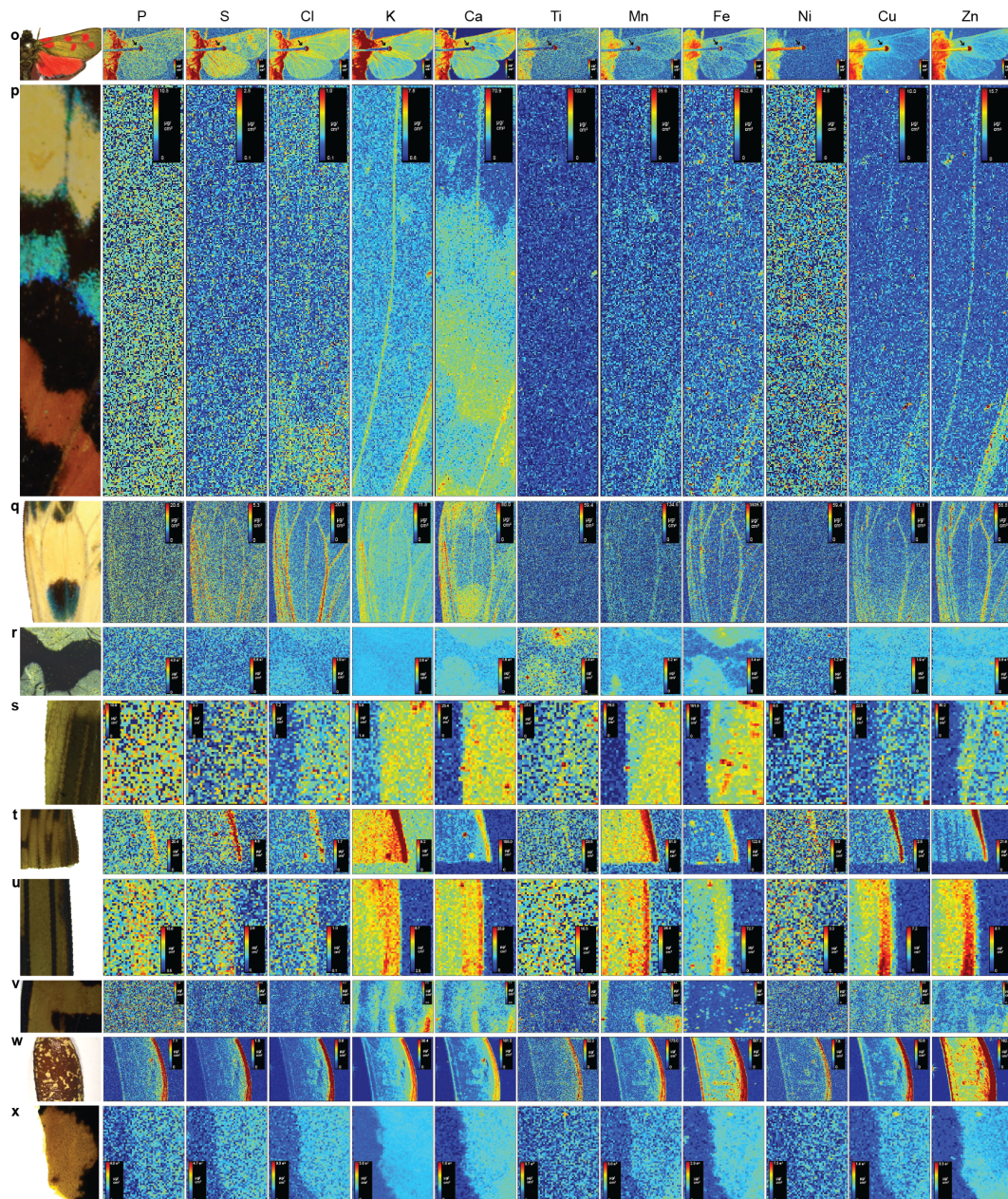
**cuticle.** Dark cuticle regions in NIGPAS NND11021-1, NIGPAS NND11021-2, NIGPAS NND11010, NIGPAS NND11023 and NIGPAS NND140539 (all Neuroptera) are enriched in Cu, Ca and Ti, whilst light regions of the cuticle in Zn and K (Fig. 5.4b, d, g, h Tables 5.3, 5.5, Supplementary Fig. 5.2j–m and Supplementary Table 5.4). In UCM 51311a (Coleoptera) dark cuticle regions are enriched in Cu and Zn, whereas light regions in the cuticle in Fe (Fig. 5.4b, d, g, h, Tables 5.3, 5.5, Supplementary Fig. 5.2h and Supplementary Table 5.4). In OCB6 dark regions of the cuticle are enriched with Ca and Ti, whereas light regions of the cuticle in Fe and K (Fig. 5.4b, d, g, h, Tables 5.3, 5.5, Supplementary Fig. 5.2h and Supplementary Table 5.4). In In YPM IP522483 (Coleoptera: Fulgoridae) dark cuticle regions are enriched in Cu, Ti and Zn, whilst light regions in Ca (Fig. 5.4b, d, g, h, Tables 5.3, 5.5, Supplementary Fig. 5.2c and Supplementary Table 5.4). The two fossils Corixidae analyzed (Hemiptera; RR1-12-07-10 and RR1-12-07-44) are enriched in Cu and Zn in dark cuticle regions and Zn and K in light regions (Fig. 5.4b, d, g, h, Tables 5.3, 5.5, Supplementary Fig. 5.2a, b and Supplementary Table 5.4). Dark regions of the cuticle of other fossil Hemiptera, such as MCZ14431 and MCZ14426, are enriched in Ti and Cu, whereas light cuticle regions in Zn and K (Fig. 5.4b, d, g, h, Tables 5.3, 5.5, Supplementary Fig. 5.2d, e and Supplementary Table 5.4). Regardless of the tight clustering of data points in fossil Hymenoptera (UAF\_GS7), there is a clear distinction between dark and light regions of the cuticle: dark regions of the cuticle are enriched in Ca, Ti and Zn, whilst light

regions in Cu and S (Fig. 5.4b, d, g, h, Tables 5.3, 5.5, Supplementary Fig. 5.2n and Supplementary Table 5.4). The fossil samples YPM IP454454 and YPM IP390330 (Coleoptera) do not display patterning in their cuticles, their black cuticles are enriched in Ti with respect with the surrounding sediment (Fig. 5.4b, d, g, h, Tables 5.3, 5.5, Supplementary Fig. 5.2g, h and Supplementary Table 5.4)

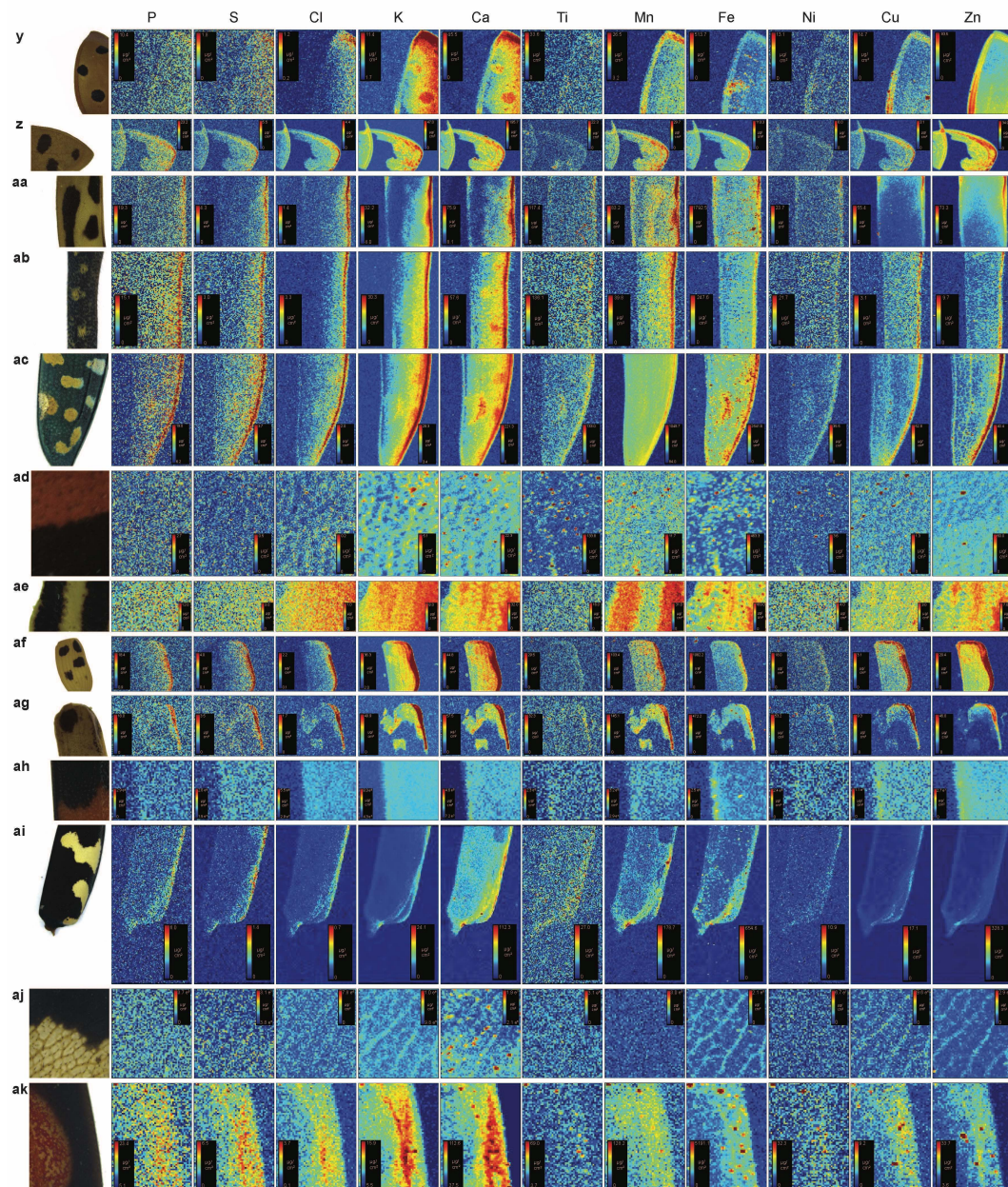
## **A5.2 Supplementary Figures:**



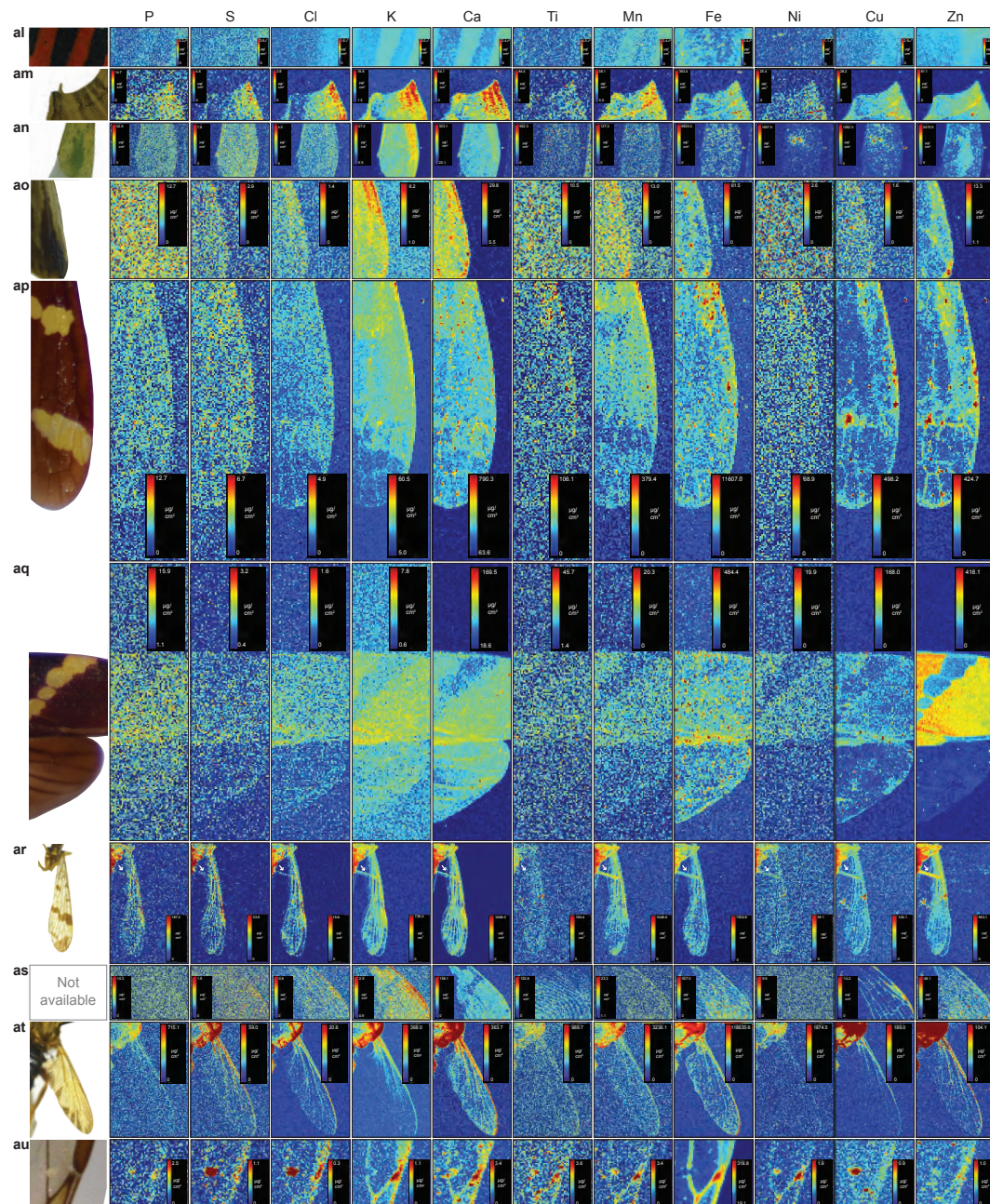




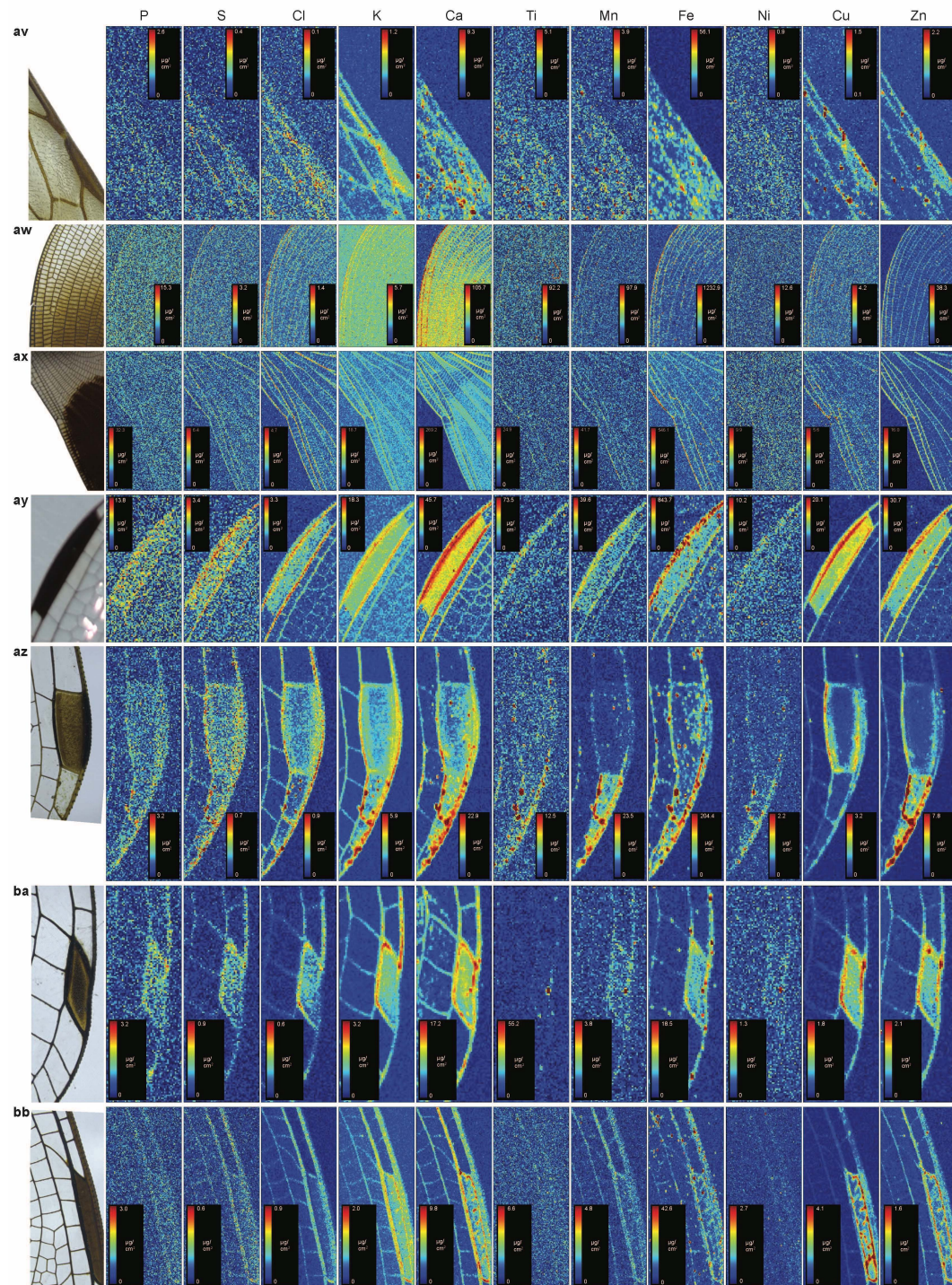


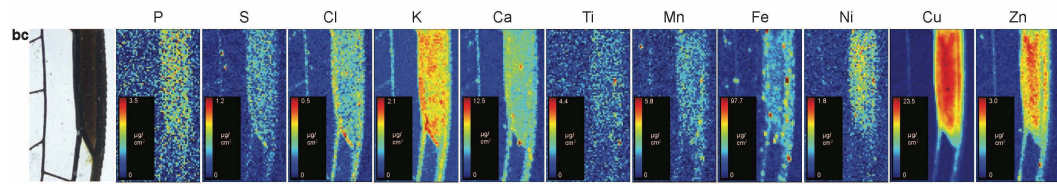








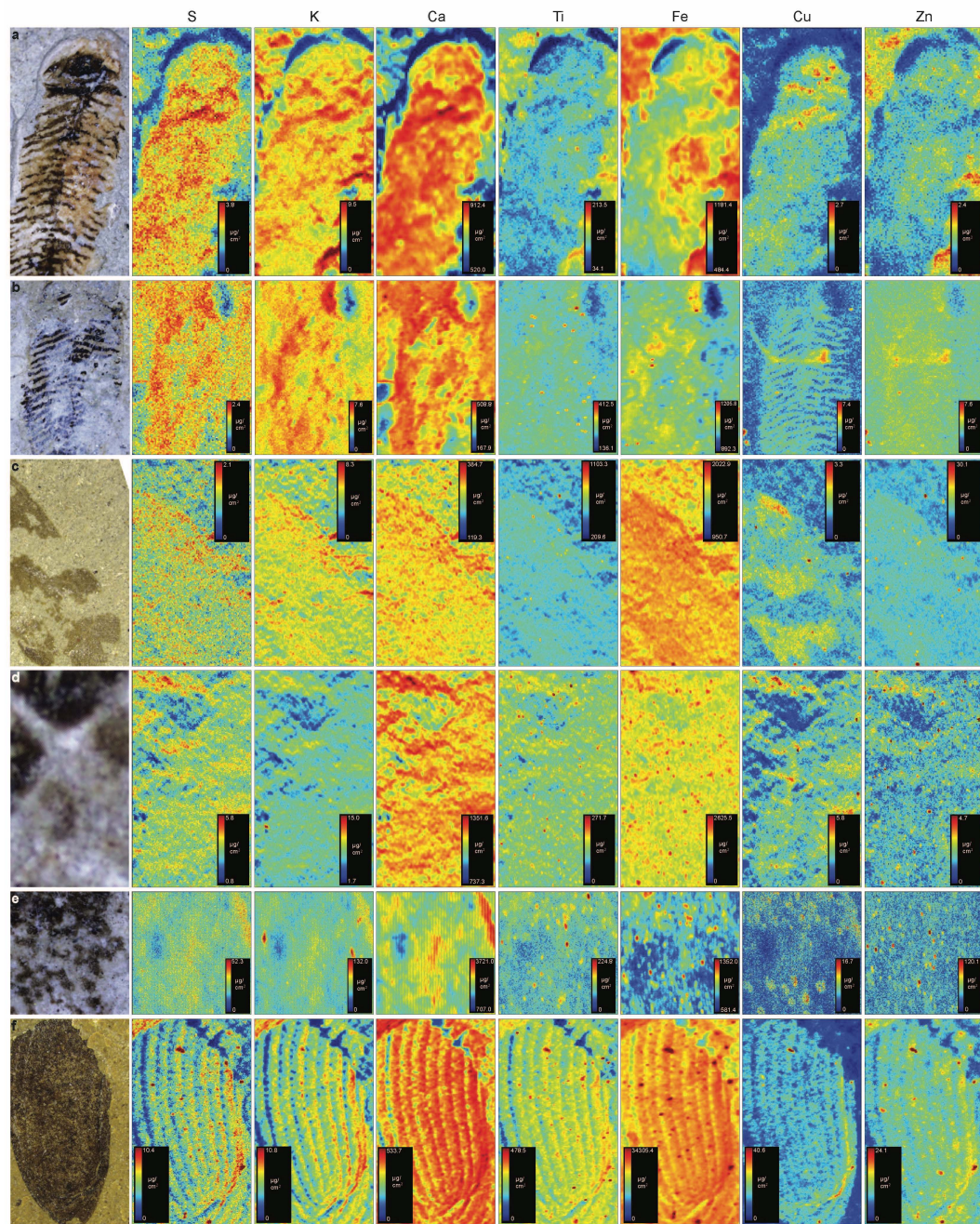




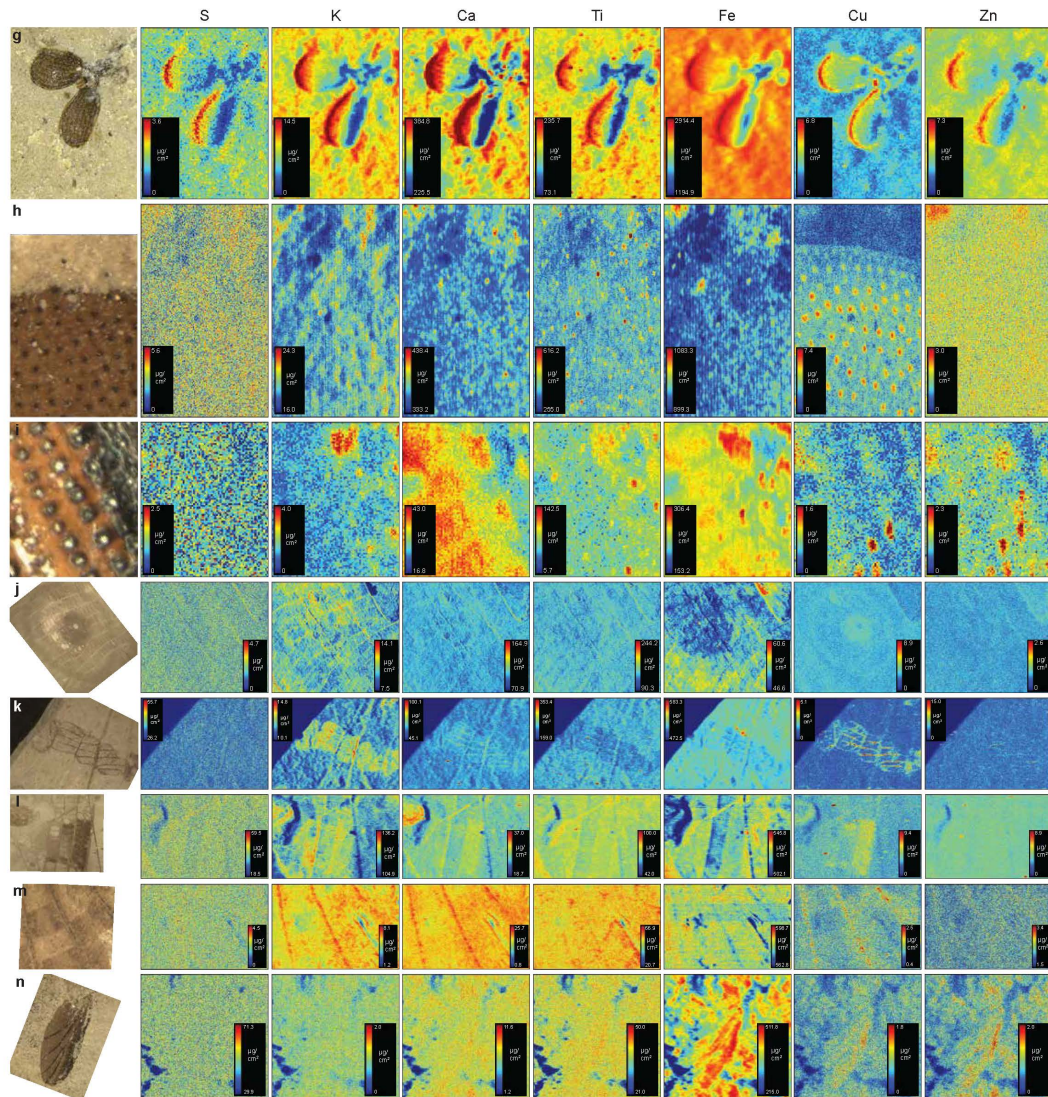
**Supplementary Figure 5.1. Caption overleaf.**

**Supplementary Figure 5.1 (previous page). X-ray fluorescence maps of extant specimens.** **a** *Macroglossum stellatarum*. **b** *Anthocharis cardamines*. **c** *Pieris rapae*. **d** *Colotis regina*. **e** *Abraxas grossulariata*. **f** *Bicyclus anynana*. **g** *Melanargia galathea*. **h** *Argynnis adippe*. **i** *Aglaia urticae*. **j** *Cupido comyntas*. **k** *Epargyreus clarus*. **l** *Parnassius apollonius*. **m** *Papilio glaucus*. **n** *Papilio nireus*. **o** *Zygaena filipendulae*. **p** *Erasmia pulchella*. **q** *Chalcidia coliadoides*. **r** *Goliathus goliathus*. **s** *Aphodius* sp. **t** *Aphodius sticticus*. **u** *Aphodius luridus*. **v** *Trichius* sp. **w** *Polyphylla fullo*. **x** *Harmonia axyridis*. **y** *Epilachna argus*. **z** *Hippodamia variegata*. **aa** *Brachyta interrogationis*. **ab** *Saperda populnea*. **ac** *Sternotomis bohemani*. **ad** Cerambycidae indet. **ae** *Leptinotarsa decemlineata*. **af** *Gonioctena decemnotata*. **ag** *Phyllobrotica quadrimaculata*. **ah** *Nicrophorus investigator*. **ai** *Cicindela hybrida*. **aj** *Therea petiveriana*. **ak** *Cercopis vulnerata*. **al** *Graphosoma lineatum*. **am** *Aelia acuminata*. **an** *Nezara robusta*. **ao** *Evacanthus interruptus*. **ap** *Deselvana fulvofasciata*. **aq** *Cicciana obliqua*. **ar** *Panorpa* sp. **as** *Panorpa lugubris*. **at** *Asilus crabroniformis*. **au** *Odynerus gracilis*. **av** *Andrena carantonica*. **aw** *Calopteryx virgo*. **ax** *Calopteryx splendens*. **ay** *Libellula quadrimaculata*. **az** *Sympetrum flaveolum*. **ba** *Pyrrhosoma nymphula*. **bb** *Aeshna caerulea*. **bc** *Cordulia aenea*. Arrows in **b**, **e**, **g**, **h**, **k**, **o** and **ar** indicate the specimen-mounting pin. Progressively higher concentrations towards the right of the maps in **t**, **u**, **w**, **y**, **z**, **aa**, **ab**, **ac**, **af**, **ag**, **ai**, **al** and **am** are an artefact and reflect curvature of the cuticle. Regions of high concentrations of Ca and Fe in **e** may represent dust particles on the wing. High concentrations of Cl, K, Ca, Ti, Fe and Cu arranged in concentric rings in **as** correspond to a fingerprint placed while mounting the specimen for analysis. High concentrations of P, S, Cl, Ti, Mn, Ni, Cu and Zn in **au** correspond to a hole in the specimen's wing. High concentration regions of Cl, K, Ca, Ti, Mn, Fe, Cu and Zn in **av**, **az**, **ba**, **bb** and **bc** correspond to dust particles on the wings. Lower concentration region of Ca and Mn in **ai** corresponds to a segment of sellotape under the specimen. Boundaries of specimens in maps and light microscopy images in **z**, **ag** and **am** do not coincide because specimens were fractured during analysis. In **w**, **y**, **ac** and **af** boundary of fossil in map does not coincide with boundary of fossil in light microscopy image due to slight variation in the orientation of the specimen during imaging. In **as** light microscopy image is not available. Scales: blue to red represents low to high concentration.



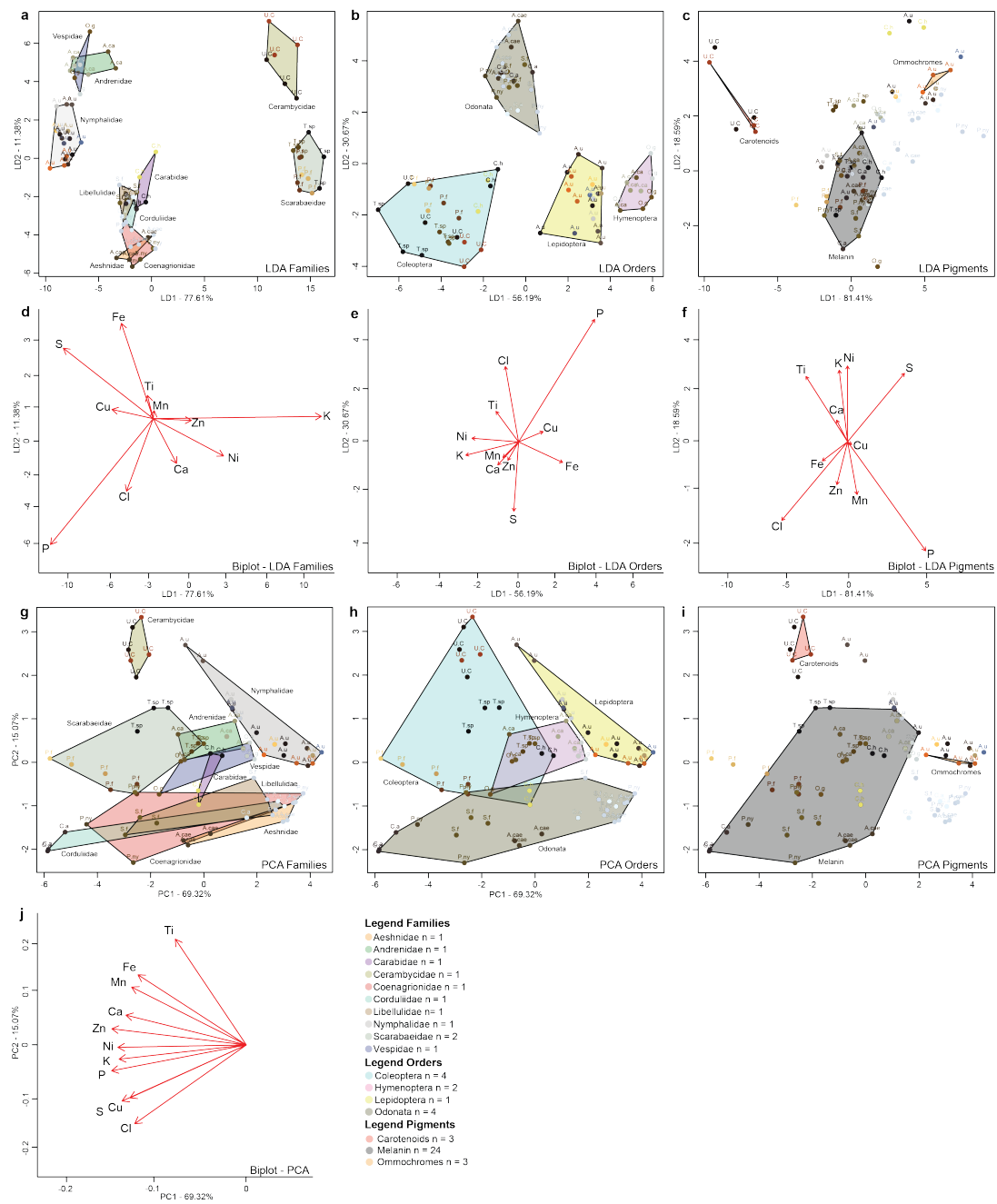






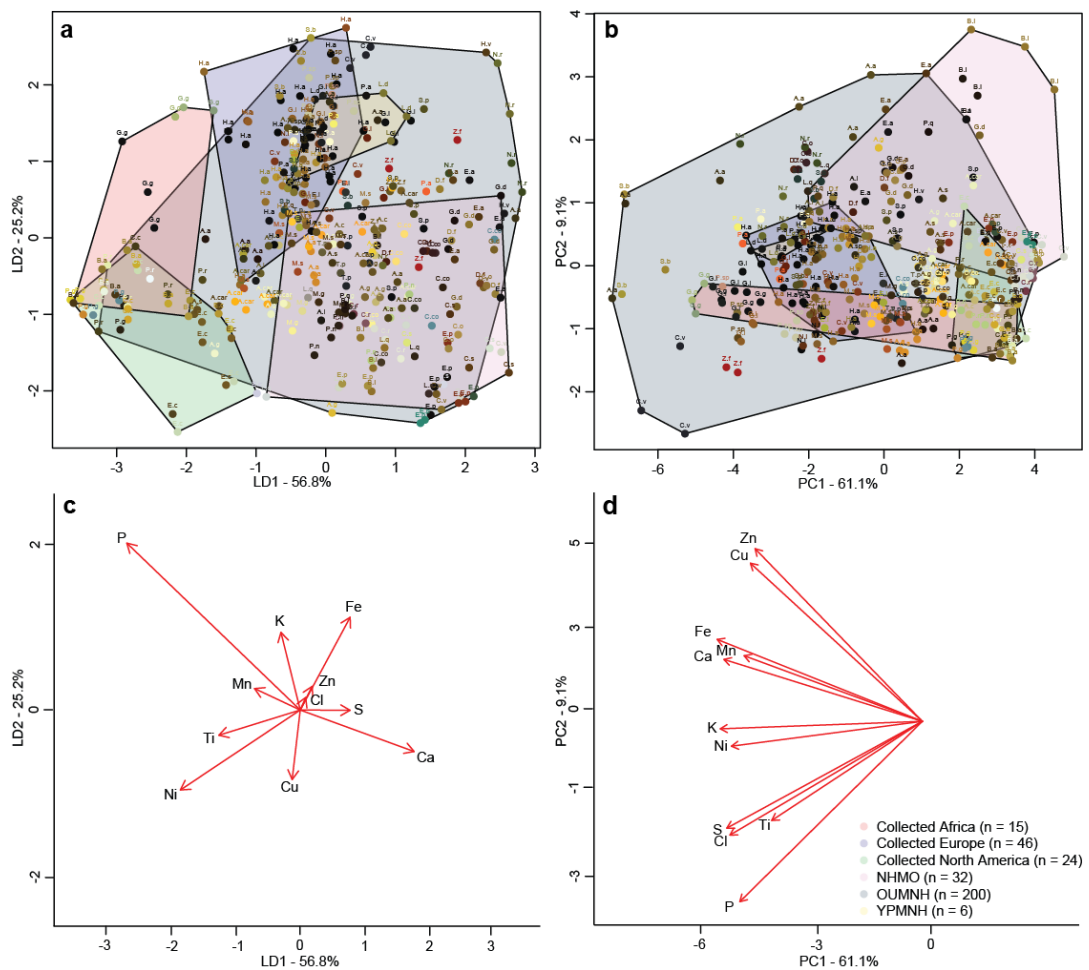
**Supplementary Figure 5.2. X-ray fluorescence maps of fossil specimens. a** RR1-12-07-10. **b** RR1-12-07-44. **c** YPMIP522483. **d** MCZ14431. **e** MCZ14426. **f** YPMIP454454. **g** YPMIP39033. **h** UCM 51311a. **i** OCB6. **j** NGPAS NND11021. **k** NGPAS NND11010. **l** NGPAS NND11023. **m** NGPAS NND140539. **n** UAF GS7. High concentration region of Cu and Zn in **b** possibly corresponds to an underlying limb. Progressively higher concentrations towards the right of the cuticle in **f** and towards the left in **g** and **i** are an artefact and reflect curvature of the cuticle. In **h** boundary of fossil in map does not coincide with boundary of fossil in light microscopy image due to sediment partially covering the specimen. Scales: blue to red represents low to high concentration.



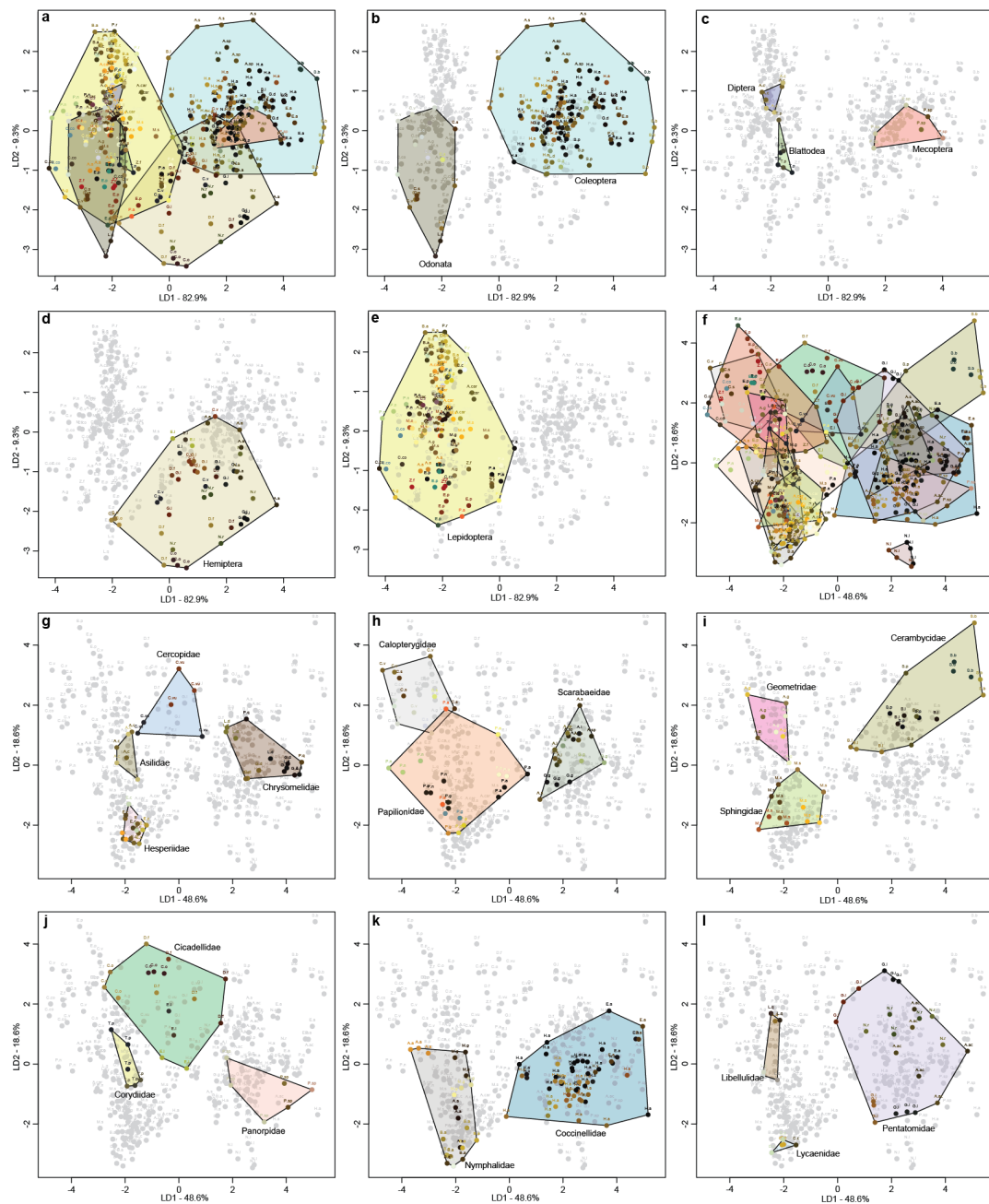


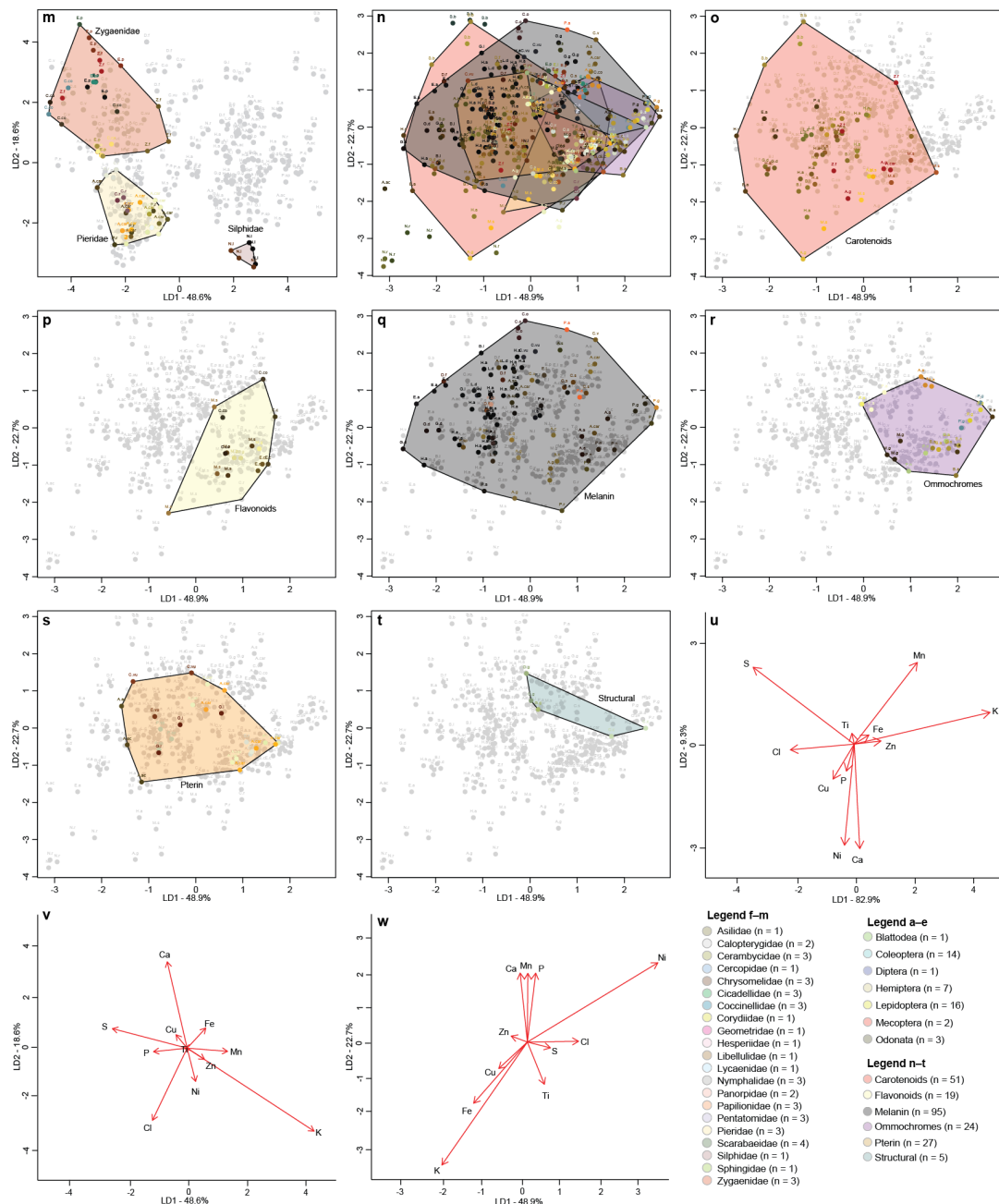
Supplementary Figure 5.3. Caption overleaf.

**Supplementary Figure 5.3 (previous page). Linear Discriminant Analysis and Principal Component Analysis of log-transformed concentrations of elements in the cuticle of extant insects from additional extant insect cuticle. a–c** LDA plots with data grouped by family (**a**), order (**b**) and coloration mechanism (**c**). **d–f** Biplot vectors showing the contribution of each element for variation in the dataset where data are grouped by family (**d**), order (**e**) and coloration mechanism (**f**). **g–i** PCA plots with data grouped by family (**g**), order (**h**) and coloration mechanism (**i**). **j** Biplot vectors showing the contribution of each element to variation in the dataset. Data point colours are mean RGB values for that region of interest in the cuticle. A.u, *Aglais urticae*; T.sp, *Trichius* sp.; P.f, *Polyphylla fullo*; U.C, *Cerambycidae* indet.; C.h, *Cicindela hybrida*; O.g, *Odynerus gracilis*; A.ca, *Andrena carantonica*; S.f, *Sympetrum flaveolum*; P.ny, *Pyrrhosoma nymphula*; A.cae, *Aeshna caerulea*; C.a, *Cordulia aenea*.



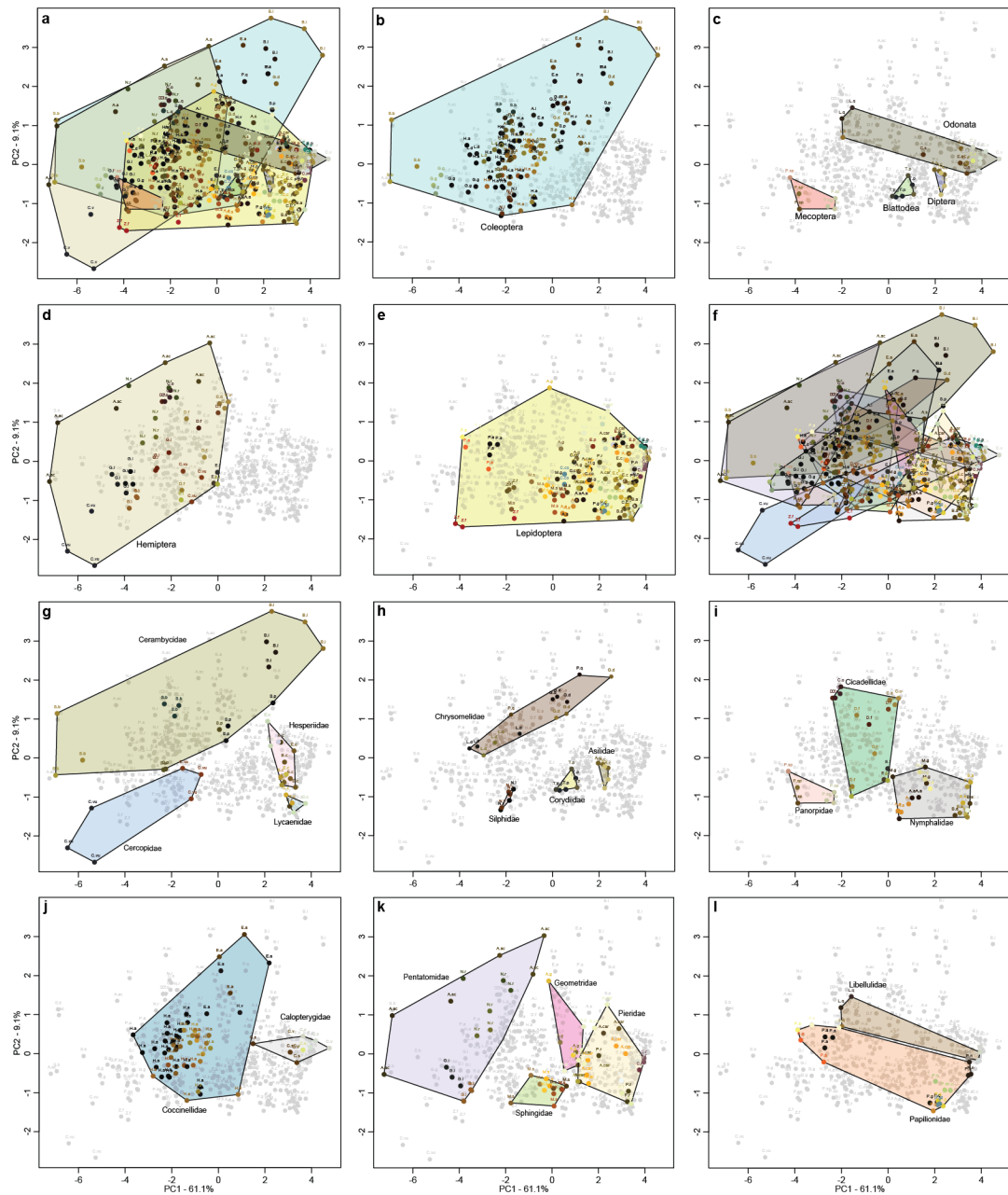
**Supplementary Figure 5.4. Linear Discriminant Analysis and Principal Component Analysis of log-transformed concentrations of elements from the cuticle of extant insects with data grouped by source of sample. a** LDA plot with data grouped by source of sample. **b** PCA plot with data grouped by source of sample. **c–d** Biplot vectors showing the contribution of each element to variation in the dataset where LDA (**c**) and PCA (**d**) data are grouped by source of sample. Abbreviations as per caption to Fig. 5.2.

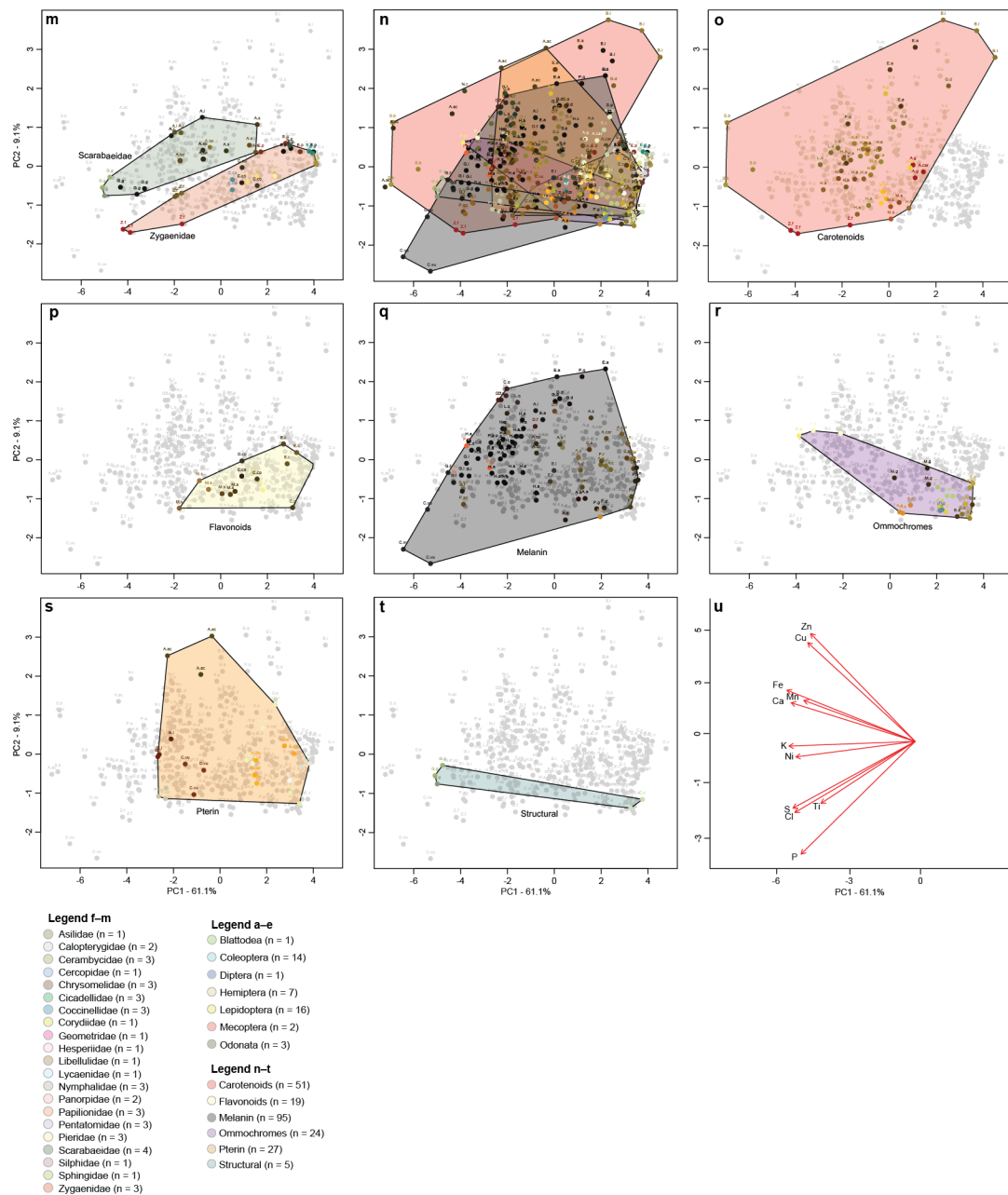




Supplementary Figure 5.5. Caption overleaf.

**Supplementary Figure 5.5 (previous page). Linear Discriminant Analysis of log-transformed concentrations of elements in the cuticle of extant insects. a–t** LDA plots with data grouped by order (**a–e**), family (**f–m**) and coloration mechanism (**n–t**). **a** All orders analyzed (n = 7). **b** Coleoptera and Odonata. **c** Mecoptera, Diptera and Blattodea. **d** Hemiptera. **e** Lepidoptera. **f** All families analyzed (n = 21). **g** Asilidae, Cercopidae, Chrysomelidae and Hesperidae. **h** Calopterygidae, Papilionidae and Scarabaeidae. **i** Cerambycidae, Geometridae and Sphingidae. **j** Cicadellidae, Corydiidae and Panorpidae. **k** Coccinellidae and Nymphalidae. **l** Libellulidae, Lycaenidae and Pentatomidae. **m** Pieridae, Silphidae and Zygaenidae. **n** All coloration mechanisms analyzed (n = 6). **o** Carotenoids. **p** Flavonoids. **q** Melanins. **r** Ommochromes. **s** Pterins. **t** Structural colours. **u–w** Biplot vectors showing the contribution of each element to variation in the dataset where data are grouped by order (**u**), family (**v**) and coloration mechanism (**w**). Data point colours are mean RGB values for that region of interest in the cuticle. Abbreviations as per caption to Fig. 5.2.

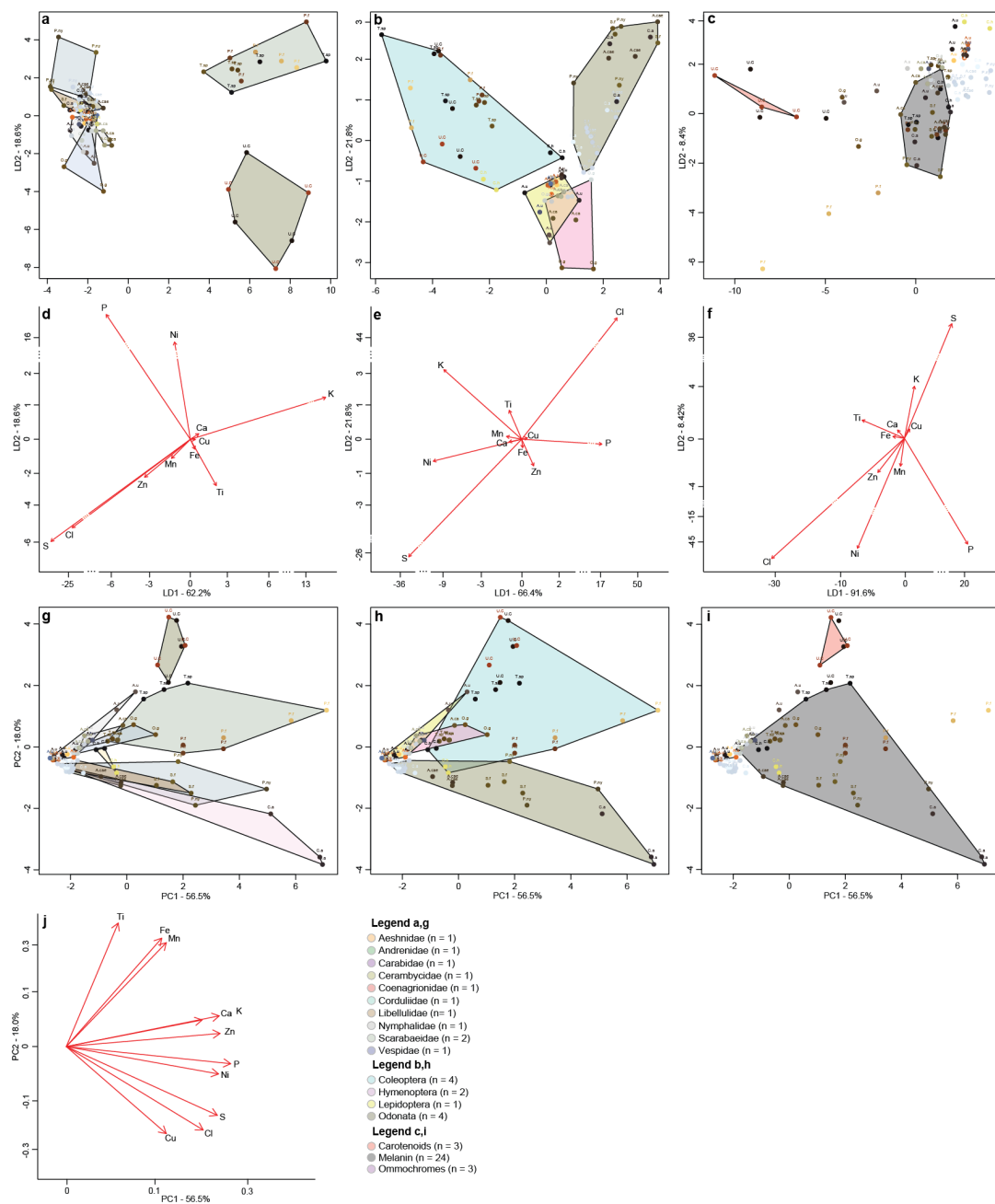




Supplementary Figure 5.6. Caption overleaf.

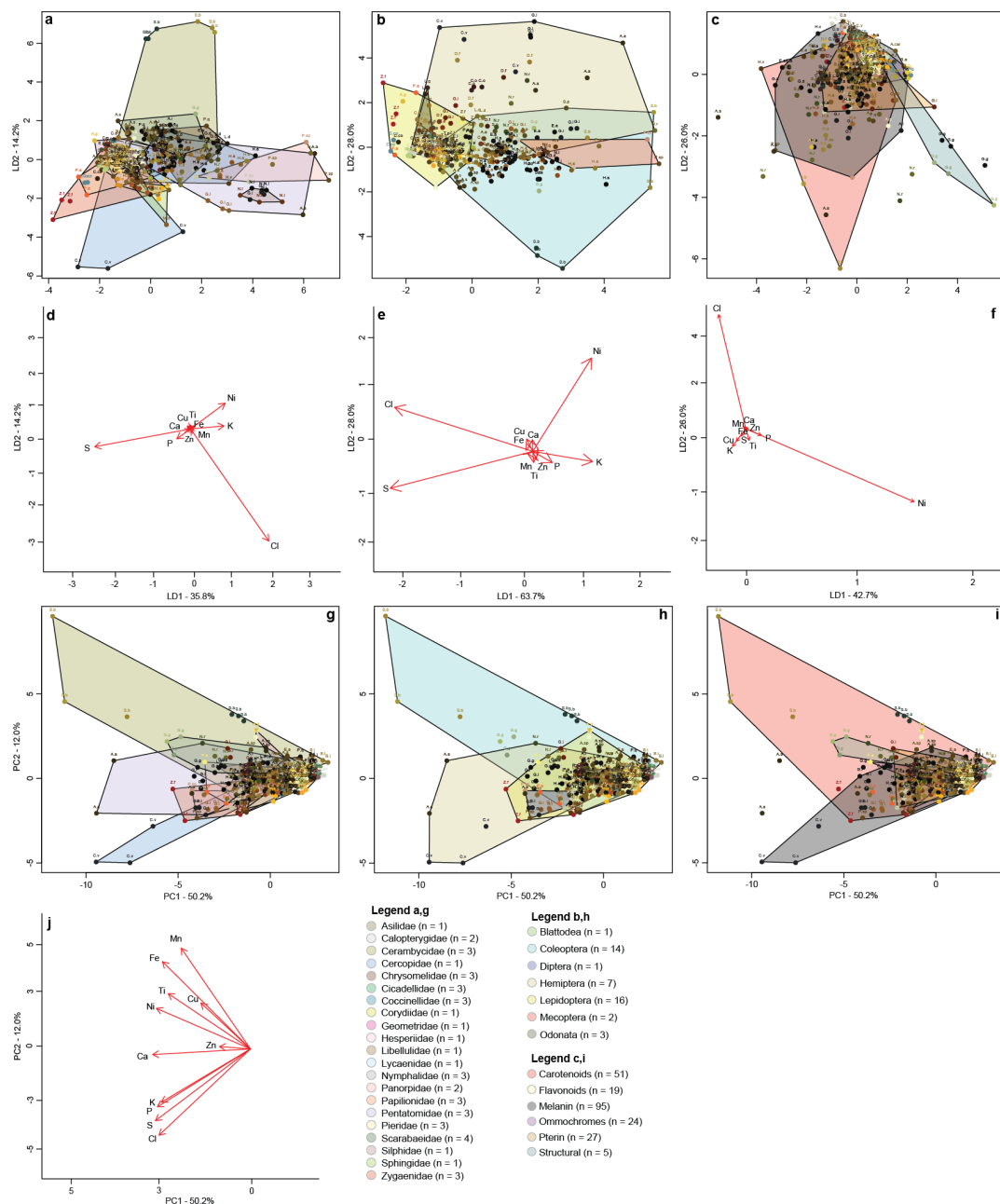


**Supplementary Figure 5.6 (previous page). Principal Component Analysis of log-transformed concentrations of elements in the cuticle of extant insects. a–t** PCA plots with data grouped by order (**a–e**), family (**f–m**) and coloration mechanisms (**n–t**). **a** All orders analyzed (n = 7). **b** Coleoptera. **c** Mecoptera, Diptera, Blattodea and Odonata. **d** Hemiptera. **e** Lepidoptera. **f** All families analyzed (n = 21). **g** Cercopidae, Cerambycidae, Hesperidae and Lycaenidae. **h** Asilidae, Chrysomelidae, Corydiidae and Silphidae. **i** Cicadellidae, Nymphalidae and Panorpidae. **j** Calopterygidae and Coccinellidae. **k** Geometridae, Pentatomidae, Pieridae and Sphingidae. **l** Libellulidae and Papilionidae. **m** Scarabaeidae and Zygaenidae. **n** All coloration mechanisms analyzed (n = 6). **o** Carotenoids. **p** Flavonoids. **q** Melanins. **r** Ommochromes. **s** Pterins. **t** Structural colours. **u** Biplot vectors showing the contribution of each element to the variation in the dataset. Data point colours are mean RGB values for that region of interest in the cuticle. Abbreviations as per caption to Fig. 5.2.



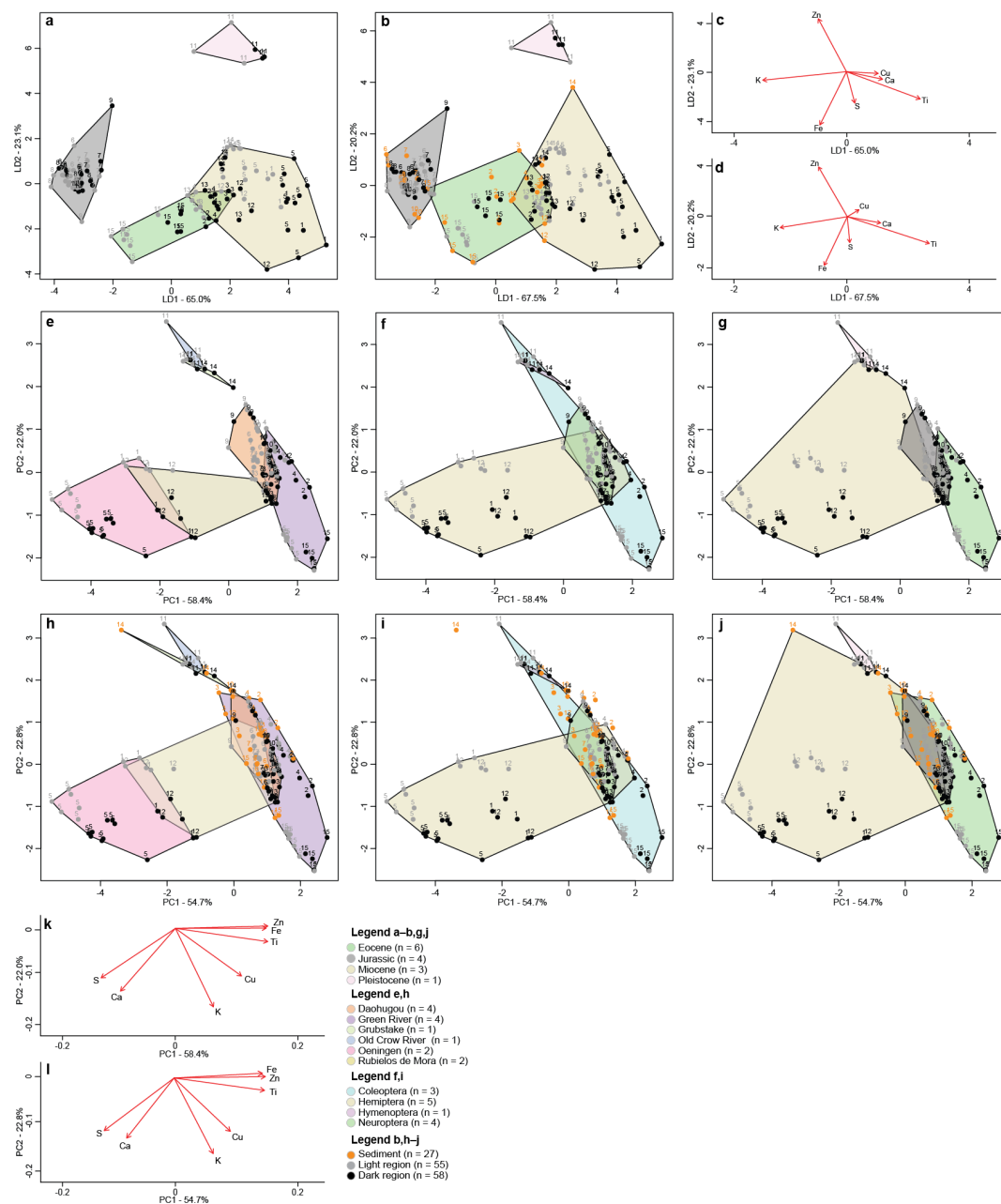
Supplementary Figure 5.7. Caption overleaf.

**Supplementary Figure 5.7 (previous page). Linear Discriminant Analysis and Principal Component Analysis of concentrations of elements from additional extant insect cuticle. a–c** LDA plots with data grouped by family (**a**), order (**b**) and coloration mechanism (**c**). **d–f** Biplot vectors showing the contribution of each element to variation in the dataset where data is grouped by family (**d**), order (**e**) and coloration mechanism (**f**). **g–i** PCA plots with data grouped by family (**g**), order (**h**) and coloration mechanism (**i**). **j** Biplot vectors showing the contribution of each element to variation in the dataset. Data point colours are mean RGB values for that region of interest in the cuticle. Abbreviations as per caption to Supplementary Fig. 5.3.

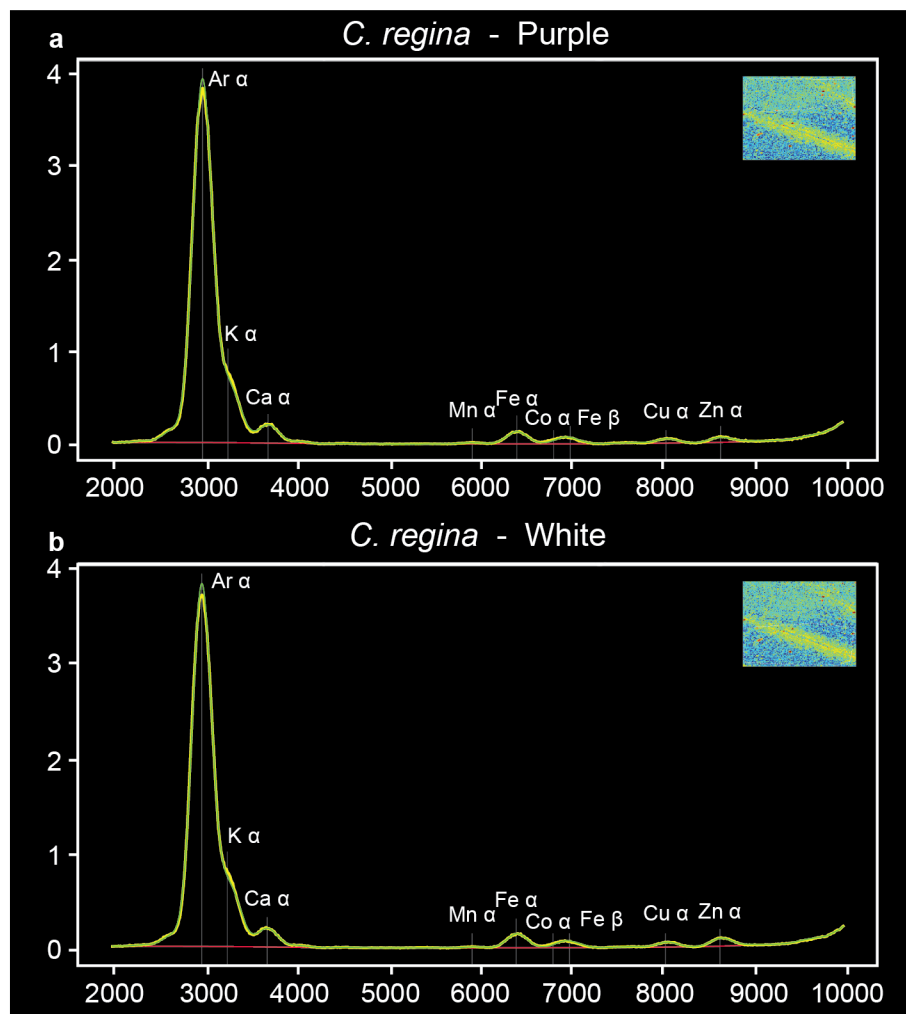


Supplementary Figure 5.8. Caption overleaf.

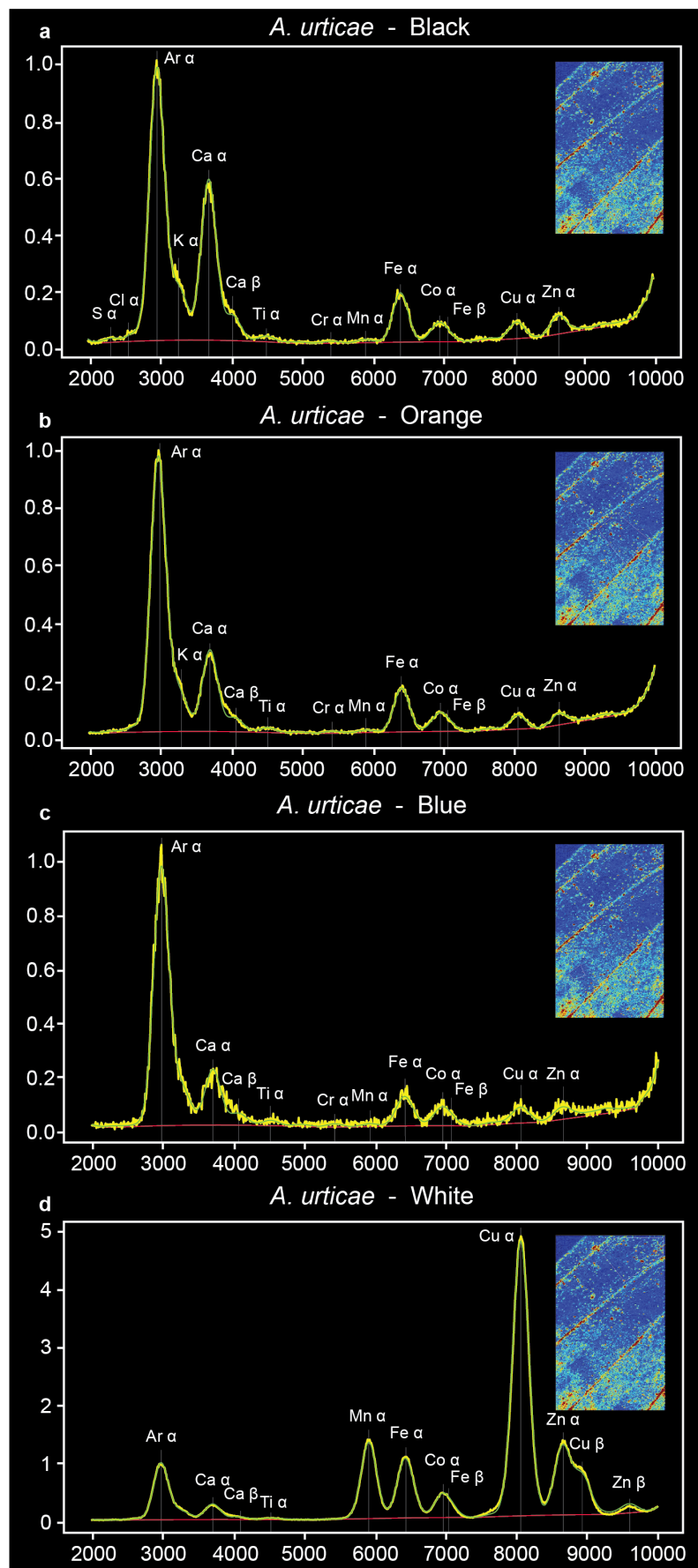
**Supplementary Figure 5.8 (previous page). Linear Discriminant Analysis and Principal Component Analysis of concentrations of elements from extant insect cuticle. a–c** LDA plots with data grouped by family (**a**), order (**b**) and coloration mechanism (**c**). **d–f** Biplot vectors showing the contribution of each element to variation in the dataset where data is grouped by family (**d**), order (**e**) and coloration mechanism (**f**). **g–i** PCA plots with data grouped by family (**g**), order (**h**) and coloration mechanism (**i**). **j** Biplot vectors showing the contribution of each element to variation in the dataset. Data point colours are mean RGB values for that region of interest in the cuticle. Abbreviations as per caption to Fig. 5.2.



**Supplementary Figure 5.9. Linear Discriminant Analysis and Principal Component Analysis of concentrations of elements in fossil insect cuticle and associated sedimentary matrix. a–b** LDA plots with data grouped by age. **c–d** Biplots showing the contribution of each element to variation in the dataset where the data are grouped by age. **e–g** PCA plots with data grouped by locality (**e**), order (**f**) and age (**g**); sediment data excluded. **h–j** PCA plots with data grouped by locality (**h**), order (**i**) and age (**j**); sediment data included. **k–l** Biplots showing the contribution of each element to variation in the dataset; sediment excluded (**k**) and sediment included (**l**). Abbreviations as per caption to Fig. 5.4.



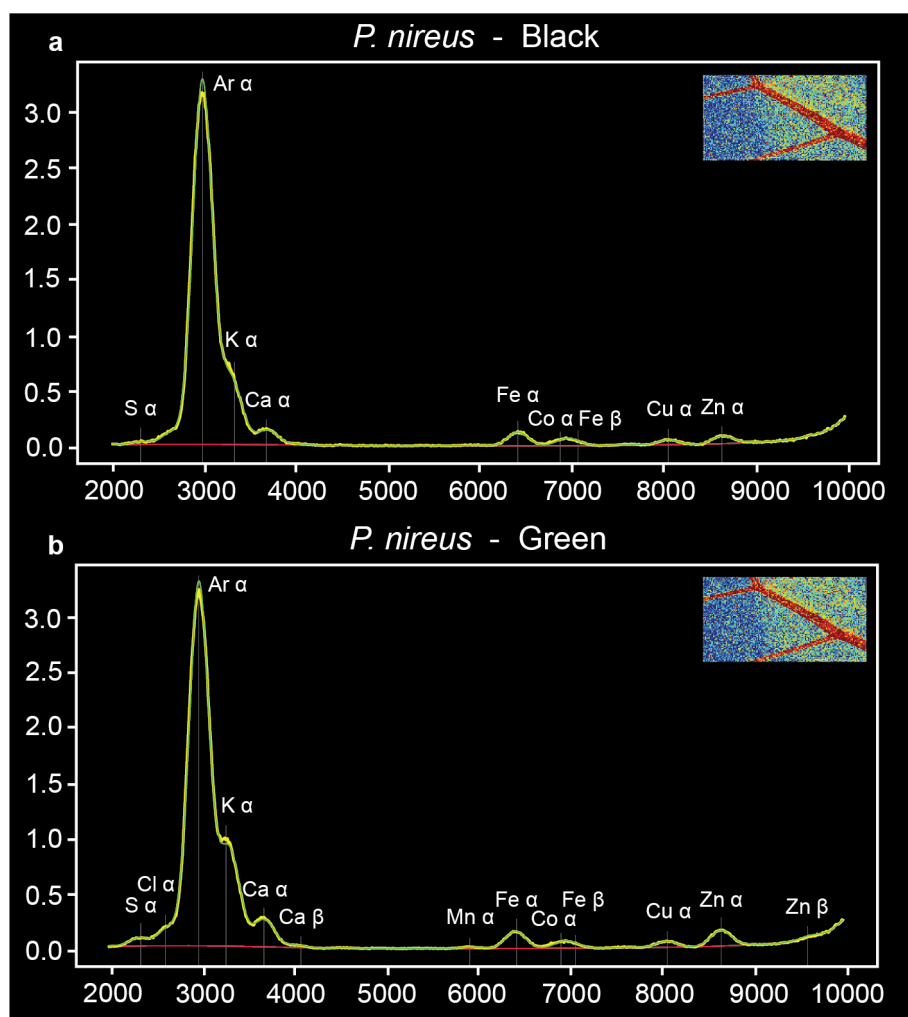
**Supplementary Figure 5.10. Multi channel analyser spectra for ROIs from *Colotis regina*, displaying purple (a) and white (b) visual colours.** X-axis represents X-ray emission energy (eV); Y-axis represents counts. Yellow line characterises the SRS-XRF spectrum analyzed, green line the fit and red line the continuum. Plotted using SMAK 1.67 <https://www.sams-xrays.com/smak/>.



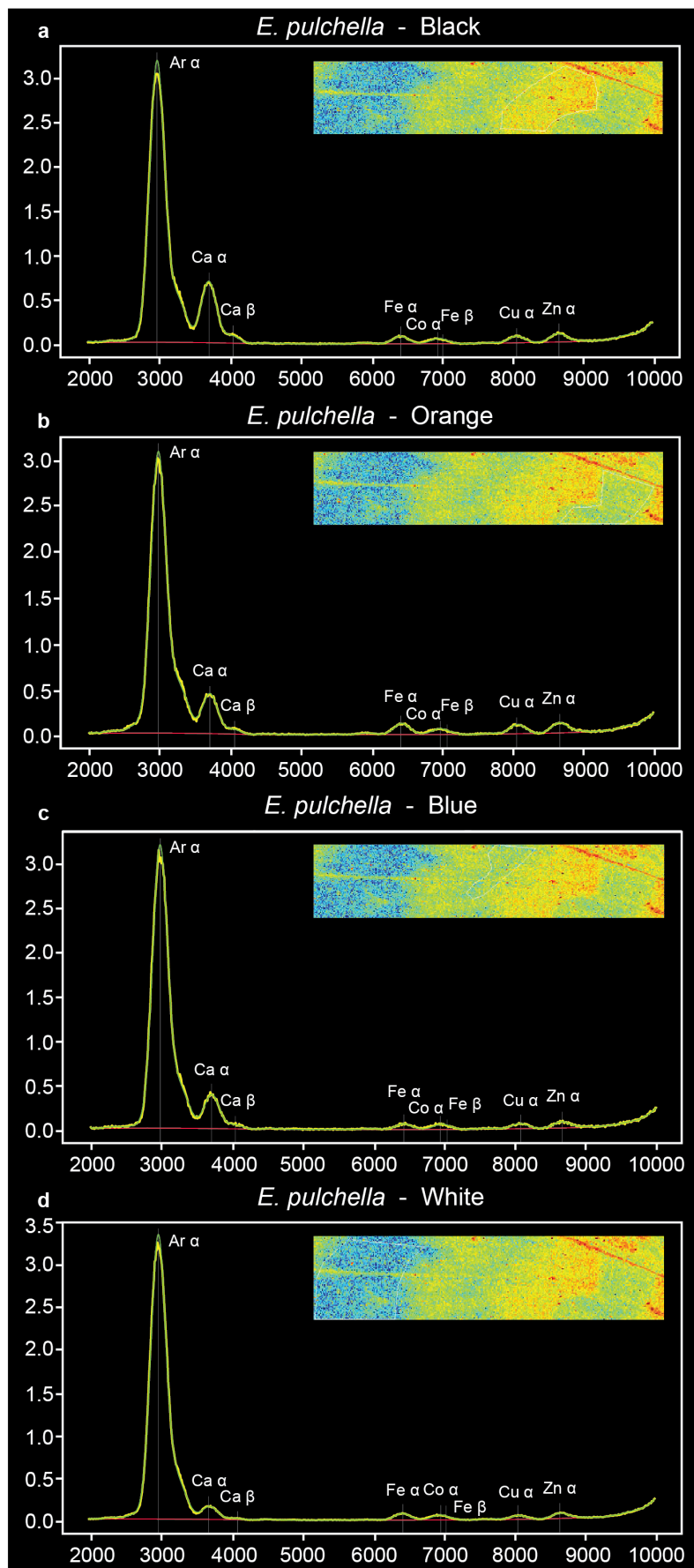
Supplementary Figure 5.11. Caption overleaf.



**Supplementary Figure 5.11 (previous page). Multi channel analyser spectra for ROIs from *Aglais urticae*, displaying black (a), orange (b), blue (c) and white (d) visual colours. X-axis represents X-ray emission energy (eV); Y-axis represents counts. Yellow line characterises the SRS-XRF spectrum analyzed, green line the fit and red line the continuum. Plotted using SMAK 1.67 <https://www.sams-xrays.com/smak/>.**

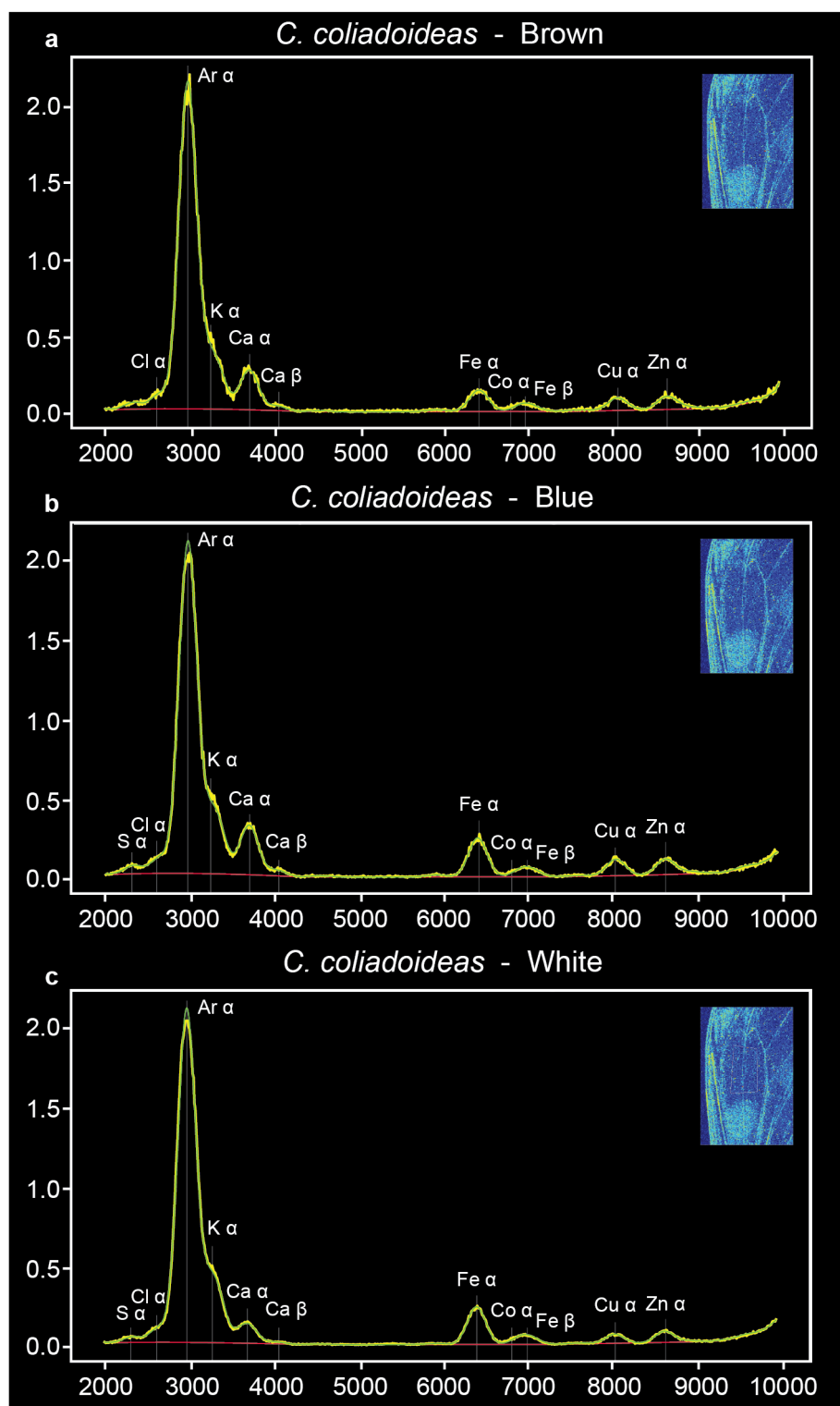


**Supplementary Figure 5.12. Multi channel analyser spectra for ROIs from *Papilio nireus*, displaying black (a) and green (b) visual colours.** X-axis represents X-ray emission energy (eV); Y-axis represents counts. Yellow line characterises the SRS-XRF spectrum analyzed, green line the fit and red line the continuum. Plotted using SMAK 1.67 <https://www.sams-xrays.com/smak/>.

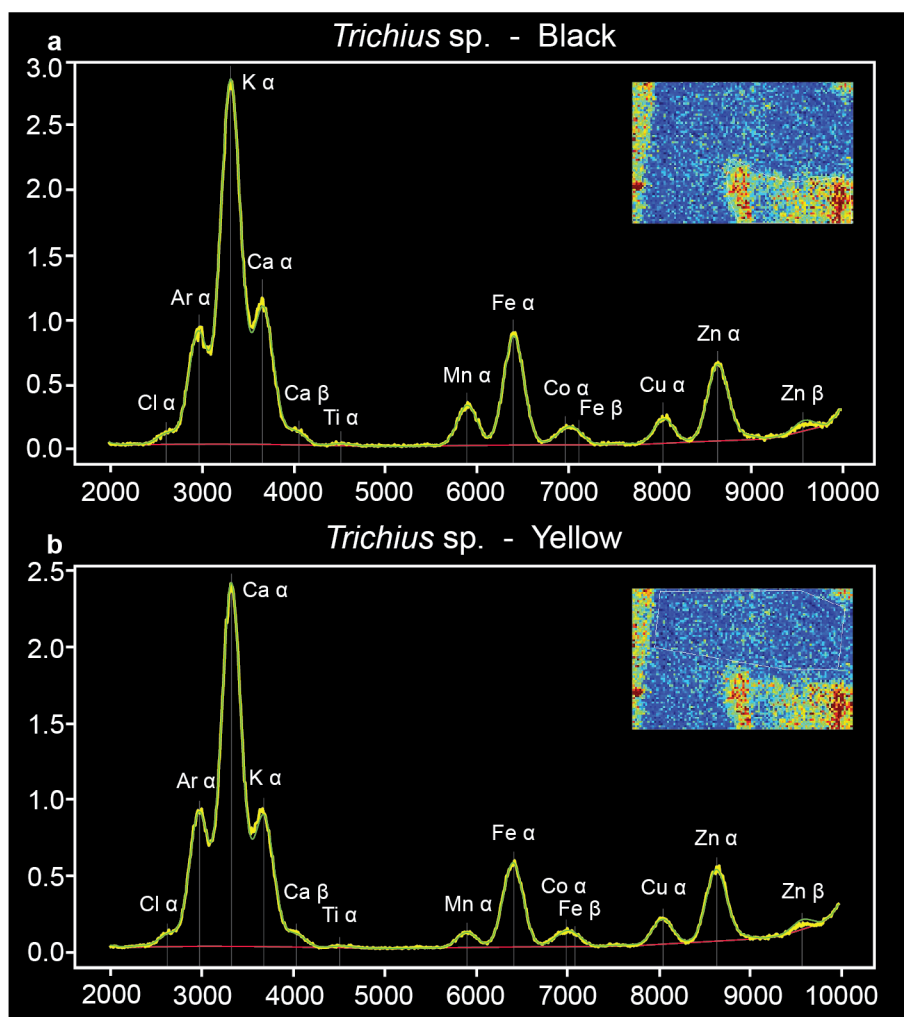


Supplementary Figure 5.13. Caption overleaf.

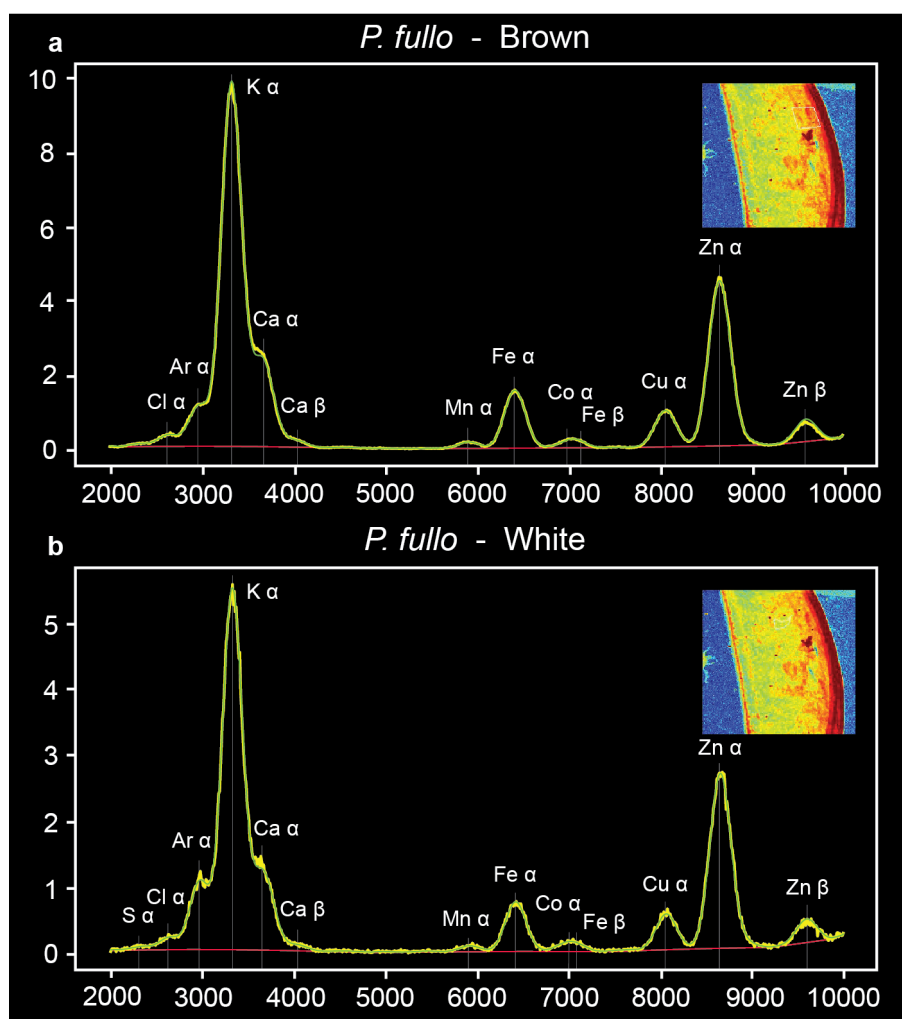
**Supplementary Figure 5.13 (previous page). Multi channel analyser spectra for ROIs from *Erasmia pulchella*,** displaying black (a), orange (b), blue (c) and white (d) visual colours. X-axis represents X-ray emission energy (eV); Y-axis represents counts. Yellow line characterises the SRS-XRF spectrum analyzed, green line the fit and red line the continuum. Plotted using SMAK 1.67 <https://www.sams-xrays.com/smak/>.



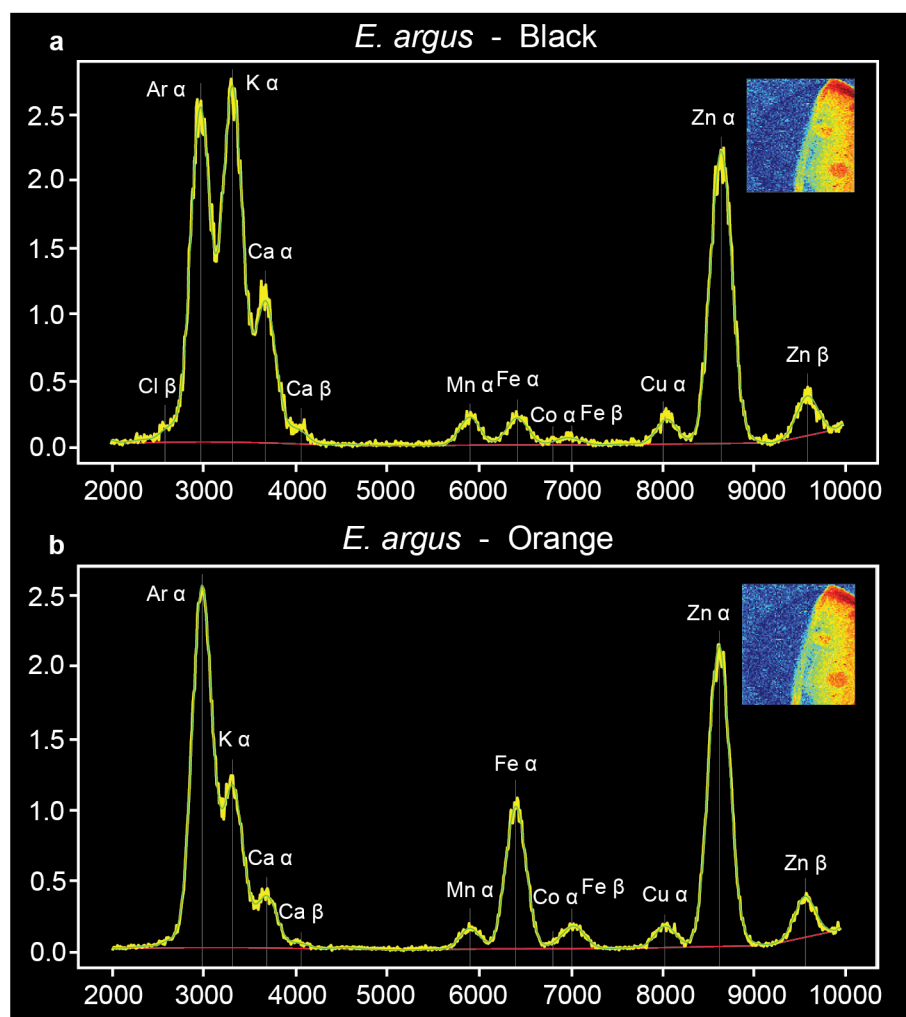
**Supplementary Figure 5.14. Multi channel analyser spectra for ROIs from *Chalcosia coliadoideas*, displaying brown (a), blue (b) and white (c) visual colours.** X-axis represents X-ray emission energy (eV); Y-axis represents counts. Yellow line characterises the SRS-XRF spectrum analyzed, green line the fit and red line the continuum. Plotted using SMAK 1.67 <https://www.sams-xrays.com/smak/>.



**Supplementary Figure 5.15. Multi channel analyser spectra for ROIs from *Trichius* sp., displaying black (a) and yellow (b) visual colours.** X-axis represents X-ray emission energy (eV); Y-axis represents counts. Yellow line characterises the SRS-XRF spectrum analyzed, green line the fit and red line the continuum. Plotted using SMAK 1.67 <https://www.sams-xrays.com/smak/>.

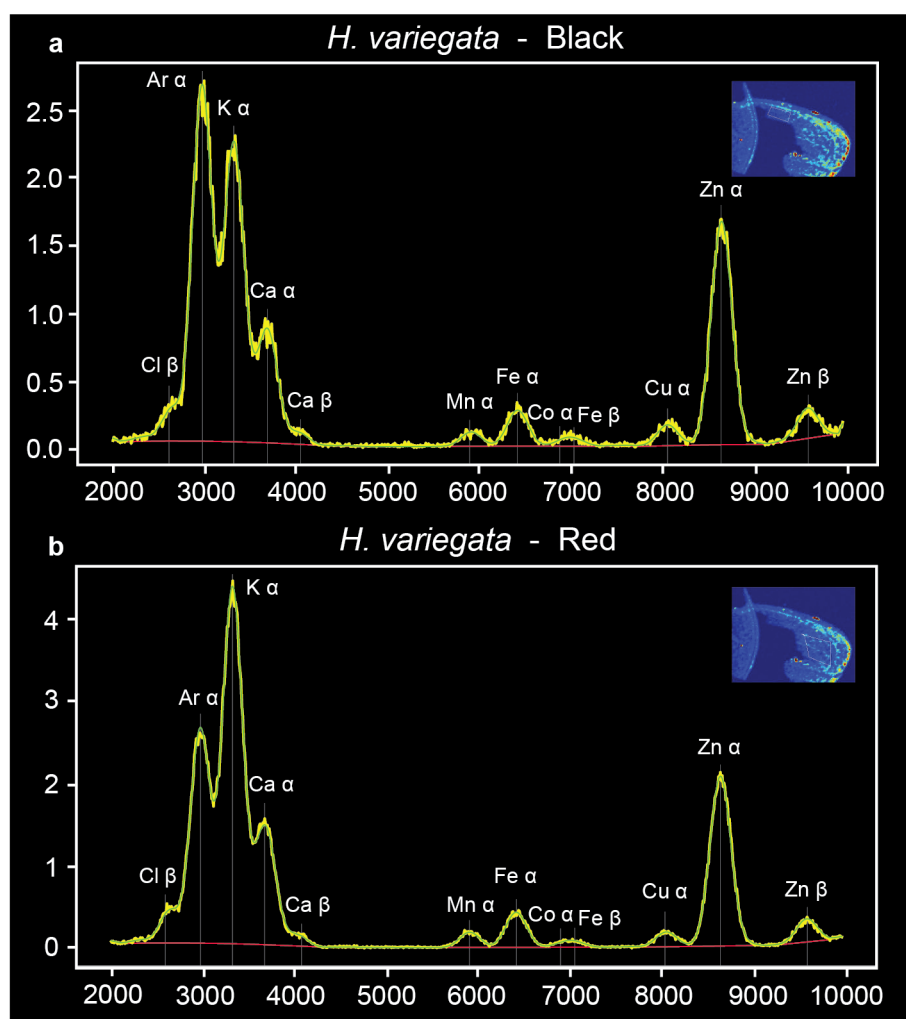


**Supplementary Figure 5.16. Multi channel analyser spectra for ROIs from *Polyphylla fullo*, displaying brown (a) and white (b) visual colours.** X-axis represents X-ray emission energy (eV); Y-axis represents counts. Yellow line characterises the SRS-XRF spectrum analyzed, green line the fit and red line the continuum. Plotted using SMAK 1.67 <https://www.sams-xrays.com/smak/>.

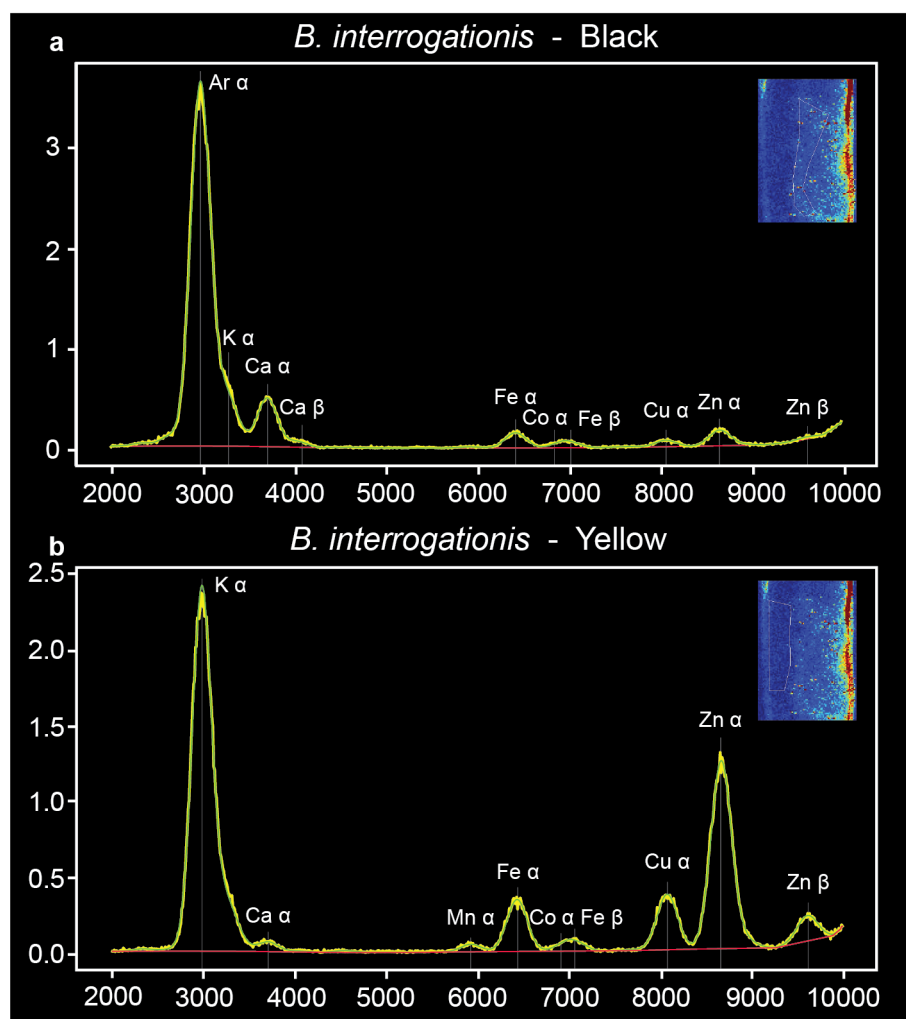


**Supplementary Figure 5.17. Multi channel analyser spectra for ROIs from *Epilachna argus*, displaying black (a) and orange (b) visual colours.** X-axis represents X-ray emission energy (eV); Y-axis represents counts. Yellow line characterises the SRS-XRF spectrum analyzed, green line the fit and red line the continuum. Plotted using SMAK 1.67 <https://www.sams-xrays.com/smak/>.

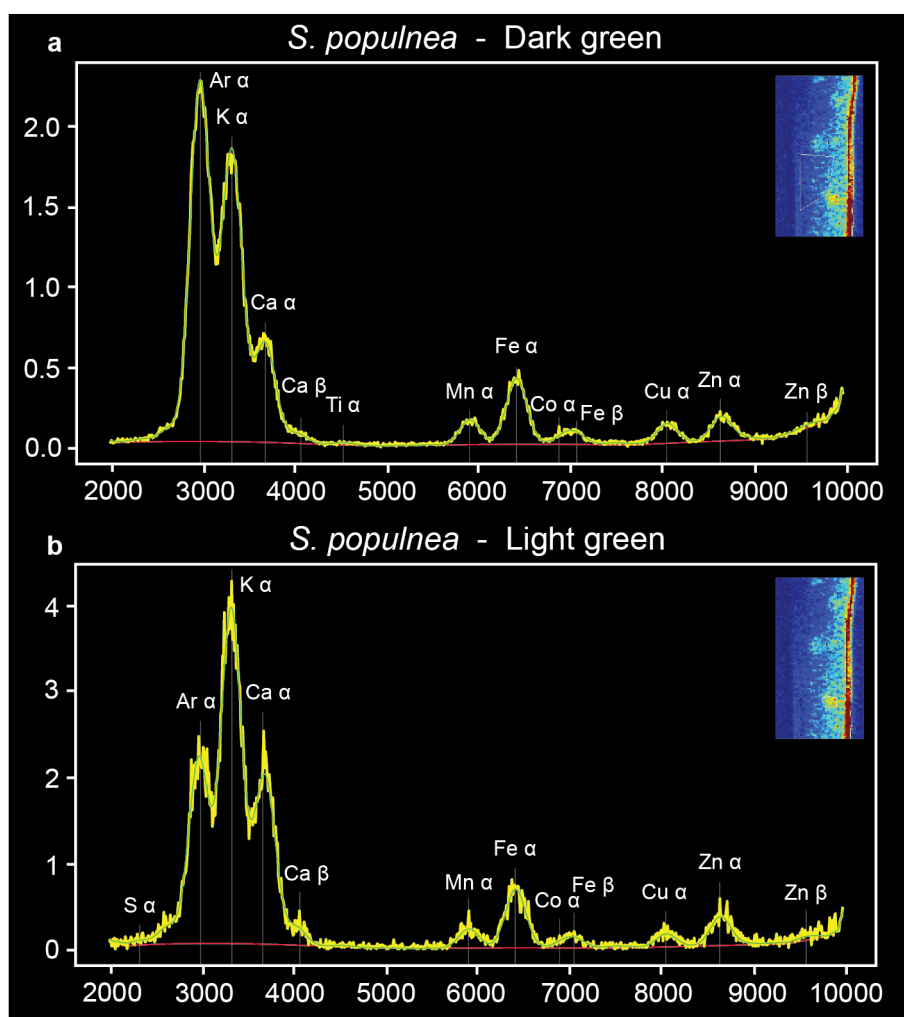




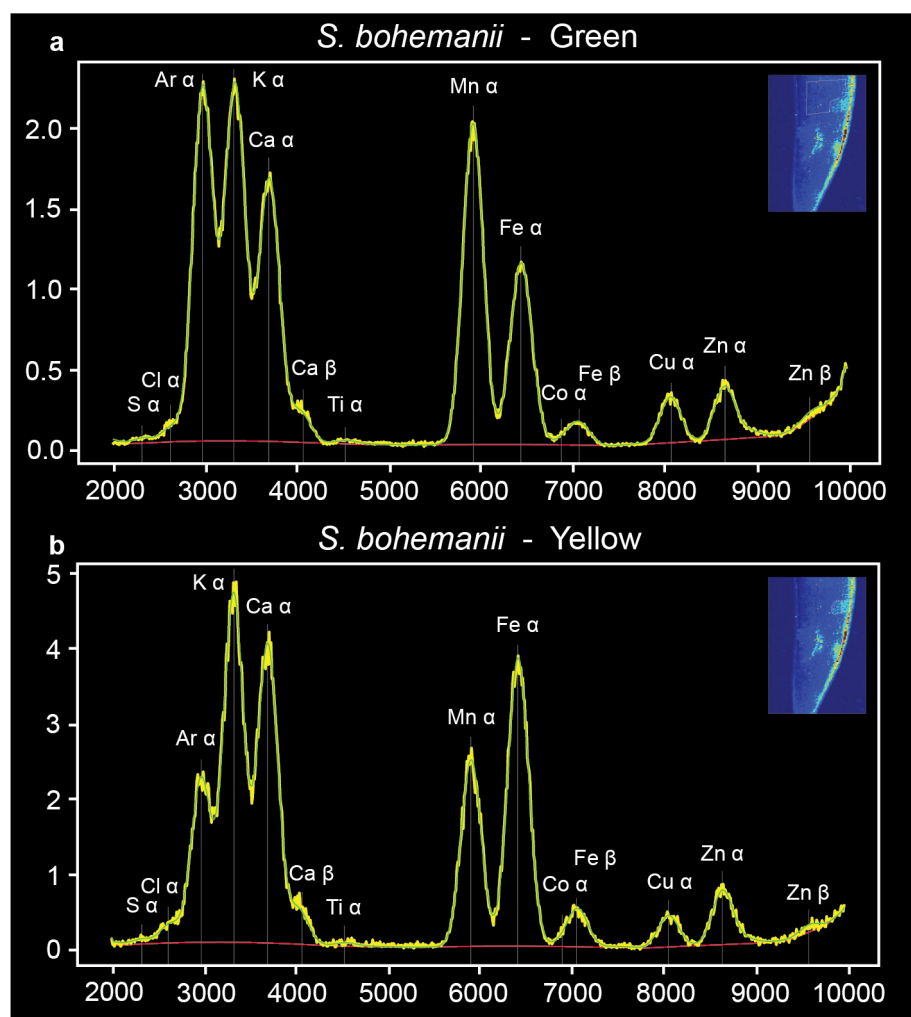
**Supplementary Figure 5.18. Multi channel analyser spectra for ROIs from *Hippodamia variegata*, displaying black (a) and red (b) visual colours.** X-axis represents X-ray emission energy (eV); Y-axis represents counts. Yellow line characterises the SRS-XRF spectrum analyzed, green line the fit and red line the continuum. Plotted using SMAK 1.67 <https://www.sams-xrays.com/smak/>.



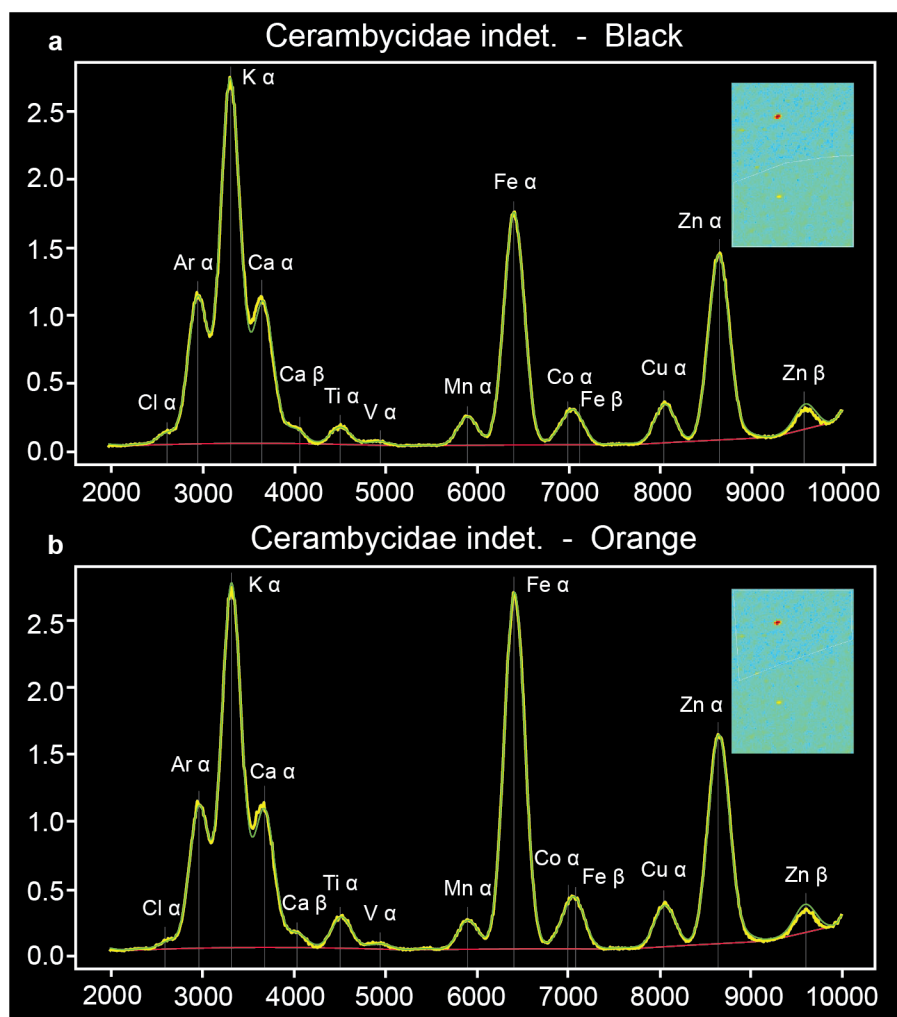
**Supplementary Figure 5.19. Multi channel analyser spectra for ROIs from *Brachyta interrogationis*, displaying black (a) and yellow (b) visual colours.** X-axis represents X-ray emission energy (eV); Y-axis represents counts. Yellow line characterises the SRS-XRF spectrum analyzed, green line the fit and red line the continuum. Plotted using SMAK 1.67 <https://www.sams-xrays.com/smak/>.



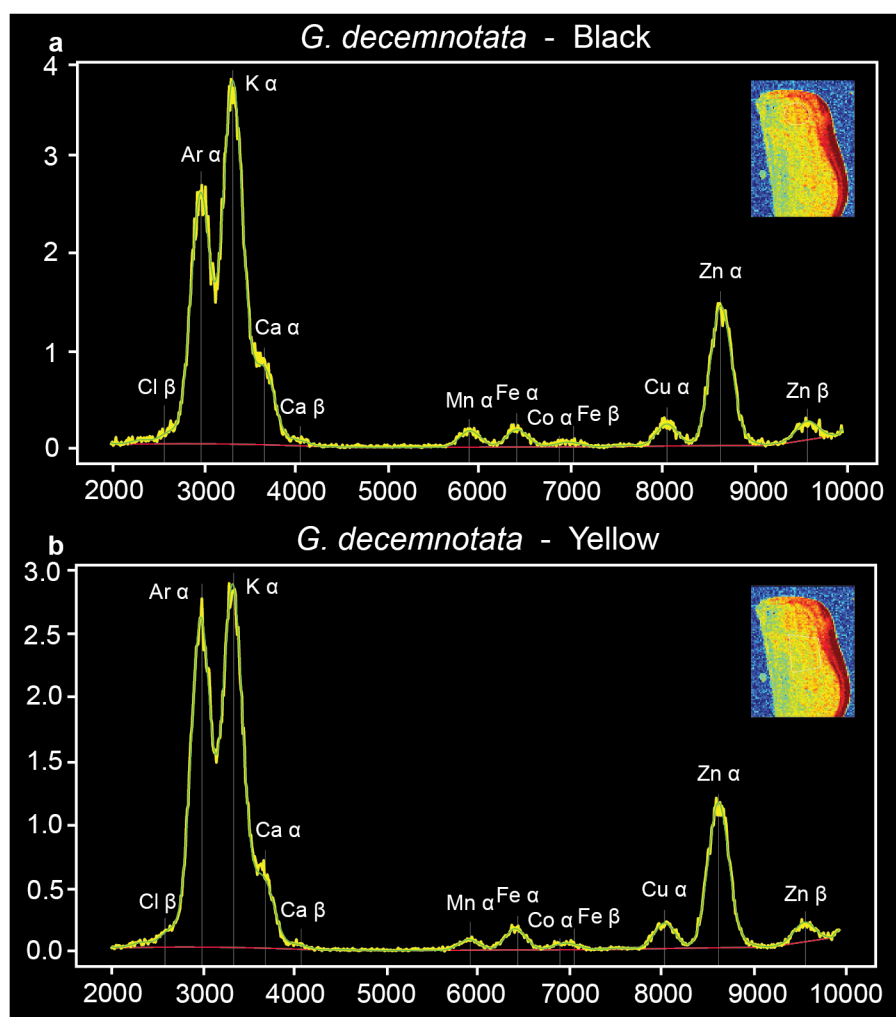
**Supplementary Figure 5.20. Multi channel analyser spectra for ROIs from *Saperda populnea*, displaying dark (a) and light green (b) visual colours. X-axis represents X-ray emission energy (eV); Y-axis represents counts. Yellow line characterises the SRS-XRF spectrum analyzed, green line the fit and red line the continuum. Plotted using SMAK 1.67 <https://www.sams-xrays.com/smak/>.**



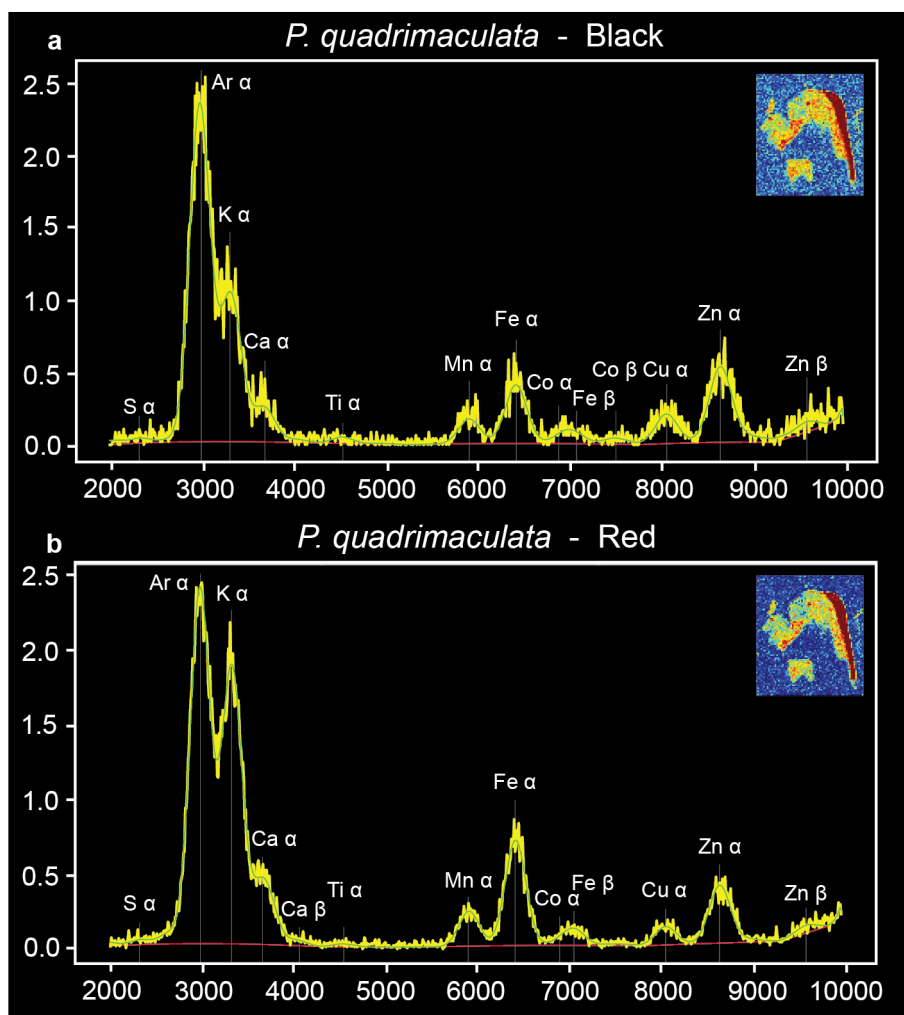
**Supplementary Figure 5.21. Multi channel analyser spectra for ROIs from *Sternotomis bohemanii*, displaying green (a) and yellow (b) visual colours.** X-axis represents X-ray emission energy (eV); Y-axis represents counts. Yellow line characterises the SRS-XRF spectrum analyzed, green line the fit and red line the continuum. Plotted using SMAK 1.67 <https://www.sams-xrays.com/smak/>.



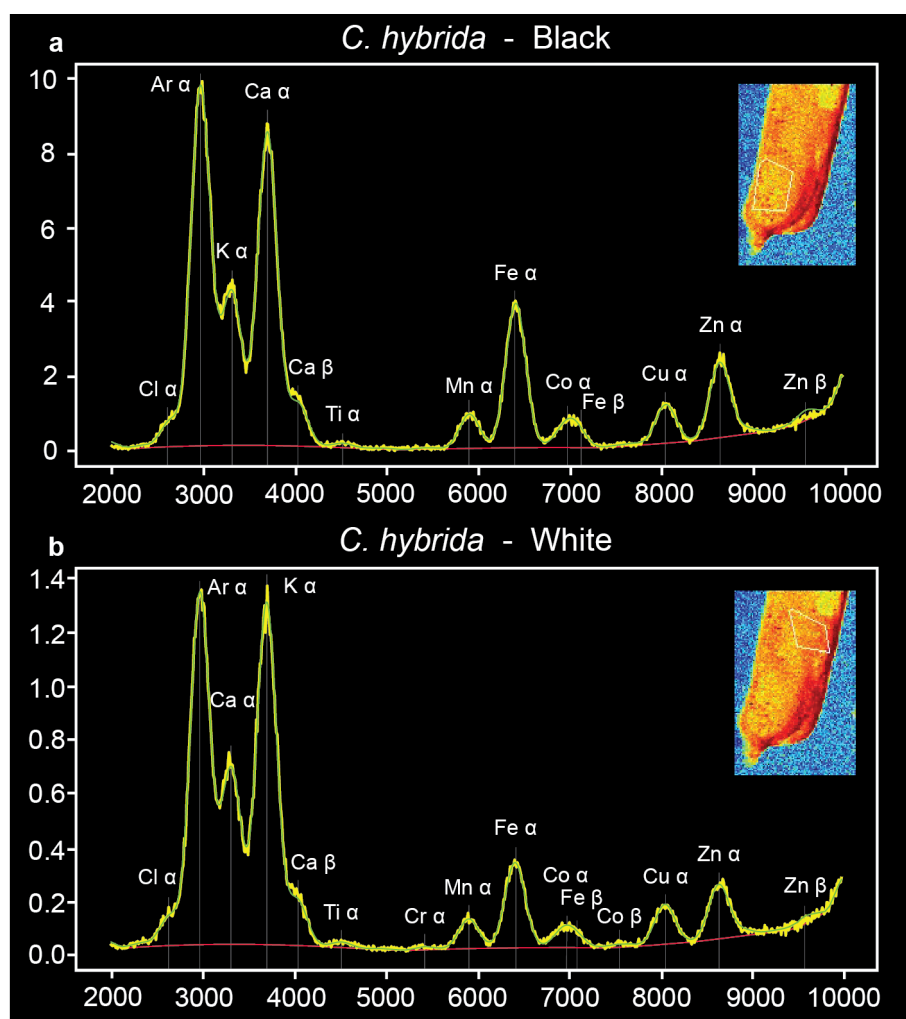
**Supplementary Figure 5.22. Multi channel analyser spectra for ROIs from *Cerambycidae indet.*, displaying black (a) and orange (b) visual colours.** X-axis represents X-ray emission energy (eV); Y-axis represents counts. Yellow line characterises the SRS-XRF spectrum analyzed, green line the fit and red line the continuum. Plotted using SMAK 1.67 <https://www.sams-xrays.com/smak/>.



**Supplementary Figure 5.23. Multi channel analyser spectra for ROIs from *Gonioctena decemnotata*, displaying black (a) and yellow (b) visual colours.** X-axis represents X-ray emission energy (eV); Y-axis represents counts. Yellow line characterises the SRS-XRF spectrum analyzed, green line the fit and red line the continuum. Plotted using SMAK 1.67 <https://www.sams-xrays.com/smak/>.

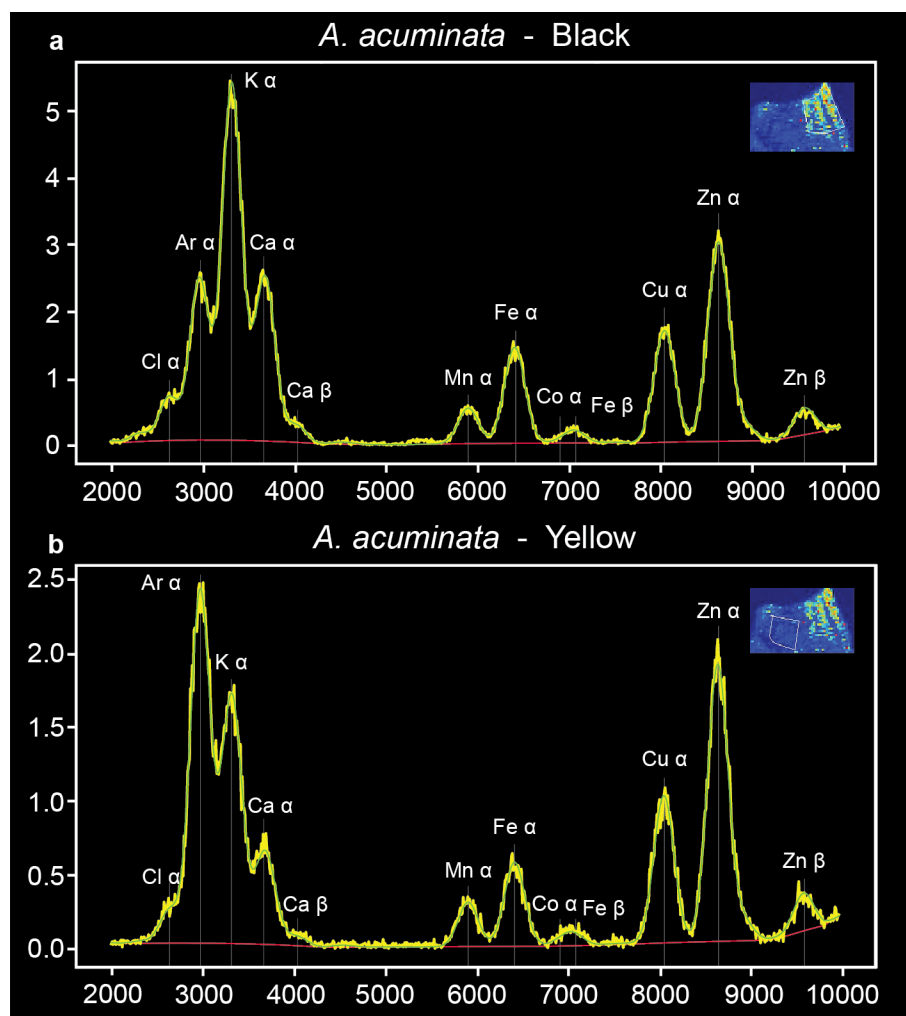


**Supplementary Figure 5.24. Multi channel analyser spectra for ROIs from *Phyllobrotica quadrimaculata*, displaying black (a) and red (b) visual colours.** X-axis represents X-ray emission energy (eV); Y-axis represents counts. Yellow line characterises the SRS-XRF spectrum analyzed, green line the fit and red line the continuum. Plotted using SMAK 1.67 <https://www.sams-xrays.com/smak/>.

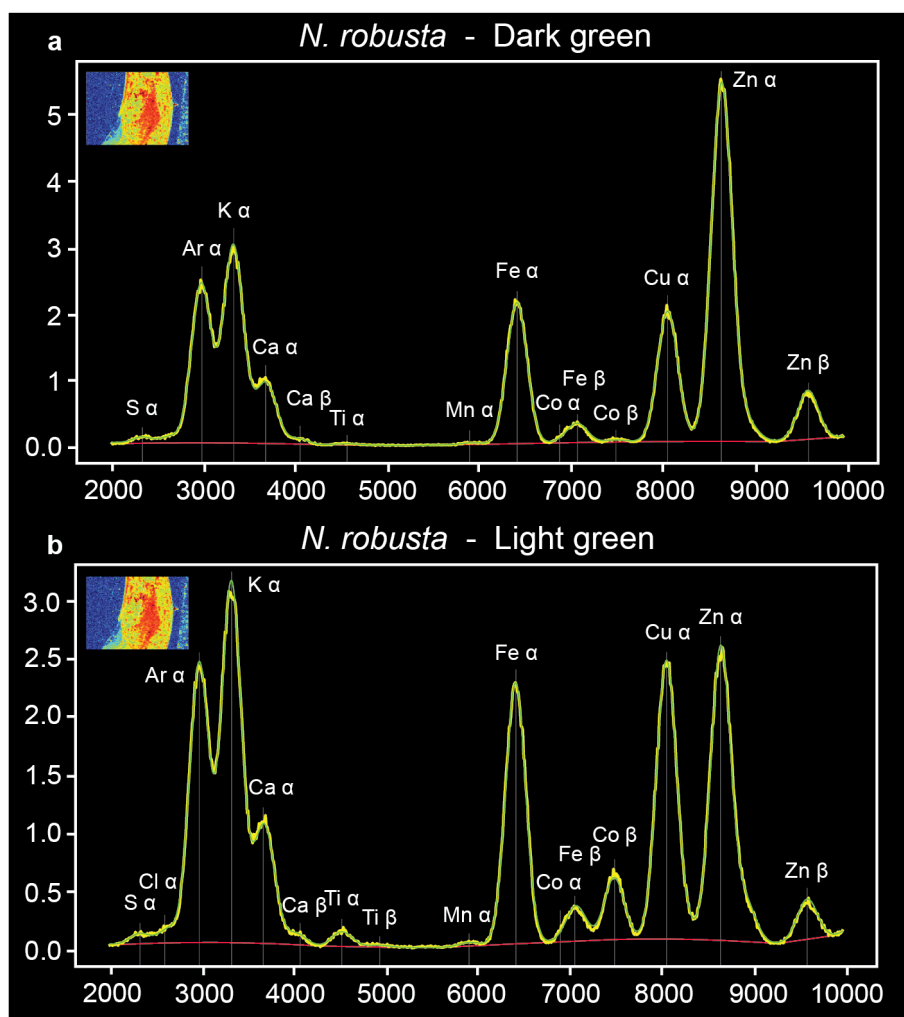


**Supplementary Figure 5.25. Multi channel analyser spectra for ROIs from *Cicindela hybrida*, displaying black (a) and white (b) visual colours.** X-axis represents X-ray emission energy (eV); Y-axis represents counts. Yellow line characterises the SRS-XRF spectrum analyzed, green line the fit and red line the continuum. Plotted using SMAK 1.67 <https://www.sams-xrays.com/smak/>.

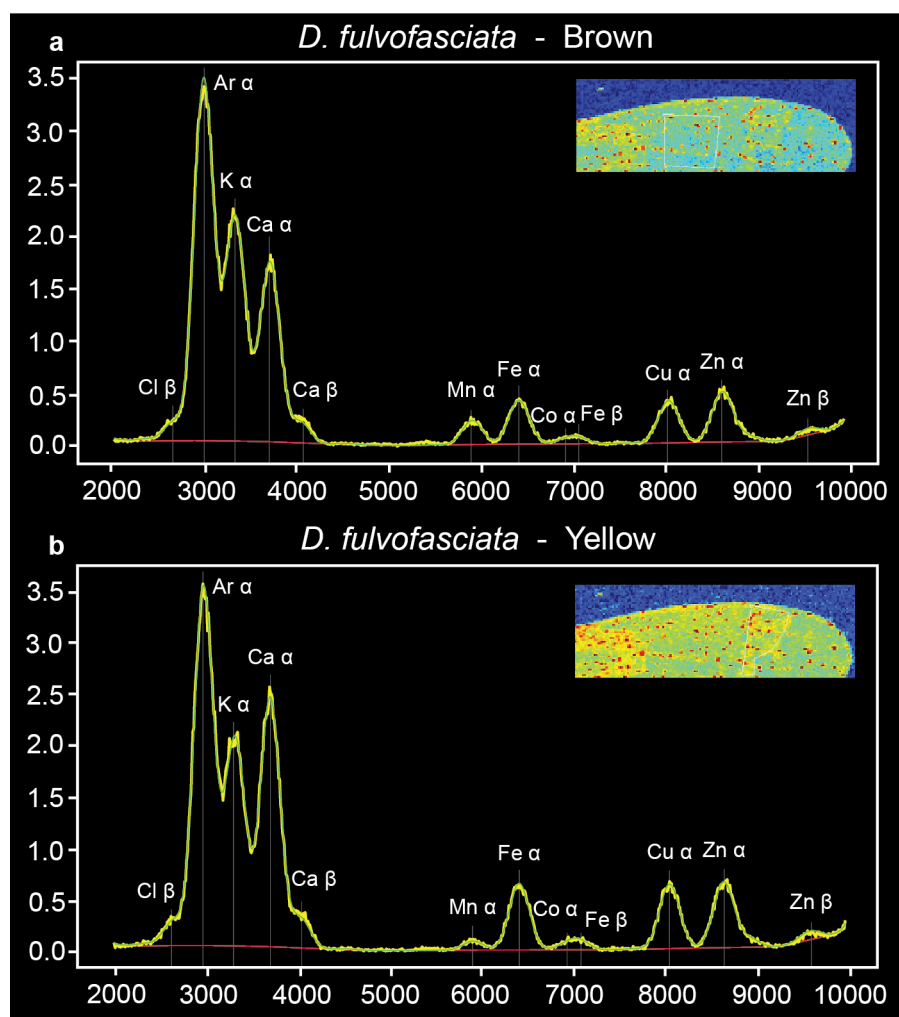




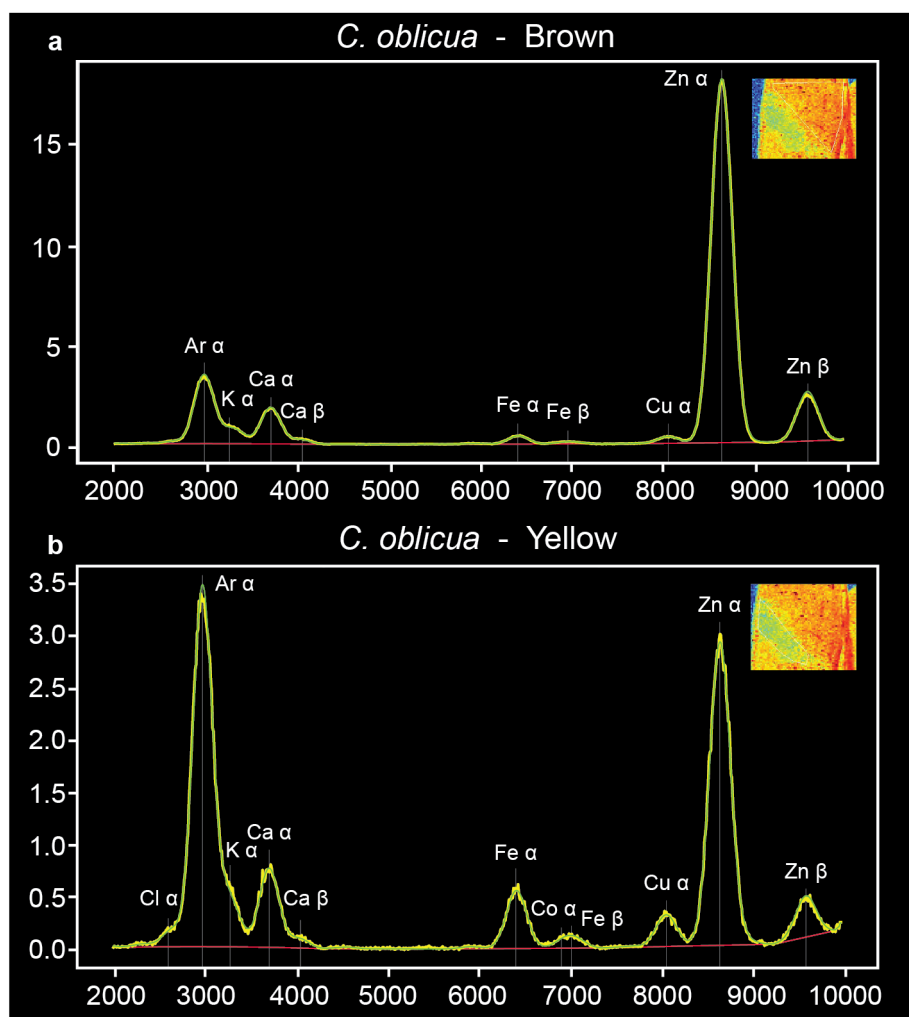
**Supplementary Figure 5.26. Multi channel analyser spectra for ROIs from *Aelia acuminata*, displaying black (a) and yellow (b) visual colours.** X-axis represents X-ray emission energy (eV); Y-axis represents counts. Yellow line characterises the SRS-XRF spectrum analyzed, green line the fit and red line the continuum. Plotted using SMAK 1.67 <https://www.sams-xrays.com/smak/>.



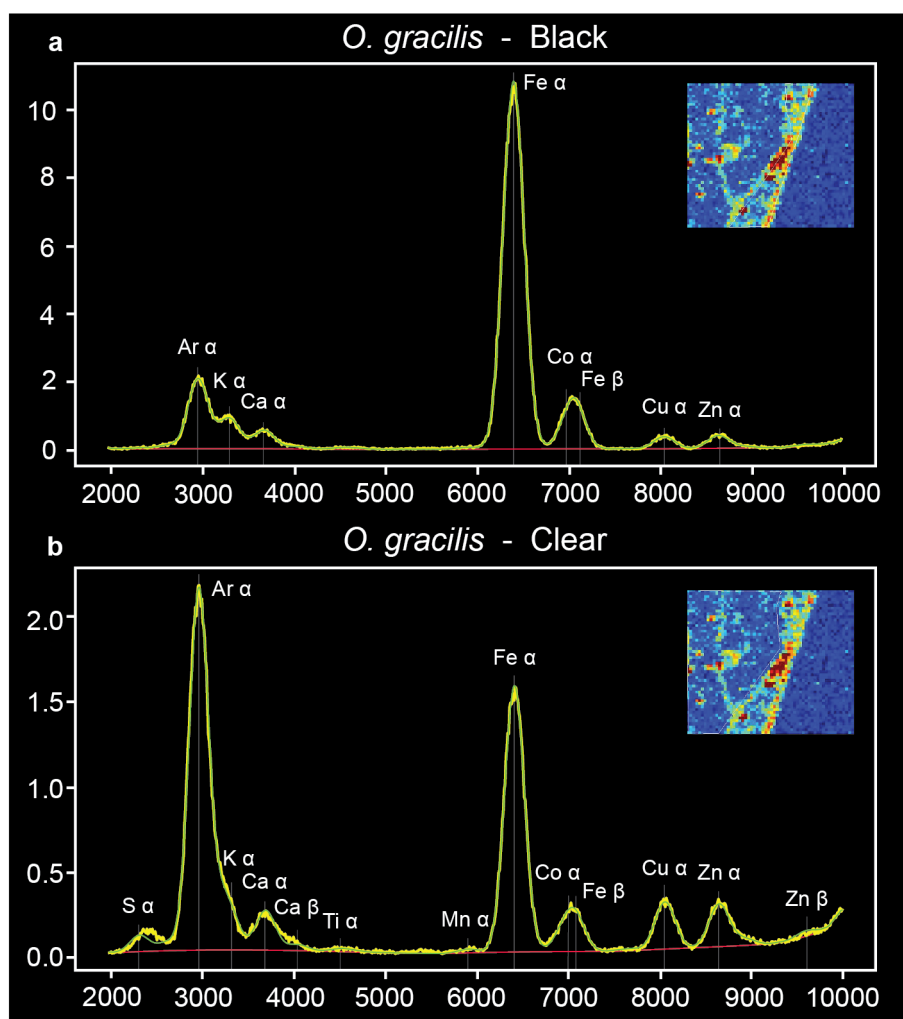
**Supplementary Figure 5.27. Multi channel analyser spectra for ROIs from *Nezara robusta*, displaying dark (a) and light green (b) visual colours.** X-axis represents X-ray emission energy (eV); Y-axis represents counts. Yellow line characterises the SRS-XRF spectrum analyzed, green line the fit and red line the continuum. Plotted using SMAK 1.67 <https://www.sams-xrays.com/smak/>.



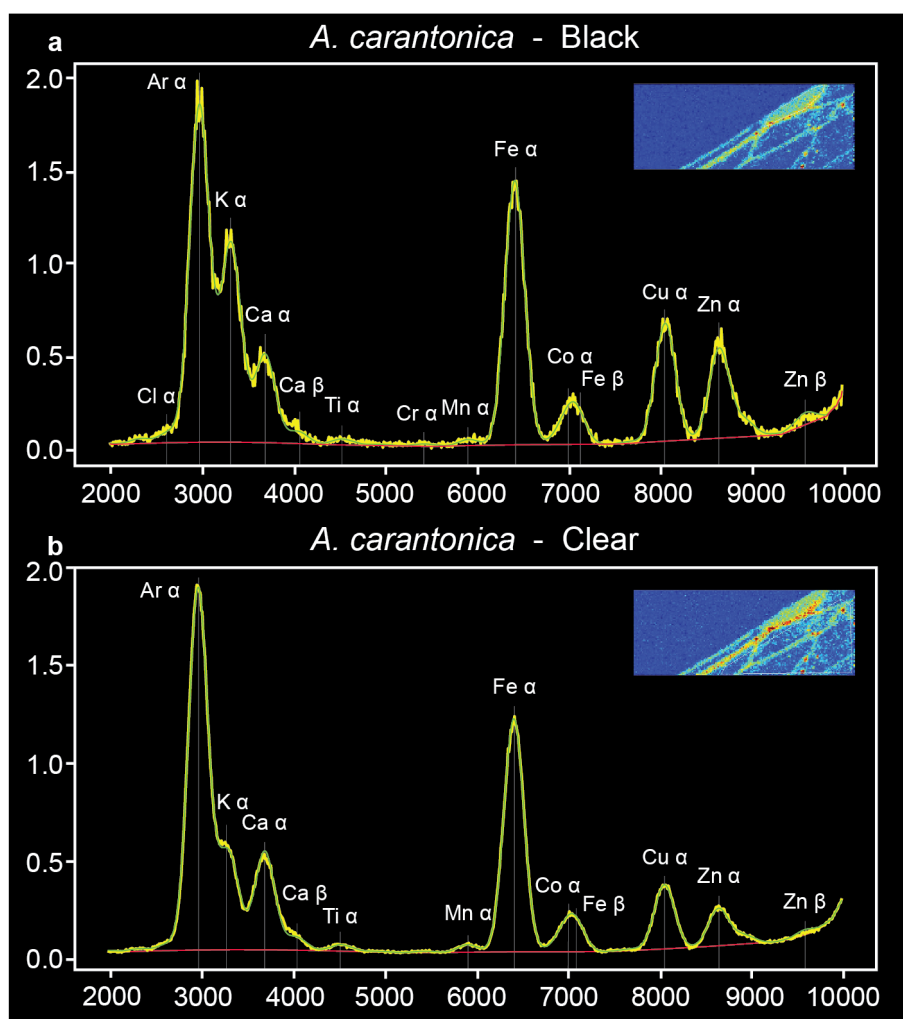
**Supplementary Figure 5.28. Multi channel analyser spectra for ROIs from *Deselvana fulvofasciata*, displaying brown (a) and yellow (b) visual colours.** X-axis represents X-ray emission energy (eV); Y-axis represents counts. Yellow line characterises the SRS-XRF spectrum analyzed, green line the fit and red line the continuum. Plotted using SMAK 1.67 <https://www.sams-xrays.com/smak/>.



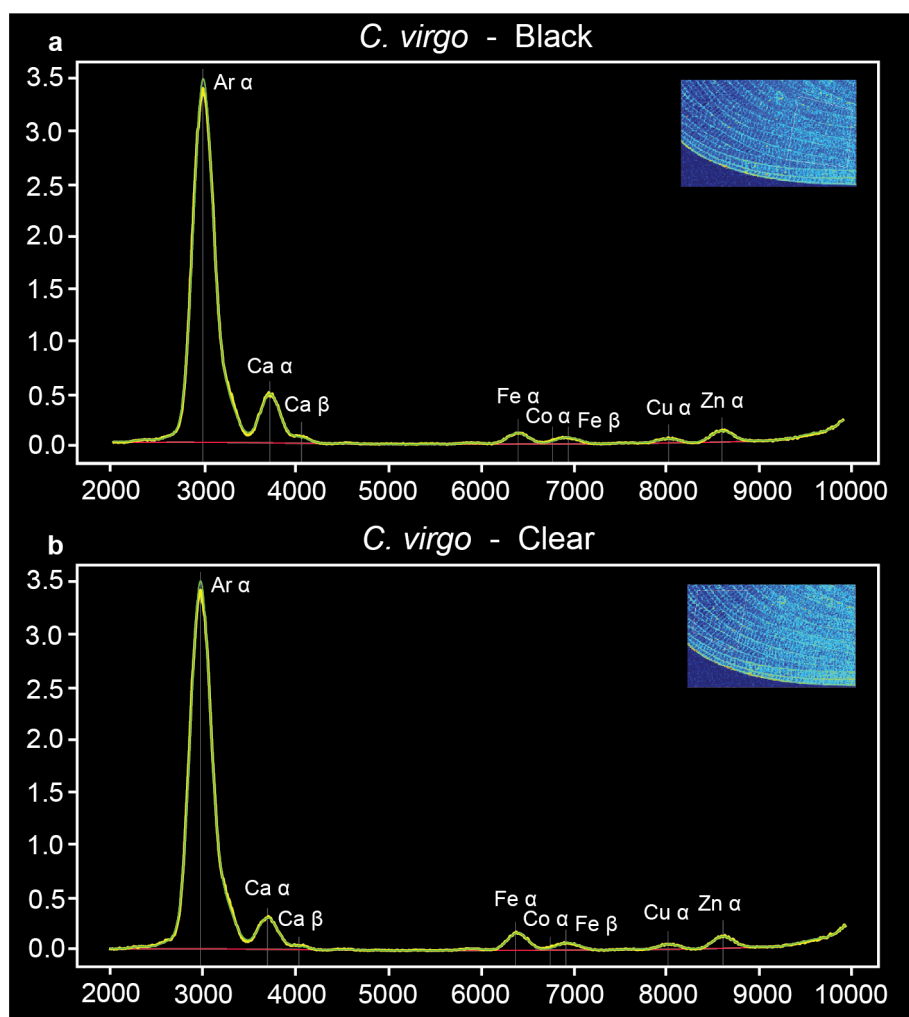
**Supplementary Figure 5.29. Multi channel analyser spectra for ROIs from *Cicciana obliqua*, displaying brown (a) and yellow (b) visual colours.** X-axis represents X-ray emission energy (eV); Y-axis represents counts. Yellow line characterises the SRS-XRF spectrum analyzed, green line the fit and red line the continuum. Plotted using SMAK 1.67 <https://www.sams-xrays.com/smak/>.



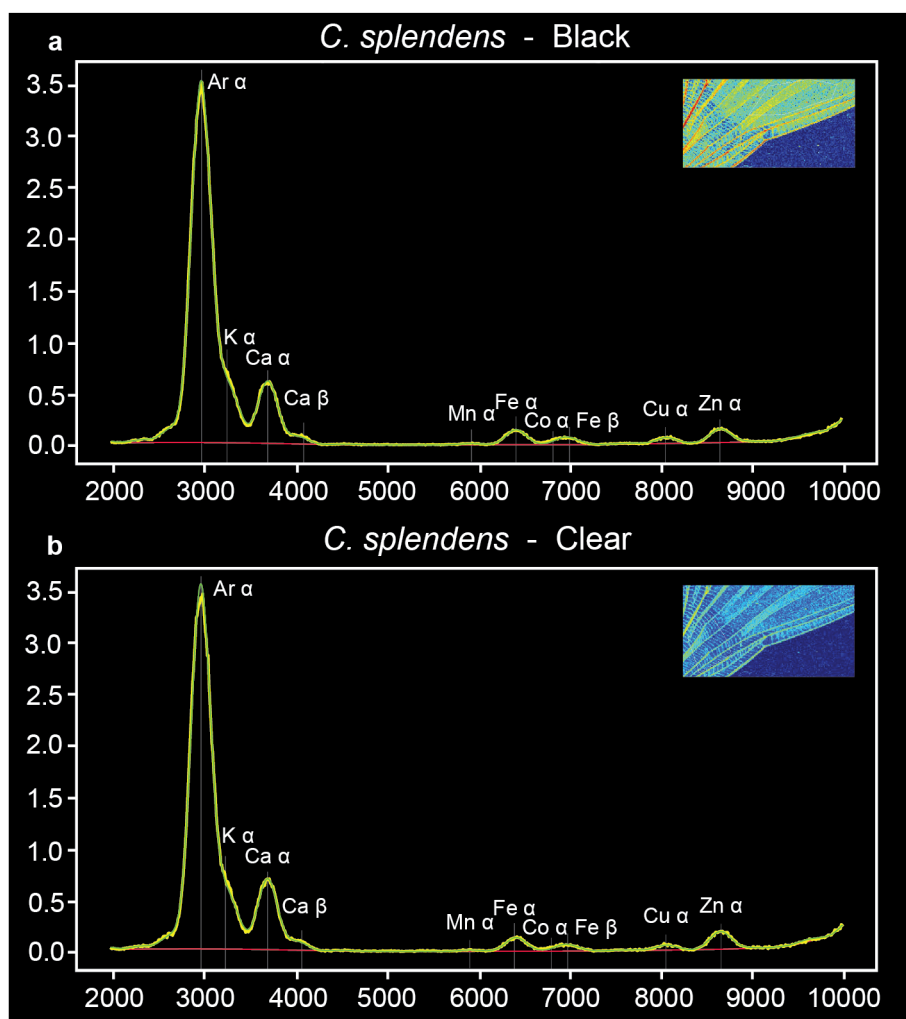
**Supplementary Figure 5.30. Multi channel analyser spectra for ROIs from *Odynerus gracilis*, displaying black (a) and clear (b) visual colours. X-axis represents X-ray emission energy (eV); Y-axis represents counts. Yellow line characterises the SRS-XRF spectrum analyzed, green line the fit and red line the continuum. Plotted using SMAK 1.67 <https://www.sams-xrays.com/smak/>.**



**Supplementary Figure 5.31. Multi channel analyser spectra for ROIs from *Andrena carantonica*, displaying black (a) and clear (b) visual colours.** X-axis represents X-ray emission energy (eV); Y-axis represents counts. Yellow line characterises the SRS-XRF spectrum analyzed, green line the fit and red line the continuum. Plotted using SMAK 1.67 <https://www.sams-xrays.com/smak/>.

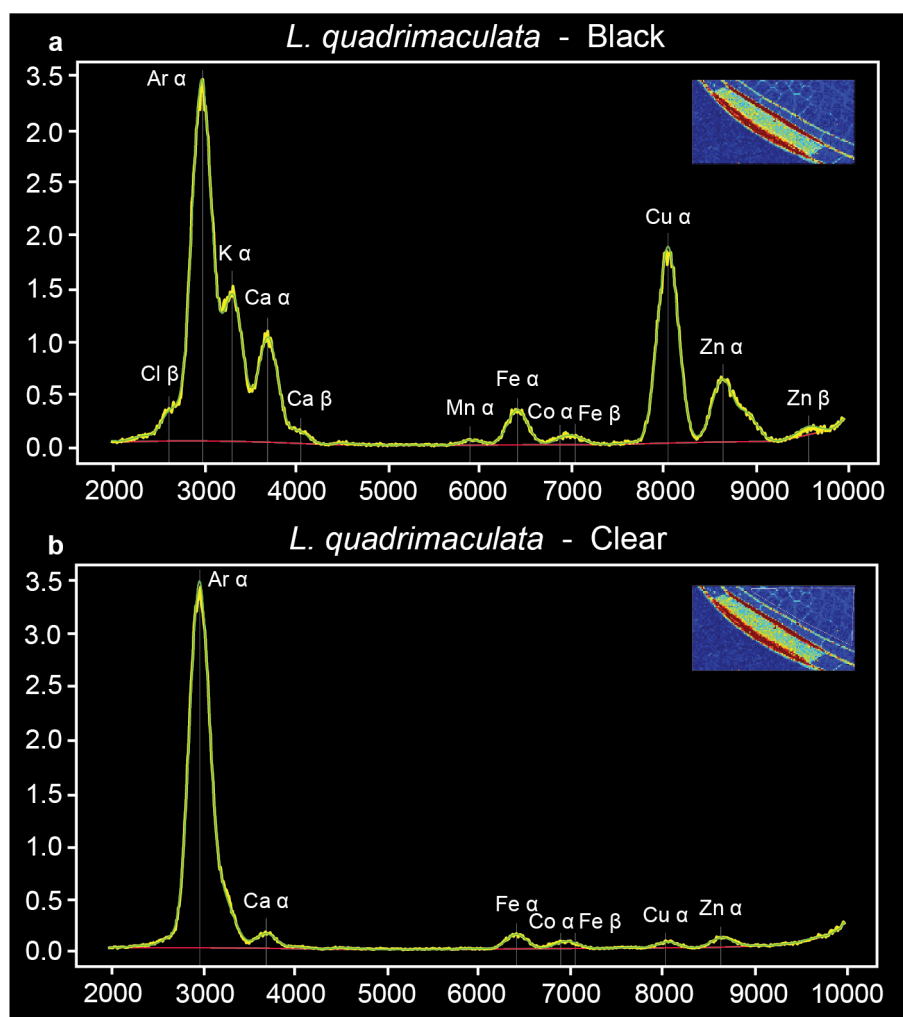


**Supplementary Figure 5.32. Multi channel analyser spectra for ROIs from *Calopteryx virgo*, displaying black (a) and clear (b) visual colours.** X-axis represents X-ray emission energy (eV); Y-axis represents counts. Yellow line characterises the SRS-XRF spectrum analyzed, green line the fit and red line the continuum. Plotted using SMAK 1.67 <https://www.sams-xrays.com/smak/>.

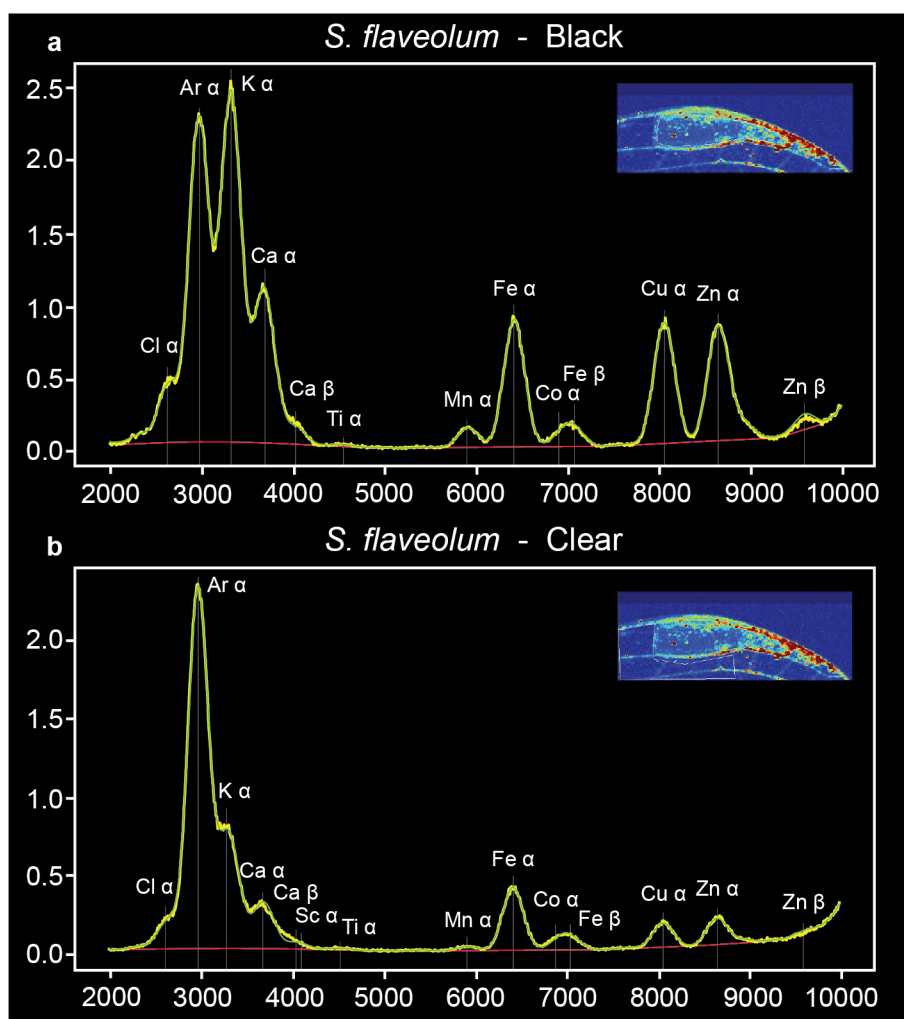


**Supplementary Figure 5.33. Multi channel analyser spectra for ROIs from *Calopteryx splendens*, displaying black (a) and clear (b) visual colours.** X-axis represents X-ray emission energy (eV); Y-axis represents counts. Yellow line characterises the SRS-XRF spectrum analyzed, green line the fit and red line the continuum. Plotted using SMAK 1.67 <https://www.sams-xrays.com/smak/>.

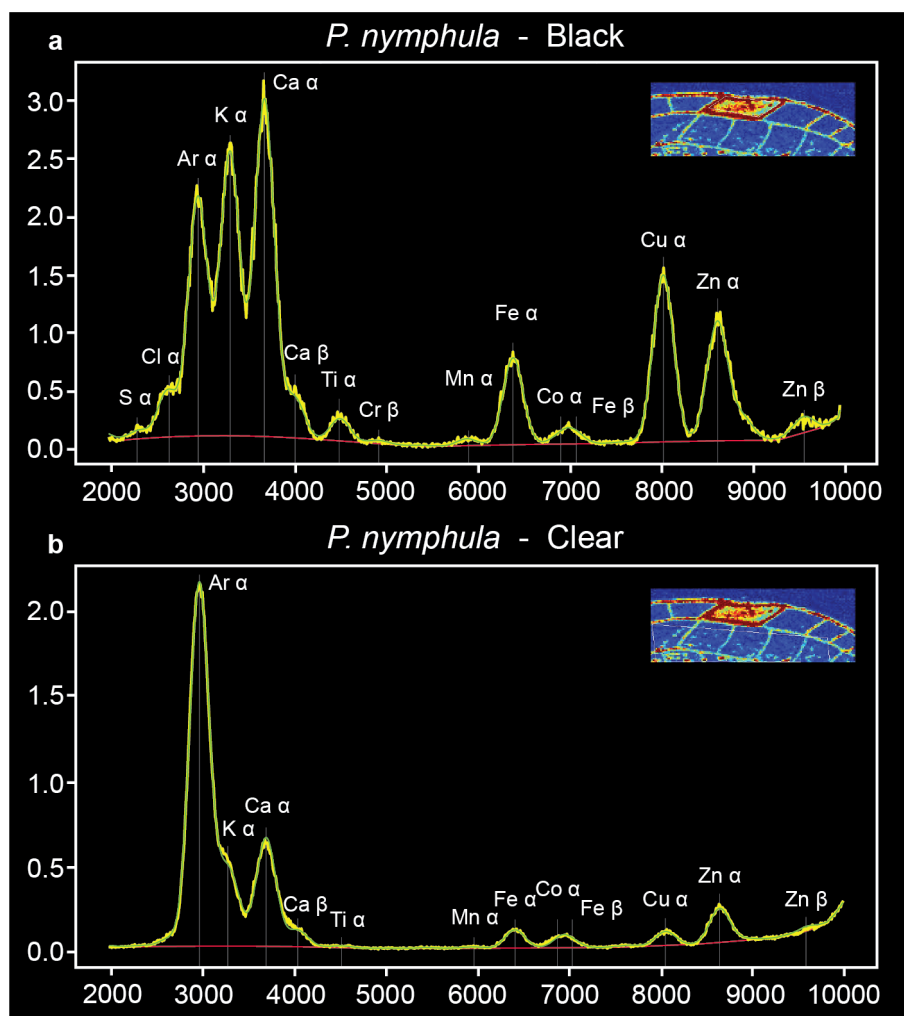




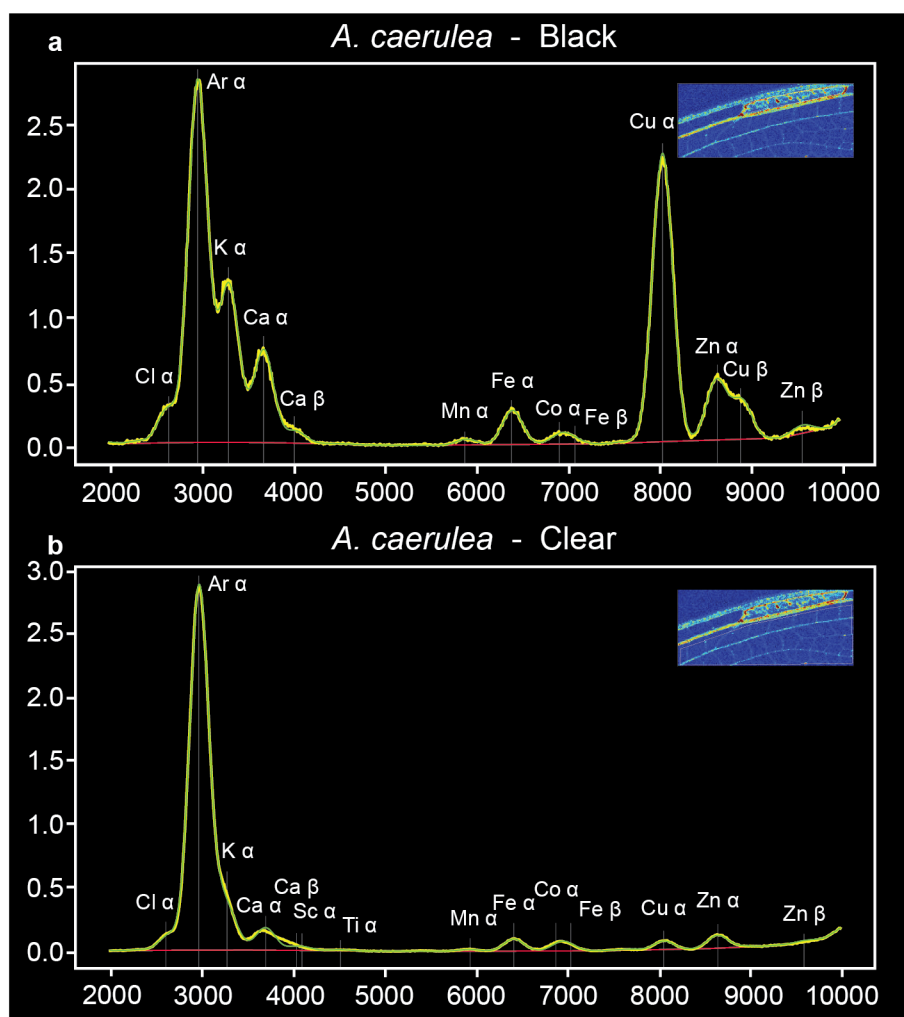
**Supplementary Figure 5.34. Multi channel analyser spectra for ROIs from *Libellula quadrimaculata*, displaying black (a) and clear (b) visual colours.** X-axis represents X-ray emission energy (eV); Y-axis represents counts. Yellow line characterises the SRS-XRF spectrum analyzed, green line the fit and red line the continuum. Plotted using SMAK 1.67 <https://www.sams-xrays.com/smak/>.



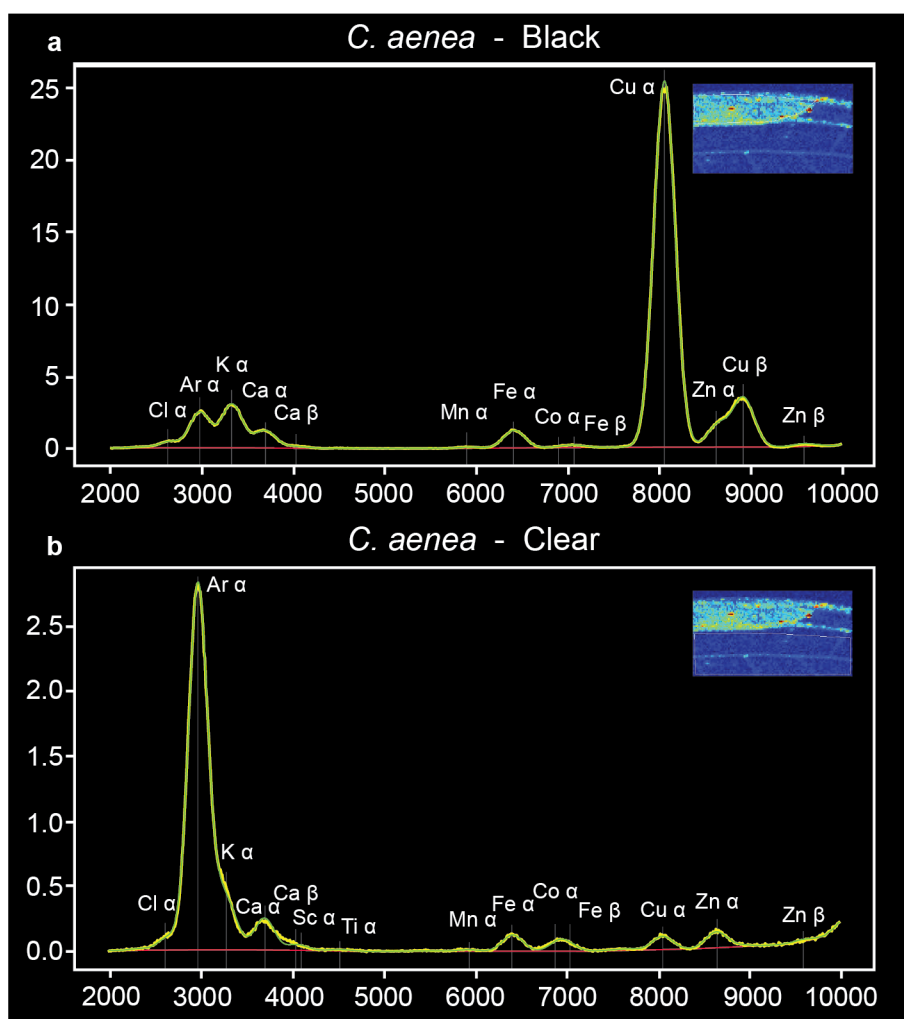
**Supplementary Figure 5.35. Multi channel analyser spectra for ROIs from *Sympetrum flaveolum*, displaying black (a) and clear (b) visual colours.** X-axis represents X-ray emission energy (eV); Y-axis represents counts. Yellow line characterises the SRS-XRF spectrum analyzed, green line the fit and red line the continuum. Plotted using SMAK 1.67 <https://www.sams-xrays.com/smak/>.



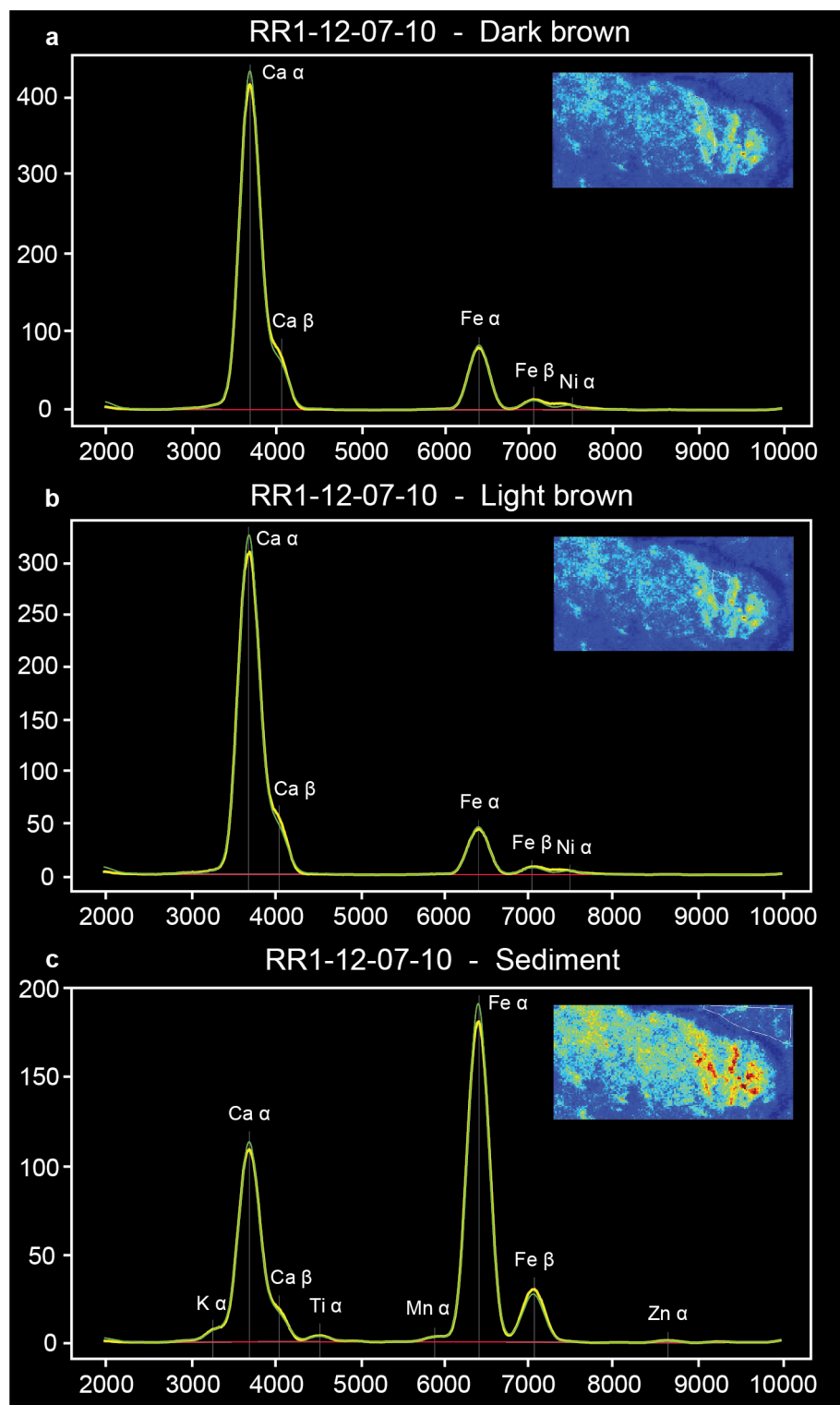
**Supplementary Figure 5.36. Multi channel analyser spectra for ROIs from *Pyrrhosoma nymphula*, displaying black (a) and clear (b) visual colours.** X-axis represents X-ray emission energy (eV); Y-axis represents counts. Yellow line characterises the SRS-XRF spectrum analyzed, green line the fit and red line the continuum. Plotted using SMAK 1.67 <https://www.sams-xrays.com/smak/>.



**Supplementary Figure 5.37. Multi channel analyser spectra for ROIs from *Aeshna caerulea*, displaying black (a) and clear (b) visual colours.** X-axis represents X-ray emission energy (eV); Y-axis represents counts. Yellow line characterises the SRS-XRF spectrum analyzed, green line the fit and red line the continuum. Plotted using SMAK 1.67 <https://www.sams-xrays.com/smak/>.

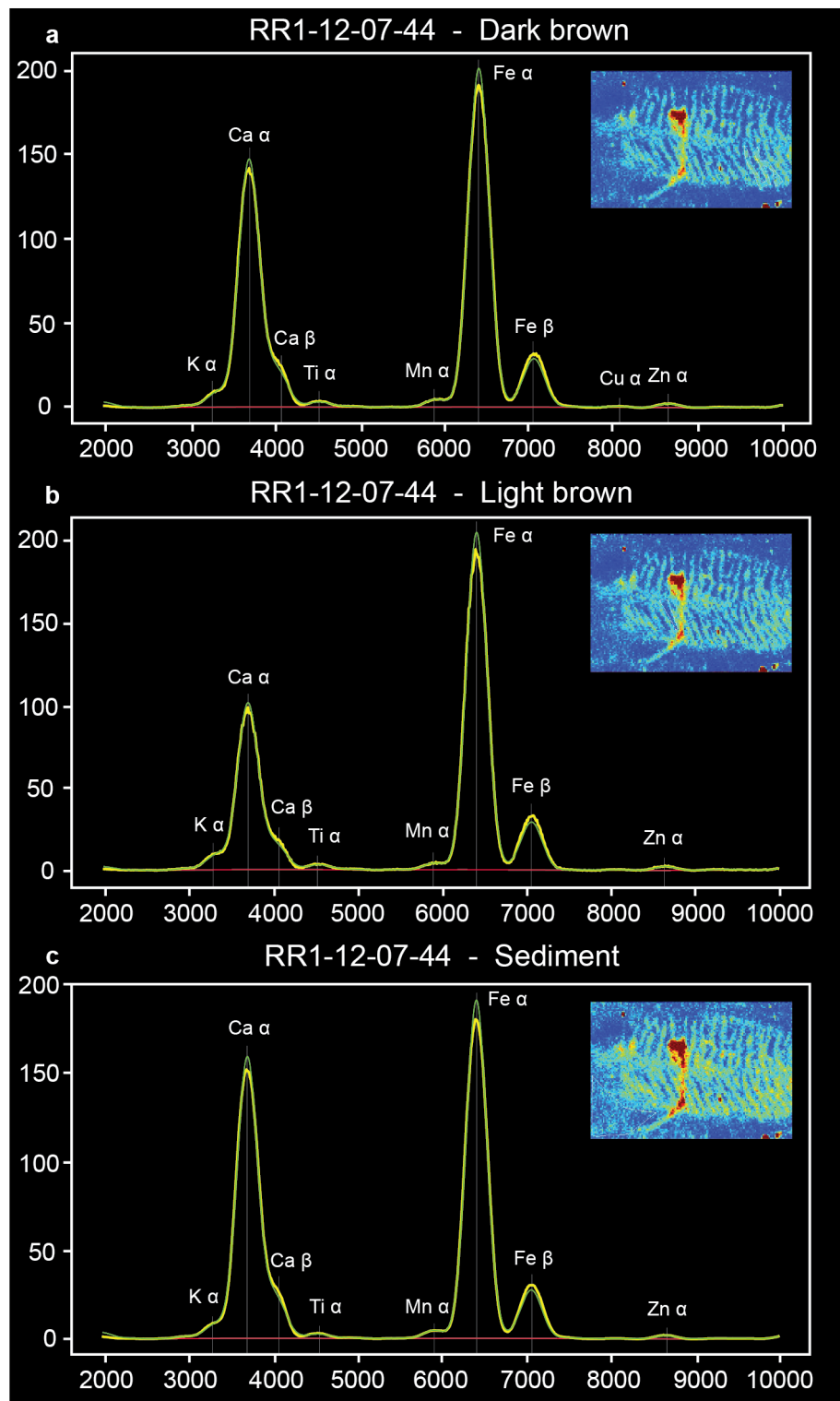


**Supplementary Figure 5.38. Multi channel analyser spectra for ROIs from *Cordulia aenea*, displaying black (a) and clear (b) visual colours.** X-axis represents X-ray emission energy (eV); Y-axis represents counts. Yellow line characterises the SRS-XRF spectrum analyzed, green line the fit and red line the continuum. Plotted using SMAK 1.67 <https://www.sams-xrays.com/smak/>.



Supplementary Figure 5.39. Caption overleaf.

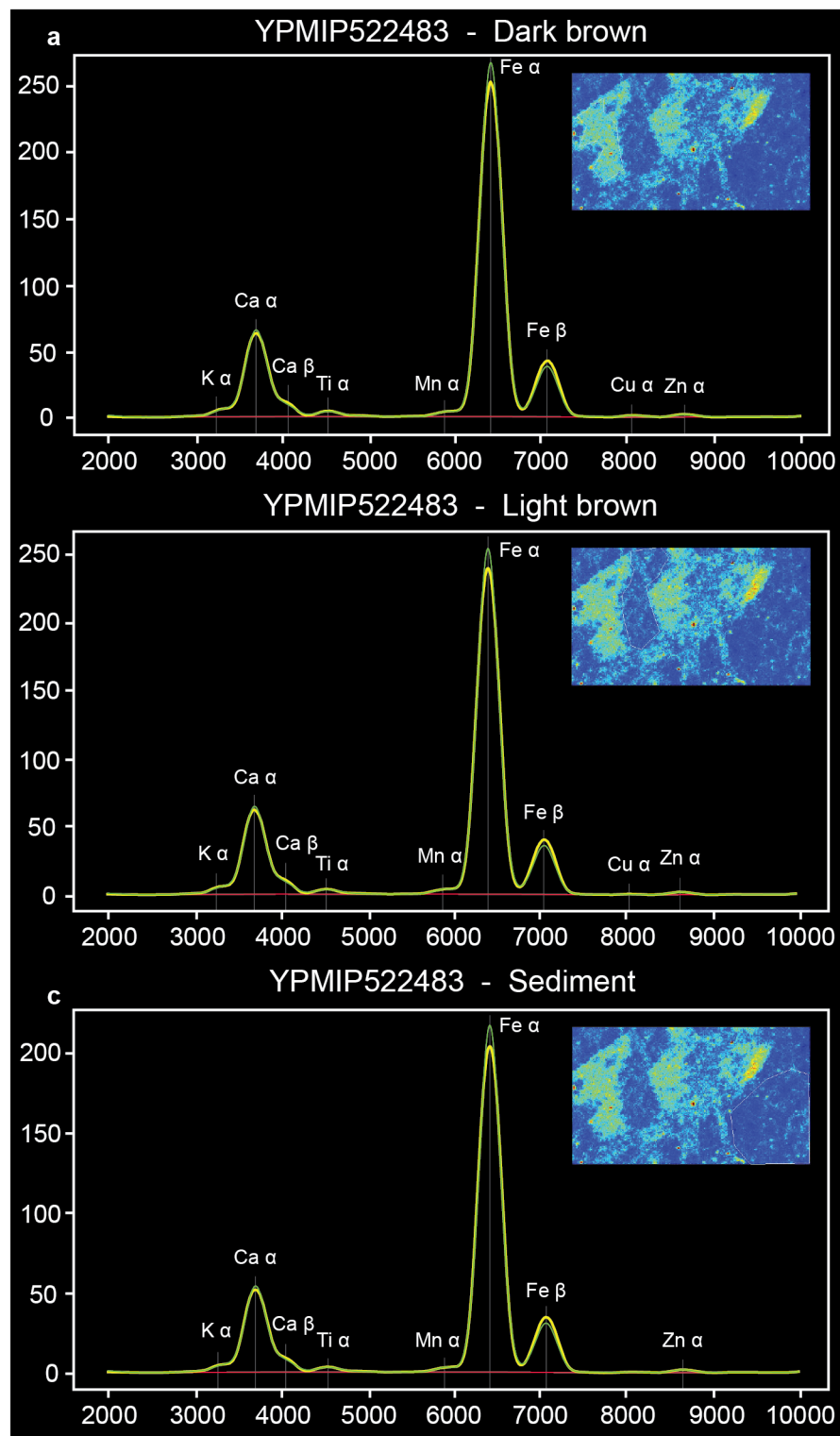
**Supplementary Figure 5.39 (previous page). Multi channel analyser spectra for ROIs from fossil Hemiptera from Rubielos de Mora (RR1-12-07-10),** representing regions displaying dark brown (**a**) and light brown (**b**) visual colours and sediment with no cuticle preserved (**c**). X-axis represents X-ray emission energy (eV); Y-axis represents counts. Yellow line characterises the SRS-XRF spectrum analyzed, green line the fit and red line the continuum. Plotted using SMAK 1.67 <https://www.sams-xrays.com/smak/>.



Supplementary Figure 5.40. Caption overleaf.

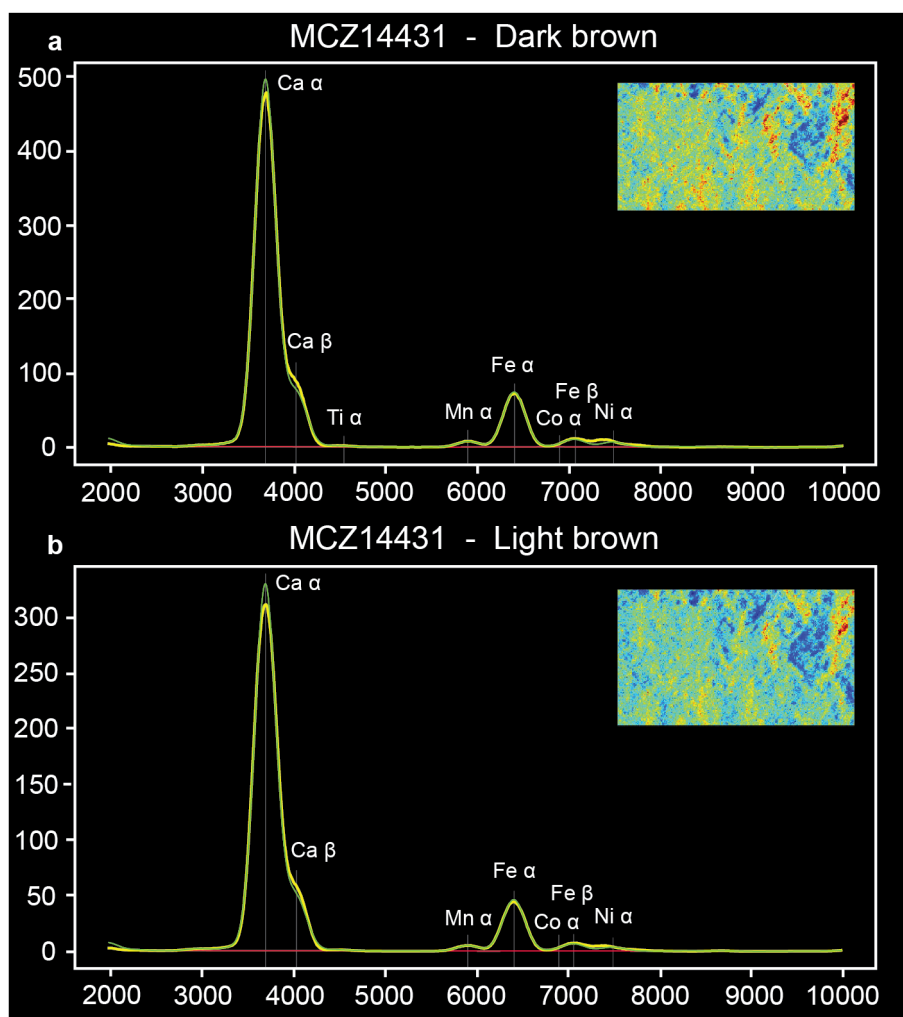


**Supplementary Figure 5.40 (previous page). Multi channel analyser spectra for ROIs from fossil Hemiptera from Rubielos de Mora (RR1-12-07-44),** representing regions displaying dark brown (**a**) and light brown (**b**) visual colours and sediment with no cuticle preserved (**c**). X-axis represents X-ray emission energy (eV); Y-axis represents counts. Yellow line characterises the SRS-XRF spectrum analyzed, green line the fit and red line the continuum. Plotted using SMAK 1.67 <https://www.sams-xrays.com/smak/>.

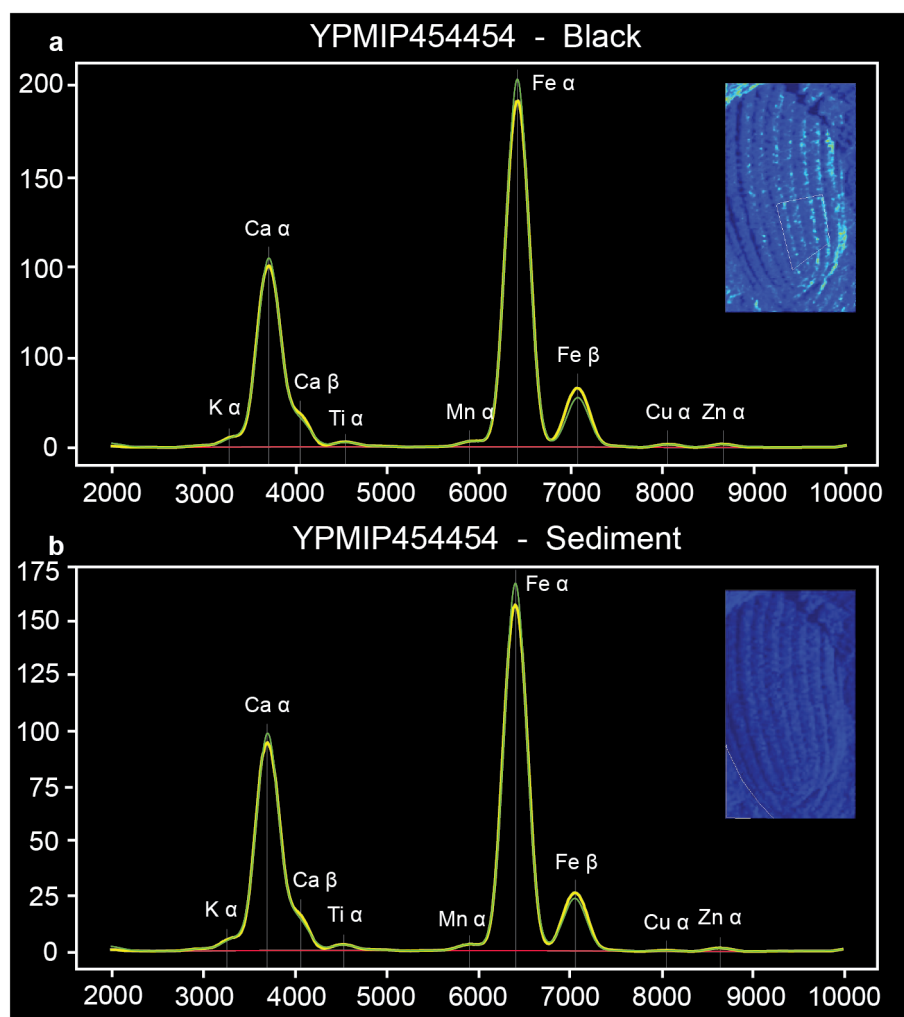


Supplementary Figure 5.41. Caption overleaf.

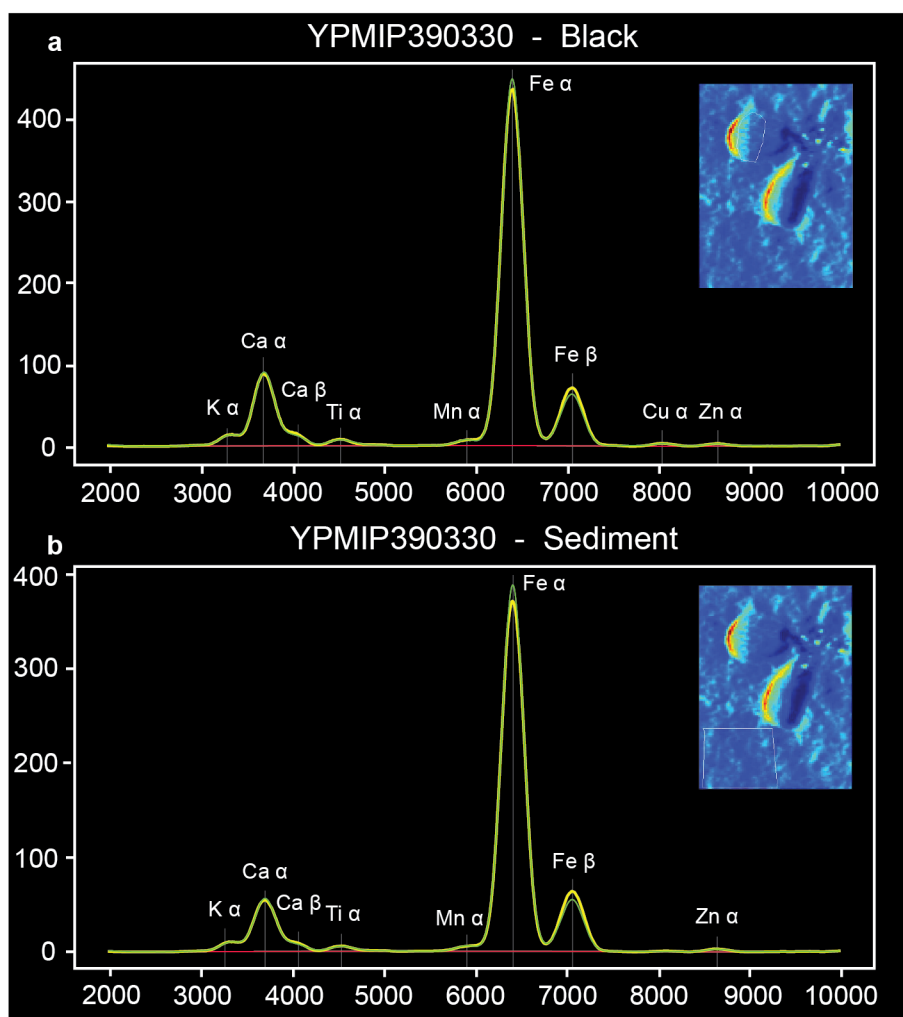
**Supplementary Figure 5.41 (previous page). Multi channel analyser spectra for ROIs from fossil Hemiptera from Green River (YPMIP522483),** representing regions displaying dark brown (**a**) and light brown (**b**) visual colours and sediment with no cuticle preserved (**c**). X-axis represents X-ray emission energy (eV); Y-axis represents counts. Yellow line characterises the SRS-XRF spectrum analyzed, green line the fit and red line the continuum. Plotted using SMAK 1.67 <https://www.sams-xrays.com/smak/>.



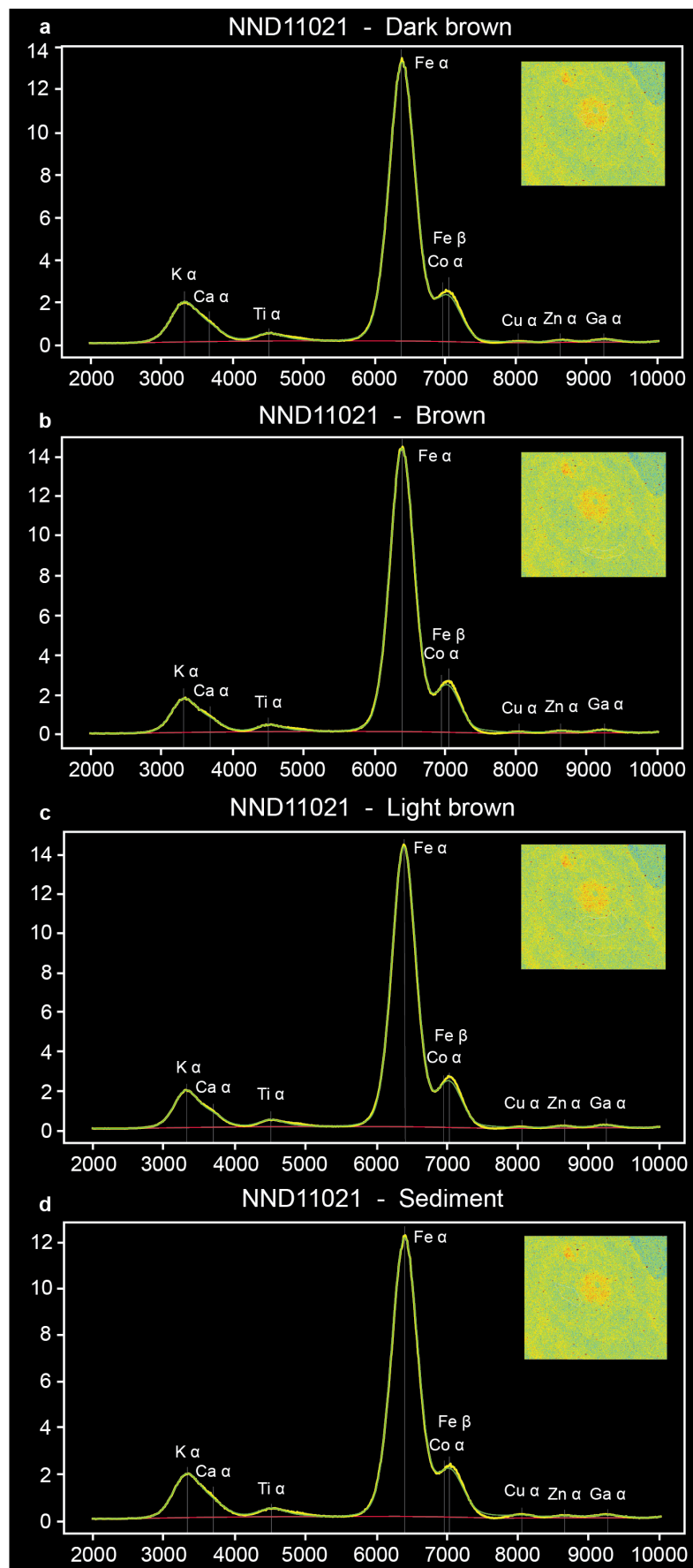
**Supplementary Figure 5.42. Multi channel analyser spectra for ROIs from fossil Hemiptera from Oeningen (MCZ14431),** representing regions displaying dark brown (**a**) and light brown (**b**) visual colours. X-axis represents X-ray emission energy (eV); Y-axis represents counts. Yellow line characterises the SRS-XRF spectrum analyzed, green line the fit and red line the continuum. Plotted using SMAK 1.67 <https://www.sams-xrays.com/smak/>.



**Supplementary Figure 5.43. Multi channel analyser spectra for ROIs from fossil Coleoptera from Green River (YPMIP454454),** representing regions displaying black visual colour (**a**) and sediment with no cuticle preserved (**b**). X-axis represents X-ray emission energy (eV); Y-axis represents counts. Yellow line characterises the SRS-XRF spectrum analyzed, green line the fit and red line the continuum. Plotted using SMAK 1.67 <https://www.sams-xrays.com/smak/>.



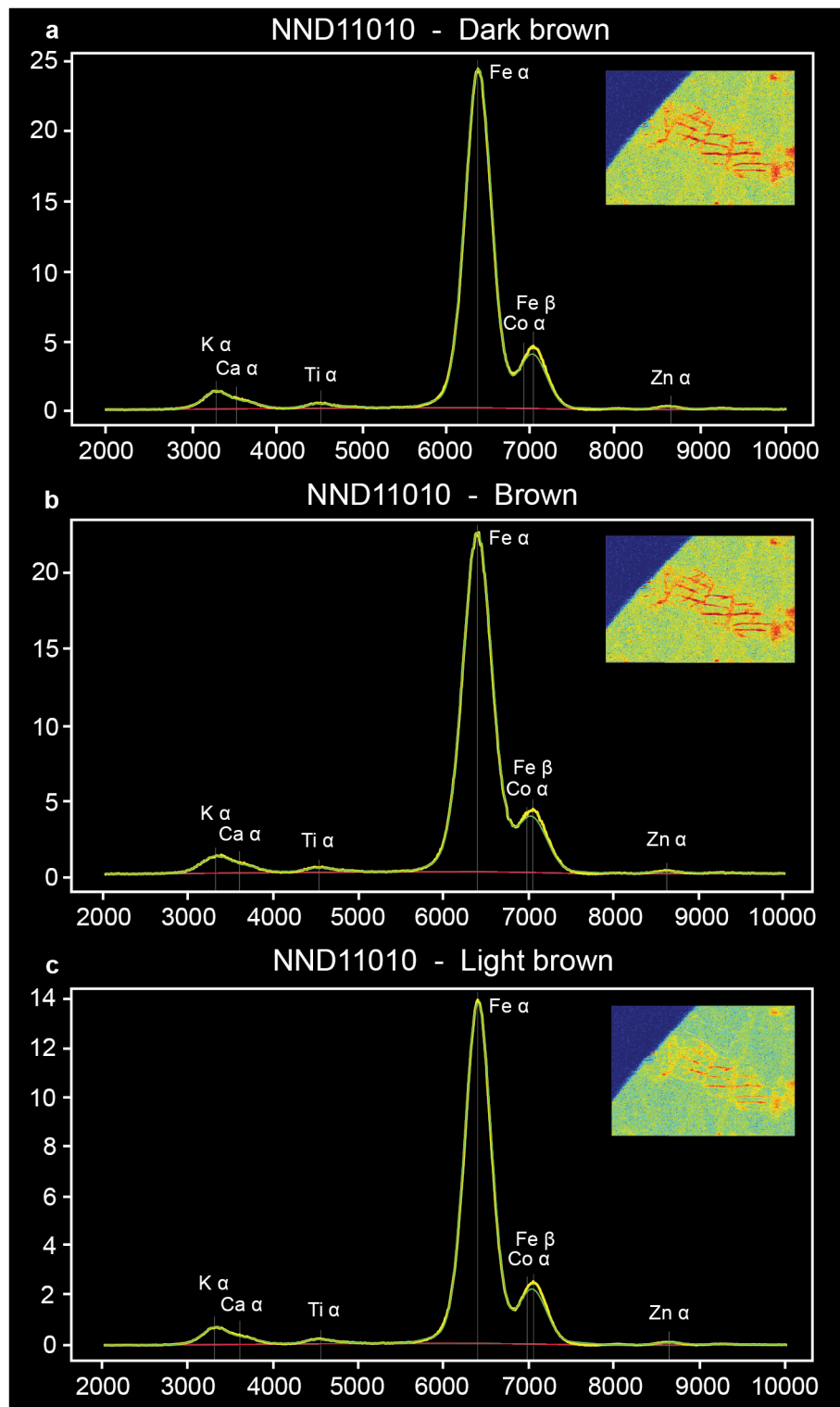
**Supplementary Figure 5.44. Multi channel analyser spectra for ROIs from fossil Coleoptera from Green River (YPMIP390330),** representing regions displaying black visual colour (**a**) and sediment with no cuticle preserved (**b**). X-axis represents X-ray emission energy (eV); Y-axis represents counts. Yellow line characterises the SRS-XRF spectrum analyzed, green line the fit and red line the continuum. Plotted using SMAK 1.67 <https://www.sams-xrays.com/smak/>.



Supplementary Figure 5.45. Caption overleaf.

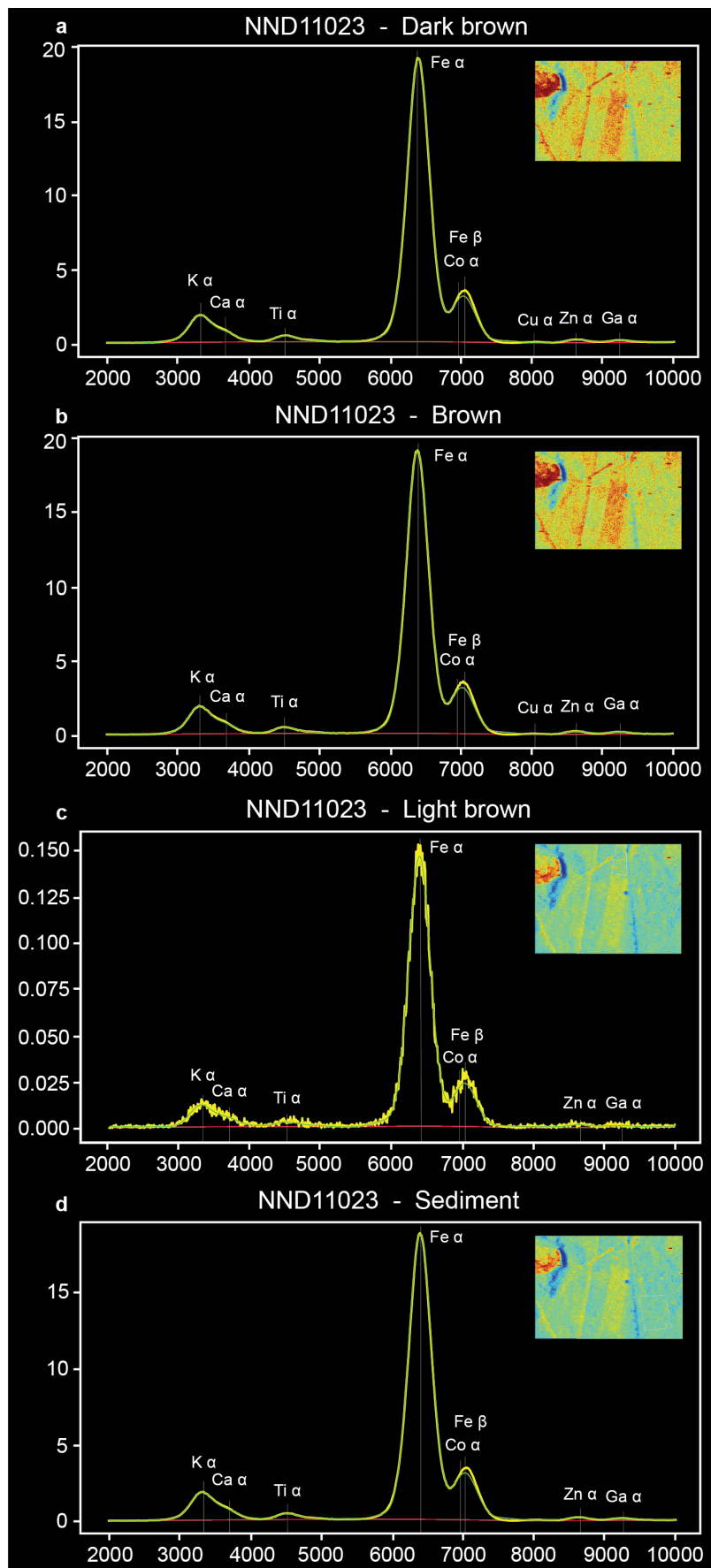
**Supplementary Figure 5.45 (previous page). Multi channel analyser spectra for ROIs from fossil Neuroptera from Daohugou (NIGPAS NND11021),** representing regions displaying dark brown (**a**), brown (**b**) and light brown (**c**) visual colours and sediment with no cuticle preserved (**d**). X-axis represents X-ray emission energy (eV); Y-axis represents counts. Yellow line characterises the SRS-XRF spectrum analyzed, green line the fit and red line the continuum. Plotted using SMAK 1.67 <https://www.sams-xrays.com/smak/>.





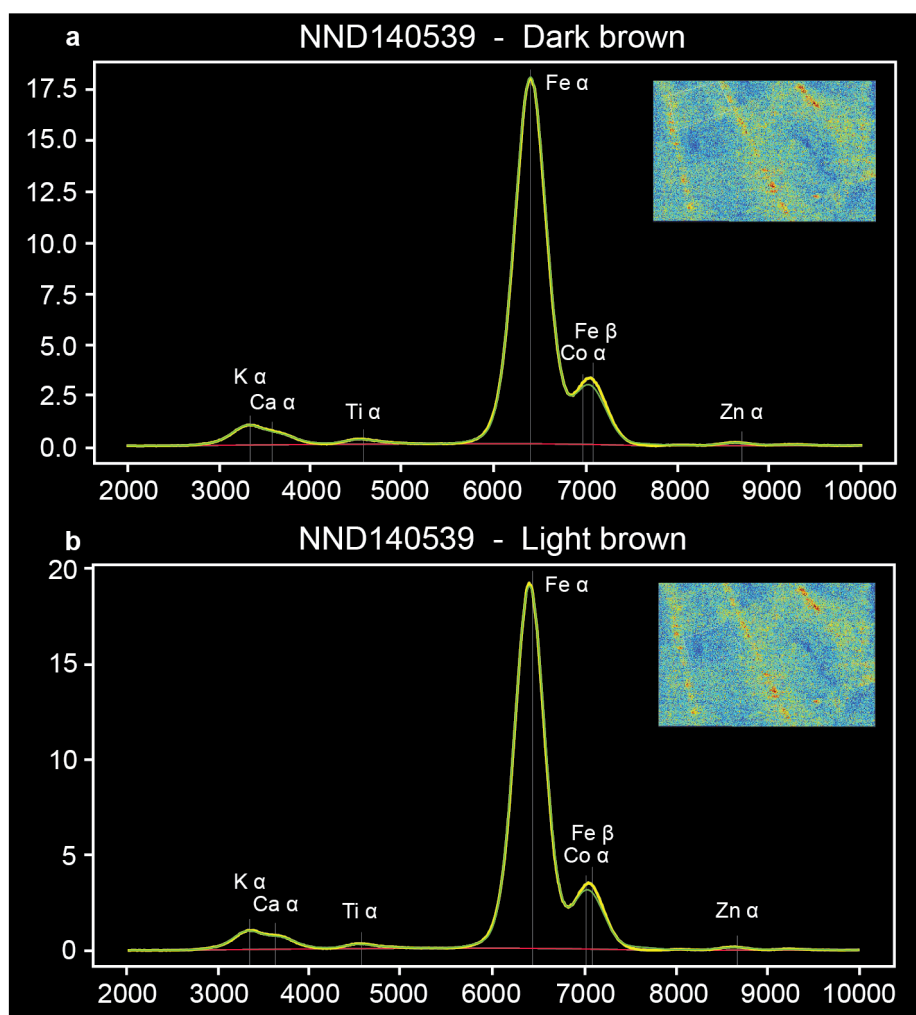
Supplementary Figure 5.46. Caption overleaf.

**Supplementary Figure 5.46 (previous page). Multi channel analyser spectra for ROIs from fossil Neuroptera from Daohugou (NIGPAS NND11010),** representing regions displaying dark brown (**a**), brown (**b**) and light brown (**c**) visual colours and sediment with no cuticle preserved (**d**). X-axis represents X-ray emission energy (eV); Y-axis represents counts. Yellow line characterises the SRS-XRF spectrum analyzed, green line the fit and red line the continuum. Plotted using SMAK 1.67 <https://www.sams-xrays.com/smak/>.

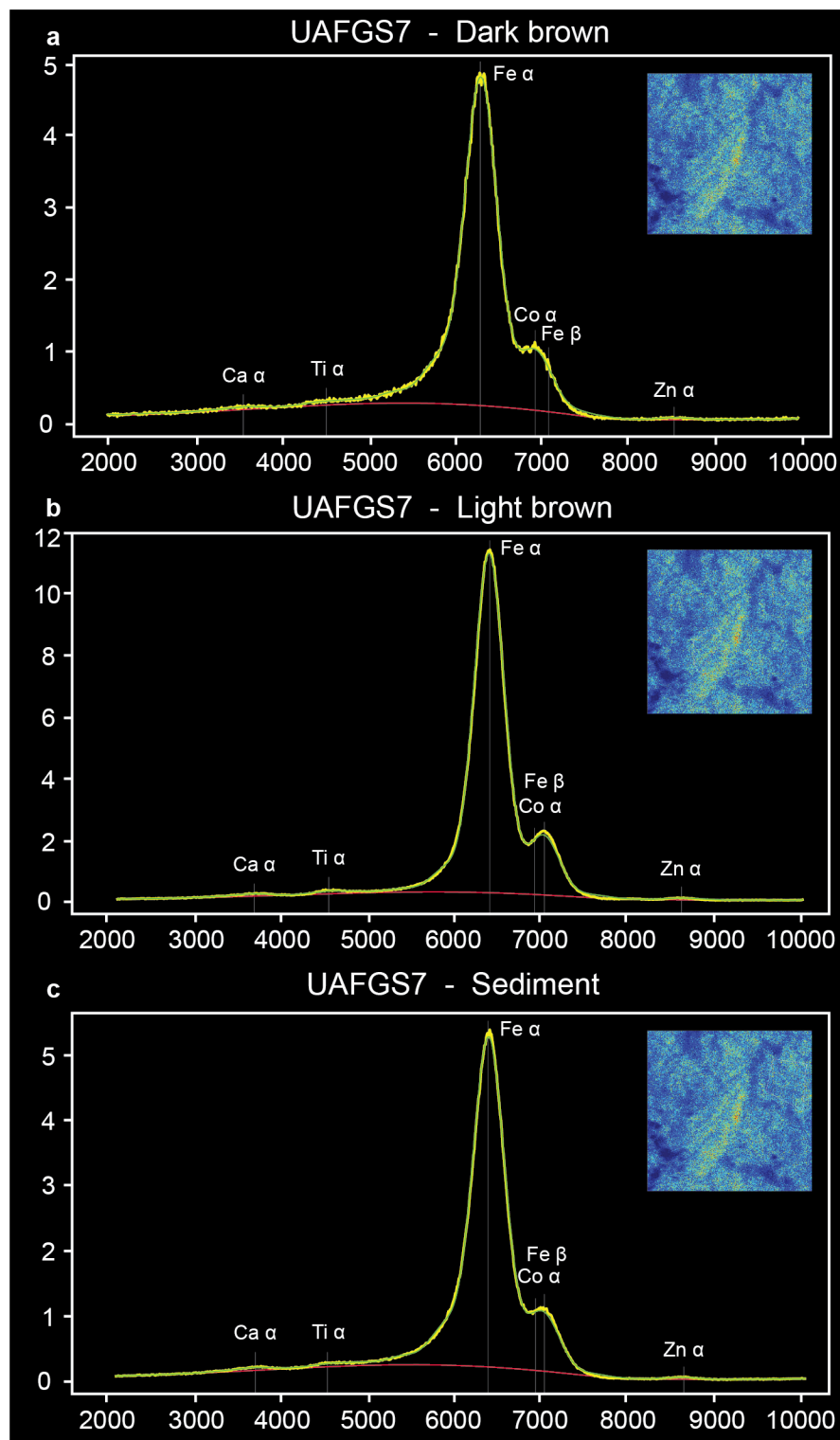


Supplementary Figure 5.47. Caption overleaf.

**Supplementary Figure 5.47 (previous page). Multi channel analyser spectra for ROIs from fossil Neuroptera from Daohugou (NIGPAS NND11023),** representing regions displaying dark brown (**a**), brown (**b**) and light brown (**c**) visual colours and sediment with no cuticle preserved (**d**). X-axis represents X-ray emission energy (eV); Y-axis represents counts. Yellow line characterises the SRS-XRF spectrum analyzed, green line the fit and red line the continuum. Plotted using SMAK 1.67 <https://www.sams-xrays.com/smak/>.



**Supplementary Figure 5.48. Multi channel analyser spectra for ROIs from fossil Neuroptera from Daohugou (NIGPAS NND140539),** representing regions displaying dark brown (**a**) and light brown (**b**) visual colours. X-axis represents X-ray emission energy (eV); Y-axis represents counts. Yellow line characterises the SRS-XRF spectrum analyzed, green line the fit and red line the continuum. Plotted using SMAK 1.67 <https://www.sams-xrays.com/smak/>.



Supplementary Figure 5.49. Caption overleaf.

**Supplementary Figure 5.49 (previous page). Multi channel analyser spectra for ROIs from fossil Hymenoptera from Grubstake (UAF\_GS7),** representing regions displaying dark brown (**a**) and light brown (**b**) visual colours and sediment with no cuticle preserved (**d**). X-axis represents X-ray emission energy (eV); Y-axis represents counts. Yellow line characterises the SRS-XRF spectrum analyzed, green line the fit and red line the continuum. Plotted using SMAK 1.67 <https://www.sams-xrays.com/smak/>.

## **A5.2. Supplementary Tables.**



| taxon                           | source                             | # specimens | # maps | map location                                           | total # ROIs                                                                                                                                                                                                                                                                                                                                                                                                       | RGB values                                                                                                                                                                                        | pixel size (μ)                          | dwelt time of XRF maps (ms) | known coloration mechanisms                                                                                                                                                                                                                                                                                                                                                                                                                                            |
|---------------------------------|------------------------------------|-------------|--------|--------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------|-----------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Lepidoptera                     |                                    |             |        |                                                        |                                                                                                                                                                                                                                                                                                                                                                                                                    |                                                                                                                                                                                                   |                                         |                             |                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |
| Sphingidae                      |                                    |             |        |                                                        |                                                                                                                                                                                                                                                                                                                                                                                                                    |                                                                                                                                                                                                   |                                         |                             |                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |
| <i>Macroglossum stellatarum</i> | OUMNH                              | 1           | 2      | Dorsal fore- and hindwing.                             | 6 (map 1); Brown 1 (77 52 130; Brown 2 (87 62 20); Brown 3 (96 67 26); 6 (map 2) Brown 4 (154 115 57); Brown 5 (130 93 43); Brown 6 (156 119 60); Yellow 1 (250 176 13); Yellow 2 (254 199 50); Yellow 3 (255 187 19); Orange 1 (166 80 12); Orange 2 (159 65 6); Orange 3 (178 81 10)                                                                                                                             |                                                                                                                                                                                                   | 0.1 x 0.1                               | 50                          | Carotenoids: Zeaxanthin, lutein, adonirubin, astaxanthin and lutein epoxide in yellow and orange regions (Czeczuga 1986). Flavonoids: Anthoxanthins in yellow regions: Anthoxanthins (Ford 1944).                                                                                                                                                                                                                                                                      |
| Pieridae                        |                                    |             |        |                                                        |                                                                                                                                                                                                                                                                                                                                                                                                                    |                                                                                                                                                                                                   |                                         |                             |                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |
| <i>Anthocharis cardamines</i>   | OUMNH                              | 1           | 2      | Dorsal fore- and hindwing. Ventral fore- and hindwing. | 6 (map 1); Dorsal: Black 1 (88 71 37); Black 2 (111 87 36); Orange 1 (255 169 36); Orange 2 (255 165 36); White 1 (241 244 187); 6 (map 2) White 2 (236 239 188); Ventral: Black 1 (87 64 19); Orange 1 (255 181 16); Orange 2 (255 175 16); Orange 3 (255 172 16); White 1 (242 244 182); White 2 (243 245 189)                                                                                                   |                                                                                                                                                                                                   | 0.1 x 0.1<br>ventral 0.15 x 0.15 dorsal | 50                          | Melanins in brown regions (Stavenga <i>et al.</i> 2004). Pterins: Ixanthopterin in wings, no colour specific (Rawson 1968). Erythropterin in orange regions (Shamin <i>et al.</i> 2014) and leucopterin/possible isoxanthopterin in yellow regions (Wijnen <i>et al.</i> 2007). Flavonoids: Anthoxanthins at family level (Ford 1944).                                                                                                                                 |
| <i>Pieris rapae</i>             | OUMNH                              | 1           | 1      | Dorsal forewing.                                       | 6                                                                                                                                                                                                                                                                                                                                                                                                                  | Black 1 (67 60 12); Black 2 (75 67 12); Black 3 (80 70 11); Yellow 1 (238 253 251); Yellow 2 (235 251 175); Yellow 3 (230 248 170)                                                                | 0.1 x 0.1                               | 50                          | Melanins in brown regions (Pfeiler 1968; Stavenga <i>et al.</i> 2006; Wijnen <i>et al.</i> 2007). Pterins: Ixanthopterin in wings, no colour specific (Rawson 1968). Leucopterin/xanthopterin in yellow regions (Stavenga <i>et al.</i> 2006; Wijnen <i>et al.</i> 2007). Carotenoids in orange regions: Torulene, β-cryptoxanthin, canthaxanthin, lutein epoxide, neoxanthin and mutatochrome (Czeczuga 1986). Flavonoids: Anthoxanthins at family level (Ford 1944). |
| <i>Colotis regina</i>           | OUMNH                              | 1           | 1      | Dorsal forewing.                                       | 6                                                                                                                                                                                                                                                                                                                                                                                                                  | White 1 (219 221 206); White 2 (231 232 211); White 3 (223 224 207); Purple 1 (127 82 93); Purple 2 (112 52 67); Purple 3 (127 90 99)                                                             | 0.03 x 0.03                             | 50                          | Pterins: Leucopterin/erythropterin in purple regions (Wijnen <i>et al.</i> 2007). Structural (Silberglied & Taylor 1973; Kemp <i>et al.</i> 2006). Flavonoids: Anthoxanthins at family level (Ford 1944).                                                                                                                                                                                                                                                              |
| Geometridae                     |                                    |             |        |                                                        |                                                                                                                                                                                                                                                                                                                                                                                                                    |                                                                                                                                                                                                   |                                         |                             |                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |
| <i>Abbraxas grossulariata</i>   | OUMNH                              | 1           | 1      | Dorsal fore- and hindwing.                             | 8                                                                                                                                                                                                                                                                                                                                                                                                                  | Brown 1 (123 101 51); Brown 2 (102 81 35); Brown 3 (141 122 70); Yellow 1 (223 187 51); Yellow 2 (239 206 51); White 1 (236 240 191); White 2 (241 246 200); White 3 (232 237 196)                | 0.1 x 0.1                               | 50                          | Melanins in black regions (Onslow 1919). Carotenoids in yellow regions: Lycopene, β-cryptoxanthin, zeaxanthin, canthaxanthin, capsanthone, lutein epoxide, neoxanthin, mutatochrome and mutatoxanthin (Czeczuga 1986).                                                                                                                                                                                                                                                 |
| Nymphalidae                     |                                    |             |        |                                                        |                                                                                                                                                                                                                                                                                                                                                                                                                    |                                                                                                                                                                                                   |                                         |                             |                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |
| <i>Bicyclus anynana</i>         | Collected by authors of this study | 1           | 1      | Dorsal forewing.                                       | 9                                                                                                                                                                                                                                                                                                                                                                                                                  | Brown 1 (74 52 1); Brown 2 (75 53 3); Brown 3 (187 170 75); Brown 4 (172 151 57); Brown 5 (156 133 38); Brown 6 (140 115 17); Yellow 1 (195 165 14); Yellow 2 (201 171 20); White 1 (233 250 224) | 0.1 x 0.1                               | 50                          | Ommochromes: Xanthommatin in brown regions and 3-OHK in yellow regions (Stavenga <i>et al.</i> 2014).                                                                                                                                                                                                                                                                                                                                                                  |
| <i>Melanargia galathea</i>      | OUMNH                              | 1           | 1      | Dorsal hindwing.                                       | 6                                                                                                                                                                                                                                                                                                                                                                                                                  | Brown 1 (63 41 9); Brown 2 (48 30 4); Brown 3 (54 38 11); Yellow 1 (250 238 148); Yellow 2 (225 217 144); Yellow 3 (223 217 150)                                                                  | 0.1 x 0.1                               | 50                          | Ommochromes: Xanthommatin in brown regions (Stavenga <i>et al.</i> 2014). Flavonoids: Anthoxanthin and quercitin in yellow and white regions(Ford 1944; Thomson 1926; Morris & Thomson 1963; Wilson 1985).                                                                                                                                                                                                                                                             |
| <i>Argynnis adippe</i>          | OUMNH                              | 1           | 1      | Dorsal forewing.                                       | 6                                                                                                                                                                                                                                                                                                                                                                                                                  | Black 1 (32 15 4); Black 2 (33 16 5); Black 3 (36 21 7); Orange 1 (214 137 1); Orange 2 (209 134 1); Orange 3 (220 144 3)                                                                         | 0.1 x 0.1                               | 50                          | Melanins in black regions (Stavenga <i>et al.</i> 2014). Ommochromes: Dihydroxanthommatin, rhodommatin and ommatin D in orange regions (Brown 1981; Stavenga <i>et al.</i> 2014). Carotenoids: Lycopene, α-carotene, β-carotene, β-cryptoxanthin, lutein, α-doradexanthin, adonirubin, astaxanthin, lutein epoxide, neoxanthin and flavochrome in orange regions (Czeczuga 1986).                                                                                      |
| <i>Aglais urticae</i>           | Collected by authors of this study | 1           | 2      | Dorsal forewing and hindwing.                          | 9 (map 1); Forewing: Black 1 (49 39 41); Black 2 (34 29 31); Brown 1 (94 80 73); Brown 2 (100 83 72); Orange 1 (228 103 35); 8 (map 2) Yellow 1 (240 189 105); White 1 (204 210 217); White 2 (204 204 202); Blue 1 (81 86 113); Hindwing: Black 1 (34 26 24); Black 2 (25 21 23); Brown 1 (60 41 33); Brown 2 (68 53 46); Orange 1 (201 97 36); Orange 2 (194 92 32); Yellow 1 (179 157 109); Blue 1 (90 111 155) |                                                                                                                                                                                                   | 0.025 x 0.025                           | 50                          | Melanins in black regions at genus level (Stavenga <i>et al.</i> 2014). Ommochromes: Xanthommatin, rhodommatin and ommatin D in orange regions (Brown 1981; Stavenga <i>et al.</i> 2014). Carotenoids: Lycopene, β-carotene, zeaxanthin, lutein, adonirubin, astaxanthin, lutein epoxide, neoxanthin and flavochrome in orange regions (Czeczuga 1986).                                                                                                                |

| taxon                         | source                             | # specimens | # maps | map location                         | total # ROIs            | RGB values                                                                                                                                                                                                                                                                                  | pixel size (μ)                             | dwelt time of XRF maps (ms) | known coloration mechanisms                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 |
|-------------------------------|------------------------------------|-------------|--------|--------------------------------------|-------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------|-----------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>Lycaenidae</b>             |                                    |             |        |                                      |                         |                                                                                                                                                                                                                                                                                             |                                            |                             |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |
| <i>Cupido comyntas</i>        | Collected by authors of this study | 1           | 1      | Ventral hindwing.                    | 5                       | Brown 1 (69 58 18); Orange 1 (206 162 24); Orange 2 (208 167 23); White 1 (201 220 176); White 2 (205 225 183)                                                                                                                                                                              | 0.05 x 0.05                                | 50                          | Flavonoids: Quercetin and kaempferol 3-glycoside in brown regions at genus level (Wilson 1987). Structural in white regions at genus level (Lobiuc & Lobiuc 2015). Carotenoids at family level: Torulene, β-cryptoxanthin, zeaxanthin, α-doradexanthin, adonirubin, astaxanthin, lutein epoxide, violaxanthin and aurochrome (Czeczuga 1986).                                                                                                                                                               |
| <b>Hesperiidae</b>            |                                    |             |        |                                      |                         |                                                                                                                                                                                                                                                                                             |                                            |                             |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |
| <i>Epargyreus clarus</i>      | Collected by authors of this study | 1           | 2      | Dorsal forewing.<br>Ventral hinwing. | 6 (map 1);<br>6 (map 2) | Forewing: Brown 1 (115 93 22); Brown 2 (118 88 14); Brown 3 (109 84 11); Yellow 1 (164 143 18); Yellow 2 (200 185 64); Yellow 3 (182 150 6); Hindwing: Brown 1 (111 86 34); Brown 2 (103 83 29); Brown 3 (80 65 30); Silver 1 (198 213 177); Silver 2 (205 205 222); Silver 3 (199 215 178) | 0.1 x 0.1<br>forewing 0.1<br>x 0.1 hinwing | 50                          | Flavonoids at family level: Anthoxanthins in brown regions (Ford 1944).                                                                                                                                                                                                                                                                                                                                                                                                                                     |
| <b>Papilionidae</b>           |                                    |             |        |                                      |                         |                                                                                                                                                                                                                                                                                             |                                            |                             |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |
| <i>Parnassius apollonius</i>  | OUMNH                              | 1           | 1      | Dorsal hindwing.                     | 8                       | Black 1 (23 17 12); Black 2 (21 18 14); Black 3 (21 17 11); Red 1 (242 95 37); Red 2 (241 96 46); Yellow 1 (253 250 204); Yellow 2 (249 244 198); Yellow 3 (251 247 103)                                                                                                                    | 0.15 x 0.15                                | 50                          | Melanins in black regions (Koch <i>et al.</i> 2000). Possibly also melanins in red regions from pigment description in other members of papilionids (Nishikawa <i>et al.</i> 2013). Ommochromes: Papiliochrome II in yellow regions (Koch <i>et al.</i> 2000). Flavonoids: Anthoxanthins at family level (Ford 1944).                                                                                                                                                                                       |
| <i>Papilio glaucus</i>        | Collected by authors of this study | 1           | 1      | Dorsal hindwing.                     | 7                       | Black 1 (38 30 5); Black 2 (36 29 5); Orange 1 (201 131 0); Yellow 1 (225 209 59); Yellow 2 (221 206 41); Blue 1 (89 136 147); Blue 2 (102 139 147)                                                                                                                                         | 0.05 x 0.05                                | 50                          | Melanins in black regions (Koch <i>et al.</i> 2000) assisted by nanostructures (Vukusic <i>et al.</i> 2004). Possibly also melanins in orange regions at genus level (Nishikawa <i>et al.</i> 2013). Ommochromes: Papiliochrome II in yellow regions (Koch <i>et al.</i> 2000) and blue regions at genus level (Wilts <i>et al.</i> 2012). Structural in blue regions (Koch <i>et al.</i> 2000). Carotenoids at genus level: Lycopene, β-cryptoxanthin, zeaxanthin, lutein and astaxanthin (Czeczuga 1986). |
| <i>Papilio nireus</i>         | OUMNH                              | 1           | 1      | Dorsal forewing.                     | 6                       | Black 1 (41 30 17); Black 2 (41 30 19); Black 3 (42 29 18); Green 1 (173 199 127); Green 2 (180 206 128); Green 3 (184 207 122)                                                                                                                                                             | 0.05 x 0.05                                | 50                          | Melanins in black regions (Koch <i>et al.</i> 2000) assisted by nanostructures (Vukusic <i>et al.</i> 2004). Ommochromes: Papiliochrome II in green/blue regions (Wilts <i>et al.</i> 2012). Carotenoids at genus level: Lycopene, β-cryptoxanthin, zeaxanthin, lutein and astaxanthin (Czeczuga 1986).                                                                                                                                                                                                     |
| <b>Zygaenidae</b>             |                                    |             |        |                                      |                         |                                                                                                                                                                                                                                                                                             |                                            |                             |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |
| <i>Zygaena filipendulae</i>   | OUMNH                              | 1           | 1      | Dorsal fore- and hindwing.           | 6                       | Brown 1 (118 90 8); Brown 2 (104 85 15); Brown 3 (117 97 12); Red 1 (165 13 4); Red 2 (165 16 4); Red 3 (173 17 3)                                                                                                                                                                          | 0.08 x 0.08                                | 50                          | Carotenoids: γ-carotene, rubixanthin, zeaxanthin, lutein, astaxanthin and mutatochrome in red regions (Czeczuga 1986).                                                                                                                                                                                                                                                                                                                                                                                      |
| <i>Erasmia pulchella</i>      | OUMNH                              | 1           | 1      | Dorsal forewing.                     | 11                      | Brown 1 (16 11 7); Brown 2 (18 13 8); Brown 3 (15 11 8); White 1 (164 146 68); White 2 (166 143 60); Orange 1 (128 47 4); Orange 2 (126 47 3); Orange 3 (130 43 4); Blue 1 (47 133 110); Blue 2 (46 132 114); Blue 3 (54 80 56)                                                             | 0.05 x 0.05                                | 50                          | Flavonoids: Anthoxanthins at family level (Ford 1944).                                                                                                                                                                                                                                                                                                                                                                                                                                                      |
| <i>Chalcosia coliadoideas</i> | OUMNH                              | 1           | 1      | Dorsal forewing.                     | 8                       | Brown 1 (46 30 13); Brown 2 (68 55 35); Brown 3 (55 51 32); White 1 (255 221 113); White 2 (254 222 116); White 3 (255 241 147); Blue 1 (61 97 84); Blue 2 (54 92 80)                                                                                                                       | 0.05 x 0.05                                | 30                          | Flavonoids: Anthoxanthins in brown regions (Ford 1944).                                                                                                                                                                                                                                                                                                                                                                                                                                                     |

| taxon                           | source     | # specimens | # maps | map location             | total # ROIs   | RGB values                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              | pixel size (μ)                 | dwelt time of XRF maps (ms) | known coloration mechanisms                                                                                                                                                                                                                                        |
|---------------------------------|------------|-------------|--------|--------------------------|----------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------|-----------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>Coleoptera</b>               |            |             |        |                          |                |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |                                |                             |                                                                                                                                                                                                                                                                    |
| <b>Scarabaeidae</b>             |            |             |        |                          |                |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |                                |                             |                                                                                                                                                                                                                                                                    |
| <i>Goliathus goliathus</i>      | Commercial | 1           | 1      | Right elytrum, centre.   | 6              | Brown 1 (18 17 18); Brown 2 (17 17 18); Brown 3 (17 17 18); White 1 (168 184 110); White 2 (131 154 118); White 3 (154 176 123)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         | 0.03 x 0.03                    | 50                          | Melanins in dark regions at family level (Hackman 1953; Kuusijärvi <i>et al.</i> 2015). Wax coloration in grey regions (Hagen 1872). Ultraviolet reflective pigment in white regions (Pope & Hinton 1977). Structural in white regions (Jiang <i>et al.</i> 2012). |
| <i>Aphodius sp.</i>             | OUMNH      | 1           | 1      | Right elytrum, anterior. | 4              | Brown 1 (45 37 5); Brown 2 (48 40 4); Yellow 1 (126 111 0); Yellow 2 (93 77 0)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          | 0.03 x 0.03                    | 50                          | Melanins in dark regions at genus level (Hackman 1953; Kuusijärvi <i>et al.</i> 2015).                                                                                                                                                                             |
| <i>Aphodius sticticus</i>       | OUMNH      | 1           | 1      | Right elytrum, anterior. | 4              | Black 1 (70 53 9); Black 2 (53 53 14); Yellow 1 (102 78 0); Yellow 2 (101 77 0)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         | 0.03 x 0.03                    | 50                          | Melanins in dark regions at genus level (Hackman 1953; Kuusijärvi <i>et al.</i> 2015).                                                                                                                                                                             |
| <i>Aphodius luridus</i>         | OUMNH      | 1           | 1      | Left elytrum, anterior.  | 4              | Black 1 (26 27 25); Black 2 (30 27 21); Yellow 1 (89 78 13); Yellow 2 (101 90 13)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | 0.03 x 0.03                    | 50                          | Melanins in dark regions at genus level (Hackman 1953; Kuusijärvi <i>et al.</i> 2015).                                                                                                                                                                             |
| <i>Trichius sp.</i>             | OUMNH      | 1           | 1      | Left elytrum, centre.    | 6              | Black 1 (12 12 15); Black 2 (19 13 13); Black 3 (19 20 25); Yellow 1 (88 61 0); Yellow 2 (95 67 0); Yellow 3 (104 80 0)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 | 0.025 x 0.025                  | 50                          | Melanins in dark regions at genus level (Hackman 1953; Kuusijärvi <i>et al.</i> 2015).                                                                                                                                                                             |
| <i>Polyphylla fullo</i>         | OUMNH      | 1           | 1      | Left elytrum, centre.    | 6              | Brown 1 (105 51 25); Brown 2 (108 60 30); Brown 3 (108 59 31); Yellow 1 (240 206 119); Yellow 2 (220 181 101); Yellow 3 (201 156 89)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    | 0.025 x 0.025                  | 50                          | Melanins in dark regions at genus level (Hackman 1953; Kuusijärvi <i>et al.</i> 2015).                                                                                                                                                                             |
| <b>Coccinellidae</b>            |            |             |        |                          |                |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |                                |                             |                                                                                                                                                                                                                                                                    |
| <i>Harmonia axyridis</i>        | Commercial | 23          | 23     | Right and left elytra.   | 2 per map = 46 | Black 1 (12 13 16); Black 2 (9 12 11); Black 3 (9 11 13); Black 4 (9 9 9); Black 5 (11 10 8); Black 6 (5 6 7); Black 7 (6 8 8); Black 8 (9 10 10); Black 9 (6 7 8); Black 10 (9 10 11); Black 11 (10 11 12); Black 12 (15 17 24); Black 13 (29 31 33); Black 14 (9 8 7); Black 15 (10 12 12); Black 16 (8 8 8); Black 17 (9 11 12); Black 18 (13 12 11); Black 19 (10 11 12); Black 20 (12 12 11); Black 21 (8 8 8); Black 22 (6 8 7); Black 23 (16 11 5); Red 1 (183 147 1); Red 2 (175 140 1); Red 3 (197 146 0); Red 4 (141 72 0); Red 5 (131 73 0); Red 6 (149 90 0); Red 7 (152 95 0); Red 8 (141 98 0); Red 9 (161 116 0); Red 10 (128 88 0); Red 11 (150 104 0); Red 12 (145 123 0); Red 13 (135 101 19); Red 14 (126 94 0); Red 15 (149 109 7); Red 16 (161 125 3); Red 17 (153 113 13); Red 18 (165 119 0); Red 19 (147 103 18); Red 20 (137 87 0); Red 21 (131 94 5); Red 22 (141 127 0); Red 23 (149 137 20) | 0.03 x 0.03 for all 27 samples | 50                          | Melanins in black regions (Bezzerdies <i>et al.</i> 2007). Carotenoids in red regions (Bezzerdies <i>et al.</i> 2007).                                                                                                                                             |
| <i>Epilachna argus</i>          | OUMNH      | 1           | 1      | Right elytrum, centre.   | 6              | Black 1 (17 16 18); Black 2 (22 20 23); Black 3 (19 18 19); Orange 1 (81 50 6); Orange 2 (90 60 7); Orange 3 (80 48 6)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  | 0.05 x 0.05                    | 50                          | Melanins in black regions at family level (Bezzerdies <i>et al.</i> 2007). Carotenoids in red regions at family level (Bezzerdies <i>et al.</i> 2007).                                                                                                             |
| <i>Hippodamia variegata</i>     | OUMNH      | 1           | 1      | Right elytrum, centre.   | 2              | Black 1 (27 25 27); Red 1 (82 55 5)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     | 0.025 x 0.025                  | 50                          | Melanins in black regions at family level (Bezzerdies <i>et al.</i> 2007). Carotenoids in red regions at family level (Bezzerdies <i>et al.</i> 2007).                                                                                                             |
| <b>Cerambycidae</b>             |            |             |        |                          |                |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |                                |                             |                                                                                                                                                                                                                                                                    |
| <i>Brachyta interrogationis</i> | NHMO       | 1           | 1      | Right elytrum, centre.   | 6              | Black 1 (32 23 12); Black 2 (31 21 11); Black 3 (31 21 11); Yellow 1 (144 111 0); Yellow 2 (147 117 0); Yellow 3 (142 112 0)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            | 0.025 x 0.025                  | 50                          | Carotenoids at family level (Czeczuga 1988).                                                                                                                                                                                                                       |
| <i>Saperda populnea</i>         | OUMNH      | 1           | 1      | Right elytrum, centre.   | 6              | Green 1 (12 14 18); Green 2 (14 15 18); Green 3 (8 10 15); Green 4 (81 75 18); Green 5 (82 74 11); Green 6 (81 76 21)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   | 0.025 x 0.025                  | 50                          | Carotenoids at family level (Czeczuga 1988).                                                                                                                                                                                                                       |
| <i>Sternotomis bohemani</i>     | OUMNH      | 1           | 1      | Right elytrum, centre.   | 6              | Green 1 (34 55 49); Green 2 (33 57 49); Green 3 (36 58 52); Yellow 1 (156 130 9); Yellow 2 (166 150 27); Yellow 3 (159 132 8)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           | 0.025 x 0.025                  | 50                          | Carotenoids at family level (Czeczuga 1988).                                                                                                                                                                                                                       |
| Cerambycidae indet.             | OUMNH      | 1           | 1      | Right elytrum, centre.   | 6              | Black 1 (26 18 12); Black 2 (25 17 10); Black 3 (33 22 16); Orange 1 (154 58 8); Orange 2 (159 56 6); Orange 3 (165 61 8)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               | 0.025 x 0.025                  | 50                          | Carotenoids at family level (Czeczuga 1988).                                                                                                                                                                                                                       |

| taxon                               | source | # specimens | # maps | map location              | total # ROIs         | RGB values                                                                                                                                                                                                                                                             | pixel size (μ)                               | dwel time of XRF maps (ms) | known coloration mechanisms                                                                                                                                                                                                              |
|-------------------------------------|--------|-------------|--------|---------------------------|----------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------|----------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>Chrysomelidae</b>                |        |             |        |                           |                      |                                                                                                                                                                                                                                                                        |                                              |                            |                                                                                                                                                                                                                                          |
| <i>Leptinotarsa decemlineata</i>    | YPMNH  | 1           | 1      | Right elytrum, centre.    | 6                    | Black 1 (22 23 22); Black 2 (16 17 18); Black 3 (17 17 17); Yellow 1 (122 115 6); Yellow 2 (118 110 5); Yellow 3 (120 110 5)                                                                                                                                           | 0.03 x 0.03                                  | 50                         | Melanins in black regions (Gortner 1911). Carotenoids: Xanthophylls (Gortner 1911; Czezuga 1971, 80, 81; Jitaru & Petcu 1972, 74; Jitaru <i>et al.</i> 73) and keto-carotenes in yellow regions (Leuenberger & Thommen 1970; Poff 1976). |
| <i>Gonioctena decemnotata</i>       | NHMO   | 1           | 1      | Right elytrum, centre.    | 4                    | Black 1 (47 48 46); Black 2 (68 56 39); Yellow 1 (163 168 35); Yellow 2 (168 177 35)                                                                                                                                                                                   | 0.025 x 0.025                                | 50                         | Melanins at family level (Gortner 1911). Carotenoids at family level (Gortner 1911; Czezuga 1971, 80, 81; Jitaru & Petcu 1972, 74; Jitaru <i>et al.</i> 73; Leuenberger & Thommen 1970; Poff 1976).                                      |
| <i>Phyllobrotica quadrimaculata</i> | NHMO   | 1           | 1      | Right elytrum, anterior.  | 6                    | Black 1 (22 11 6); Black 2 (22 11 4); Black 3 (26 11 3); Yellow 1 (91 66 2); Yellow 2 (91 68 2); Yellow 3 (91 64 2)                                                                                                                                                    | 0.1 x 0.1                                    | 50                         | Melanins at family level (Gortner 1911). Carotenoids at family level (Gortner 1911; Czezuga 1971, 80, 81; Jitaru & Petcu 1972, 74; Jitaru <i>et al.</i> 73; Leuenberger & Thommen 1970; Poff 1976).                                      |
| <b>Silphidae</b>                    |        |             |        |                           |                      |                                                                                                                                                                                                                                                                        |                                              |                            |                                                                                                                                                                                                                                          |
| <i>Nicrophorus investigator</i>     | OUMNH  | 1           | 1      | Right elytrum, centre.    | 6                    | Black 1 (9 10 11); Black 2 (9 10 11); Black 3 (10 10 10); Brown 1 (99 46 10); Brown 2 (107 50 11); Brown 3 (97 47 13)                                                                                                                                                  | 0.03 x 0.03                                  | 50                         | AKH red pigment-concentrating hormone at family level (Gäde <i>et al.</i> 2015).                                                                                                                                                         |
| <b>Carabidae</b>                    |        |             |        |                           |                      |                                                                                                                                                                                                                                                                        |                                              |                            |                                                                                                                                                                                                                                          |
| <i>Cicindela hybrida</i>            | OUMNH  | 1           | 1      | Right elytrum, centre.    | 4                    | Black 1 (20 19 22); Black 2 (19 19 22); Yellow 1 (230 221 106); Yellow 2 (230 223 110)                                                                                                                                                                                 | 0.03 x 0.03                                  | 50                         | Melanins in dark regions (Schultz 1986).                                                                                                                                                                                                 |
| <b>Blattodea</b>                    |        |             |        |                           |                      |                                                                                                                                                                                                                                                                        |                                              |                            |                                                                                                                                                                                                                                          |
| <b>Corydiidae</b>                   |        |             |        |                           |                      |                                                                                                                                                                                                                                                                        |                                              |                            |                                                                                                                                                                                                                                          |
| <i>Therea petiveriana</i>           | OUMNH  | 1           | 1      | Right elytrum, centre.    | 6                    | Black 1 (31 36 42); Black 2 (33 36 41); Black 3 (30 34 40); Yellow 1 (61 60 48); Yellow 2 (84 79 53); Yellow 3 (102 94 56)                                                                                                                                             | 0.025 x 0.025                                | 50                         | AKH red pigment-concentrating hormone at order level (Gäde <i>et al.</i> 2015).                                                                                                                                                          |
| <b>Hemiptera</b>                    |        |             |        |                           |                      |                                                                                                                                                                                                                                                                        |                                              |                            |                                                                                                                                                                                                                                          |
| <b>Cercopidae</b>                   |        |             |        |                           |                      |                                                                                                                                                                                                                                                                        |                                              |                            |                                                                                                                                                                                                                                          |
| <i>Cercopis vulnerata</i>           | OUMNH  | 1           | 1      | Right elytrum, anterior.  | 6                    | Black 1 (33 35 42); Black 2 (36 36 43); Black 3 (35 36 42); Red 1 (121 52 18); Red 2 (128 53 16); Red 3 (121 54 15)                                                                                                                                                    | 0.03 x 0.03                                  | 50                         | Pterins: Isoxanthopterin, violapterin and erythropterin at family level (Merlini & Nasini 1966).                                                                                                                                         |
| <b>Pentatomidae</b>                 |        |             |        |                           |                      |                                                                                                                                                                                                                                                                        |                                              |                            |                                                                                                                                                                                                                                          |
| <i>Graphosoma lineatum</i>          | OUMNH  | 2           | 2      | Centre of elytrum.        | 6 (map 1); 6 (map 2) | Specimen 1: Black 1 (12 14 14); Black 2 (13 15 15); Black 3 (13 15 14); Red 1 (104 30 3); Red 2 (102 29 2); Red 3 (106 32 9); Specimen 2: Black 1 (27 30 29); Black 2 (29 32 30); Black 3 (28 31 30); Orange 1 (137 90 13); Orange 2 (149 91 14); Orange 3 (142 91 14) | 0.03 x 0.03 sample 1<br>0.03 x 0.03 sample 2 | 50                         | AKH red pigment concentrating hormone (Gäde <i>et al.</i> 2003). Pterins: Isoxanthopterin and erythropterin at genus level (Merlini & Nasini 1966), erythropterin at family level (Niva & Takeda 2002).                                  |
| <i>Aelia acuminata</i>              | OUMNH  | 1           | 1      | Centre of thorax.         | 6                    | Brown 1 (56 48 14); Brown 2 (52 41 8); Brown 3 (58 48 10); Yellow 1 (84 67 8); Yellow 2 (87 73 8); Yellow 3 (79 65 8)                                                                                                                                                  | 0.05 x 0.05                                  | 50                         | Pterins: Isoxanthopterin and erythropterin at family level (Merlini & Nasini 1966), erythropterin at family level (Niva & Takeda 2002).                                                                                                  |
| <i>Nezara robusta</i>               | OUMNH  | 1           | 1      | Right elytrum, centre.    | 6                    | Dark green 1 (56 75 7); Dark green 2 (56 72 7); Dark green 3 (56 71 7); Light green 1 (109 104 22); Light green 2 (100 102 35); Light green 3 (98 104 42)                                                                                                              | 0.05 x 0.05                                  | 50                         | Pterins: Isoxanthopterin and erythropterin at family level (Merlini & Nasini 1966), erythropterin at family level (Niva & Takeda 2002).                                                                                                  |
| <b>Cicadellidae</b>                 |        |             |        |                           |                      |                                                                                                                                                                                                                                                                        |                                              |                            |                                                                                                                                                                                                                                          |
| <i>Evacanthus interruptus</i>       | OUMNH  | 1           | 1      | Left elytrum, centre.     | 4                    | Black 1 (47 48 46); Black 2 (68 56 39); Yellow 1 (163 168 35); Yellow 2 (168 177 35)                                                                                                                                                                                   | 0.03 x 0.03                                  | 50                         | Melanins in black and red regions at family level (Timmons <i>et al.</i> 2011).                                                                                                                                                          |
| <i>Deselvana fulvofasciata</i>      | OUMNH  | 1           | 1      | Right elytrum, posterior. | 6                    | Brown 1 (112 62 32); Brown 2 (81 33 17); Brown 3 (73 26 7); Yellow 1 (161 137 58); Yellow 2 (150 127 55); Yellow 3 (152 123 14)                                                                                                                                        | 0.05 x 0.05                                  | 50                         | Melanins in black and red regions at family level (Timmons <i>et al.</i> 2011).                                                                                                                                                          |
| <i>Cicciana obliqua</i>             | OUMNH  | 1           | 1      | Right elytrum, posterior. | 6                    | Brown 1 (58 35 39); Brown 2 (56 26 27); Brown 3 (54 27 29); Yellow 1 (172 141 68); Yellow 2 (157 132 65); Yellow 3 (146 116 55)                                                                                                                                        | 0.05 x 0.05                                  | 50                         | Melanins in black and red regions at family level (Timmons <i>et al.</i> 2011).                                                                                                                                                          |
| <b>Mecoptera</b>                    |        |             |        |                           |                      |                                                                                                                                                                                                                                                                        |                                              |                            |                                                                                                                                                                                                                                          |
| <b>Panorpidae</b>                   |        |             |        |                           |                      |                                                                                                                                                                                                                                                                        |                                              |                            |                                                                                                                                                                                                                                          |
| <i>Panora sp.</i>                   | OUMNH  | 1           | 1      | Dorsal forewing.          | 6                    | Brown 1 (127 105 46); Brown 2 (195 142 118); Brown 3 (95 67 9); Brown 4 (202 199 161); Brown 5 (198 199 162); Brown 6 (199 199 161)                                                                                                                                    | 0.1 x 0.1                                    | 50                         | Melanins in black regions at genus level (Nakagoshi <i>et al.</i> 1984). Pterins: sepiapterin in yellow regions (Nakagoshi <i>et al.</i> 1984).                                                                                          |
| <i>Panorpa lugubris</i>             | OUMNH  | 1           | 1      | Dorsal forewing.          | 6                    | Brown 1 (127 105 46); Brown 2 (195 142 118); Brown 3 (95 67 9); Brown 4 (202 199 161); Brown 5 (198 199 162); Brown 6 (199 199 161)                                                                                                                                    | 0.05 x 0.05                                  | 50                         | Melanins in black regions at genus level (Nakagoshi <i>et al.</i> 1984). Pterins: sepiapterin in yellow regions (Nakagoshi <i>et al.</i> 1984).                                                                                          |

| taxon                           | source | # specimens | # maps | map location           | total # ROIs | RGB values                                                                                                                                         | pixel size (μ) | dwell time of XRF maps (ms) | known coloration mechanisms                                                                                                                                                                                                            |
|---------------------------------|--------|-------------|--------|------------------------|--------------|----------------------------------------------------------------------------------------------------------------------------------------------------|----------------|-----------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>Diptera</b>                  |        |             |        |                        |              |                                                                                                                                                    |                |                             |                                                                                                                                                                                                                                        |
| <b>Asilidae</b>                 |        |             |        |                        |              |                                                                                                                                                    |                |                             |                                                                                                                                                                                                                                        |
| <i>Asilus crabroniformis</i>    | OUMNH  | 1           | 1      | Dorsal forewing.       | 6            | Brown 1 (122 102 46); Brown 2 (114 95 38); Brown 3 (116 97 44); Brown 4 (195 182 112); Brown 5 (207 193 117); brown 6 (198 187 116)                | 0.1 x 0.1      | 50                          | AKH red pigment concentrating hormone at order level (Gäde <i>et al.</i> 1990). Melanins in black regions at order level (O'Grady & DeSalle 2000; Wittkopp <i>et al.</i> 2003; Gompel <i>et al.</i> 2005; Parchem <i>et al.</i> 2007). |
| <b>Hymenoptera</b>              |        |             |        |                        |              |                                                                                                                                                    |                |                             |                                                                                                                                                                                                                                        |
| <b>Vespidae</b>                 |        |             |        |                        |              |                                                                                                                                                    |                |                             |                                                                                                                                                                                                                                        |
| <i>Odynerus gracilis</i>        | NHMO   | 1           | 1      | Right wing.            | 5            | Brown 1 (107 77 23); Brown 2 (107 77 25); Colourless 1 (201 207 199); Colourless 2 (207 216 216); Colourless 3 (208 213 212)                       | 0.025 x 0.025  | 50                          | Pterins: xanthopterin in yellow regions (Shamim <i>et al.</i> 2014).                                                                                                                                                                   |
| <b>Andrenidae</b>               |        |             |        |                        |              |                                                                                                                                                    |                |                             |                                                                                                                                                                                                                                        |
| <i>Andrena carantonica</i>      | NHMO   | 1           | 1      | Right wing.            | 5            | Brown 1 (103 83 43); Brown 2 (103 85 42); Colourless 1 (172 172 146); Colourless 2 (162 158 120); Colourless 3 (178 180 150)                       | 0.025 x 0.025  | 50                          | Melanins in dark regions at superfamily level (Polidori <i>et al.</i> 2017)                                                                                                                                                            |
| <b>Odonata</b>                  |        |             |        |                        |              |                                                                                                                                                    |                |                             |                                                                                                                                                                                                                                        |
| <b>Calopterygidae</b>           |        |             |        |                        |              |                                                                                                                                                    |                |                             |                                                                                                                                                                                                                                        |
| <i>Calopteryx virgo</i>         | NHMO   | 1           | 1      | Right wing, posterior. | 6            | Brown 1 (114 86 15); Brown 2 (120 93 17); Brown 3 (132 107 27); Colourless 1 (210 217 204); Colourless 2 (211 214 201); Colourless 3 (210 212 192) | 0.025 x 0.025  | 50                          | Melanins in dark regions at genus level (Fitzstephens & Getty 2000). AKH red pigment concentrating hormone at geus level (Gäde 2009). Structural in dark regions at genus level (Fitzstephens & Getty 2000; Stavenga 2009).            |
| <i>Calopteryx splendens</i>     | NHMO   | 1           | 1      | Right wing, posterior. | 6            | Brown 1 (92 57 11); Brown 2 (91 56 12); Brown 3 (80 45 8); Colourless 1 (214 225 207); Colourless 2 (213 214 225); Colourless 3 (222 230 120)      | 0.025 x 0.025  | 50                          | Melanins in dark regions at genus level (Fitzstephens & Getty 2000). AKH red pigment concentrating hormone at geus level (Gäde 2009). Structural in dark regions at genus level (Fitzstephens & Getty 2000; Stavenga 2009).            |
| <b>Libellulidae</b>             |        |             |        |                        |              |                                                                                                                                                    |                |                             |                                                                                                                                                                                                                                        |
| <i>Libellula quadrimaculata</i> | NHMO   | 1           | 1      | Right wing, posterior. | 6            | Brown 1 (56 41 38); Brown 2 (144 109 17); Brown 3 (47 35 21); Colourless 1 (210 217 213); Colourless 2 (209 215 194); Colourless 3 (151 146 137)   | 0.05 x 0.05    | 50                          | Melanins in dark regions at genus level (Fitzstephens & Getty 2000). AKH red pigment concentrating hormone at geus level (Gäde 2009). Structural in dark regions at genus level (Fitzstephens & Getty 2000; Stavenga 2009).            |
| <i>Sympetrum flaveolum</i>      | NHMO   | 1           | 1      | Right wing, posterior. | 6            | Brown 1 (110 92 34); Brown 2 (101 81 25); Brown 3 (97 80 32); Colourless 1 (205 215 229); Colourless 2 (205 216 229); Colourless 3 (200 210 221)   | 0.025 x 0.025  | 50                          | Melanins in dark regions at genus level (Fitzstephens & Getty 2000). AKH red pigment concentrating hormone at geus level (Gäde 2009). Structural in dark regions at genus level (Fitzstephens & Getty 2000; Stavenga 2009).            |
| <b>Coenagrionidae</b>           |        |             |        |                        |              |                                                                                                                                                    |                |                             |                                                                                                                                                                                                                                        |
| <i>Pyrrhosoma nymphula</i>      | NHMO   | 1           | 1      | Right wing, posterior. | 6            | Brown 1 (76 63 35); Brown 2 (90 76 36); Brown 3 (116 97 30); Colourless 1 (203 213 226); Colourless 2 (205 216 232); Colourless 3 (204 215 229)    | 0.025 x 0.025  | 50                          | Melanins in dark regions at genus level (Fitzstephens & Getty 2000). AKH red pigment concentrating hormone at geus level (Gäde 2009). Structural in dark regions at genus level (Fitzstephens & Getty 2000; Stavenga 2009).            |
| <b>Aeshnidae</b>                |        |             |        |                        |              |                                                                                                                                                    |                |                             |                                                                                                                                                                                                                                        |
| <i>Aeshna caerulea</i>          | NHMO   | 1           | 1      | Right wing, posterior. | 6            | Brown 1 (70 55 38); Brown 2 (77 62 39); Brown 3 (71 56 38); Colourless 1 (208 220 230); Colourless 2 (206 218 230); Colourless 3 (202 214 226)     | 0.025 x 0.025  | 50                          | Melanins in dark regions at genus level (Fitzstephens & Getty 2000). Structural in dark regions at genus level (Fitzstephens & Getty 2000; Stavenga 2009).                                                                             |
| <b>Corduliidae</b>              |        |             |        |                        |              |                                                                                                                                                    |                |                             |                                                                                                                                                                                                                                        |
| <i>Cordulia aenea</i>           | NHMO   | 1           | 1      | Right wing, posterior. | 6            | Brown 1 (52 42 43); Brown 2 (48 41 45); Brown 3 (62 49 46); Colourless 1 (223 235 243); Colourless 2 (231 245 253); Colourless 3 (231 245 253)     | 0.025 x 0.025  | 50                          | Melanins in dark regions at genus level (Fitzstephens & Getty 2000). Structural in dark regions at genus level (Fitzstephens & Getty 2000; Stavenga 2009).                                                                             |

**Supplementary Table 5.1 (previous page). Details of extant specimens analyzed using synchrotron rapid scanning X-ray fluorescence.** OUMNH, Oxford University Museum of Natural History; NHMO, Natural History Museum of Oslo; YPMNH, Yale Peabody Museum of Natural History.

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| species                  | P      | S      | Cl     | K      | concentration (µg/cm <sup>2</sup> ) |        |        |        |        |        | Cu     | Zn     | visual colour | coloration mechanism                     | specific coloration                                                                                                                 | RGB values  | websafe | hex           | tribe          | subfamily      | family     | suborder    | order       | run    |
|--------------------------|--------|--------|--------|--------|-------------------------------------|--------|--------|--------|--------|--------|--------|--------|---------------|------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------|-------------|---------|---------------|----------------|----------------|------------|-------------|-------------|--------|
|                          |        |        |        |        | Ca                                  | Ti     | Mn     | Fe     | Ni     |        |        |        |               |                                          |                                                                                                                                     |             |         |               |                |                |            |             |             |        |
| Macroglossum stellatarum | 2.2447 | 0.7199 | 0.5895 | 2.2611 | 2.4153                              | 1.5217 | 1.3299 | 4.0884 | 0.5344 | 0.4064 | 0.7074 | Brown  | flavonoids    | anthoxanthin                             | 77 52 13                                                                                                                            | #663300     | #4d340d | Macroglossina | Macroglossinae | Sphingidae     | Ditrysia   | Lepidoptera | Feb-16      |        |
| Macroglossum stellatarum | 2.4372 | 0.6353 | 0.5736 | 2.0922 | 2.3578                              | 1.7366 | 1.5652 | 4.0564 | 0.655  | 0.3713 | 0.8291 | Brown  | flavonoids    | anthoxanthin                             | 87 62 20                                                                                                                            | #663300     | #573e14 | Macroglossina | Macroglossinae | Sphingidae     | Ditrysia   | Lepidoptera | Feb-16      |        |
| Macroglossum stellatarum | 2.4982 | 0.702  | 0.6338 | 2.435  | 3.4271                              | 1.9751 | 1.6537 | 3.819  | 0.5441 | 0.4564 | 0.9043 | Brown  | flavonoids    | anthoxanthin                             | 96 67 26                                                                                                                            | #663333     | #60431a | Macroglossina | Macroglossinae | Sphingidae     | Ditrysia   | Lepidoptera | Feb-16      |        |
| Macroglossum stellatarum | 2.9862 | 0.7283 | 0.6667 |        | 3.1252                              | 4.4938 | 1.4399 | 3.1488 | 4.5023 | 0.6812 | 0.576  | 1.4254 | Brown         | flavonoids                               | anthoxanthin                                                                                                                        | 154 115 57  | #996633 | #9a7339       | Macroglossina  | Macroglossinae | Sphingidae | Ditrysia    | Lepidoptera | Feb-16 |
| Macroglossum stellatarum | 3.235  | 1.0176 | 1.0461 |        | 3.7893                              | 4.6037 | 2.2554 | 1.8005 | 4.2246 | 0.6355 | 0.9318 | 1.3294 | Brown         | flavonoids                               | anthoxanthin                                                                                                                        | 130 93 43   | #996633 | #825d2b       | Macroglossina  | Macroglossinae | Sphingidae | Ditrysia    | Lepidoptera | Feb-16 |
| Macroglossum stellatarum | 2.7368 | 0.7003 | 0.6886 |        | 3.2571                              | 4.5486 | 2.2683 | 1.4671 | 6.632  | 0.4631 | 0.6692 | 0.7796 | Brown         | flavonoids                               | anthoxanthin                                                                                                                        | 156 119 60  | #996633 | #9c773c       | Macroglossina  | Macroglossinae | Sphingidae | Ditrysia    | Lepidoptera | Feb-16 |
| Macroglossum stellatarum | 2.739  | 0.7412 | 0.6228 |        | 3.668                               | 3.1607 | 1.565  | 1.4596 | 2.5453 | 0.5342 | 0.6201 | 1.0434 | Yellow        | carotenoids                              | zeaxanthin, lutein, adonirubin, astaxanthin and lutein epoxide                                                                      | 250 176 13  | #ff9900 | #fab00d       | Macroglossina  | Macroglossinae | Sphingidae | Ditrysia    | Lepidoptera | Feb-16 |
| Macroglossum stellatarum | 2.8579 | 0.7448 | 0.5985 |        | 3.9418                              | 3.021  | 1.9319 | 1.514  | 3.3108 | 0.5375 | 0.622  | 0.9679 | Yellow        | carotenoids                              | zeaxanthin, lutein, adonirubin, astaxanthin and lutein epoxide                                                                      | 254 199 50  | #ffcc33 | #fec732       | Macroglossina  | Macroglossinae | Sphingidae | Ditrysia    | Lepidoptera | Feb-16 |
| Macroglossum stellatarum | 2.5866 | 0.904  | 0.6973 |        | 3.8683                              | 3.31   | 1.3345 | 1.2762 | 4.8855 | 0.4193 | 0.6403 | 1.1311 | Yellow        | carotenoids                              | zeaxanthin, lutein, adonirubin, astaxanthin and lutein epoxide                                                                      | 255 187 19  | #ffcc00 | #ffbb13       | Macroglossina  | Macroglossinae | Sphingidae | Ditrysia    | Lepidoptera | Feb-16 |
| Macroglossum stellatarum | 2.7065 | 0.6959 | 0.6611 |        | 2.7062                              | 2.2509 | 1.8553 | 1.5417 | 3.5714 | 0.5435 | 0.556  | 0.7877 | Orange        | carotenoids                              | zeaxanthin, lutein, adonirubin, astaxanthin and lutein epoxide                                                                      | 166 80 12   | #996600 | #a6500c       | Macroglossina  | Macroglossinae | Sphingidae | Ditrysia    | Lepidoptera | Feb-16 |
| Macroglossum stellatarum | 2.5112 | 0.7226 | 0.6106 |        | 2.4695                              | 2.2882 | 1.4436 | 1.2873 | 2.6592 | 0.5849 | 0.5316 | 0.7791 | Orange        | carotenoids                              | zeaxanthin, lutein, adonirubin, astaxanthin and lutein epoxide                                                                      | 159 65 6    | #993300 | #9f4106       | Macroglossina  | Macroglossinae | Sphingidae | Ditrysia    | Lepidoptera | Feb-16 |
| Macroglossum stellatarum | 2.5954 | 0.8602 | 0.5779 |        | 2.5084                              | 1.9252 | 2.0753 | 1.1862 | 2.4824 | 0.6962 | 0.5437 | 0.7332 | Orange        | carotenoids                              | zeaxanthin, lutein, adonirubin, astaxanthin and lutein epoxide                                                                      | 178 81 10   | #996600 | #b2510a       | Macroglossina  | Macroglossinae | Sphingidae | Ditrysia    | Lepidoptera | Feb-16 |
| Anthocharis cardamines   | 2.6983 | 0.7127 | 0.3835 |        | 2.2022                              | 1.3662 | 0.1196 | 1.4464 | 2.3132 | 0.4842 | 0.5357 | 0.8508 | Brown         | melanins                                 | leucopterin and possible isoxanthopterin                                                                                            | 88 71 37    | #663333 | #584725       | Anthocharini   | Pierinae       | Pieridae   | Ditrysia    | Lepidoptera | Feb-16 |
| Anthocharis cardamines   | 2.0793 | 0.5368 | 0.3342 |        | 1.6803                              | 0.9817 | 0.1288 | 1.176  | 1.8767 | 0.7645 | 0.3228 | 0.9457 | Brown         | melanins                                 | leucopterin and possible isoxanthopterin                                                                                            | 111 87 36   | #666633 | #6f572a       | Anthocharini   | Pierinae       | Pieridae   | Ditrysia    | Lepidoptera | Feb-16 |
| Anthocharis cardamines   | 2.644  | 0.6641 | 0.2673 |        | 2.0165                              | 0.8311 | 0.1757 | 1.3138 | 1.0891 | 0.453  | 0.3289 | 0.7054 | Orange        | pterins                                  | erythropterin                                                                                                                       | 255 169 36  | #ff9933 | #ffa924       | Anthocharini   | Pierinae       | Pieridae   | Ditrysia    | Lepidoptera | Feb-16 |
| Anthocharis cardamines   | 2.5424 | 0.8149 | 0.2869 |        | 2.2387                              | 0.9357 | 0.1541 | 1.2521 | 1.3536 | 0.453  | 0.4288 | 0.7857 | Orange        | pterins                                  | erythropterin                                                                                                                       | 255 165 36  | #ff9933 | #ffa524       | Anthocharini   | Pierinae       | Pieridae   | Ditrysia    | Lepidoptera | Feb-16 |
| Anthocharis cardamines   | 2.869  | 0.6585 | 0.3042 |        | 2.7374                              | 1.3528 | 0.1532 | 1.4374 | 1.5135 | 0.6626 | 0.6751 | 1.2796 | White         | pterins                                  | leucopterin and possible isoxanthopterin                                                                                            | 241 244 187 | #ffccff | #f114bb       | Anthocharini   | Pierinae       | Pieridae   | Ditrysia    | Lepidoptera | Feb-16 |
| Anthocharis cardamines   | 2.1806 | 0.595  | 0.296  |        | 2.5396                              | 1.5446 | 0.1313 | 1.0596 | 1.5264 | 0.6001 | 0.8036 | 1.1408 | White         | pterins                                  | Leucopterin and possible isoxanthopterin                                                                                            | 236 239 188 | #ffccff | #ecfcbf       | Anthocharini   | Pierinae       | Pieridae   | Ditrysia    | Lepidoptera | Feb-16 |
| Anthocharis cardamines   | 2.6655 | 0.7919 | 0.2701 |        | 1.7646                              | 1.3638 | 1.6446 | 1.3433 | 1.7937 | 0.622  | 0.5209 | 0.9104 | Brown         | melanins                                 | leucopterin                                                                                                                         | 87 64 19    | #663300 | #574013       | Anthocharini   | Pierinae       | Pieridae   | Ditrysia    | Lepidoptera | Feb-16 |
| Anthocharis cardamines   | 2.111  | 0.7305 | 0.3013 |        | 2.1377                              | 1.1924 | 1.364  | 1.2617 | 1.627  | 0.7048 | 0.2972 | 0.8906 | Orange        | pterins                                  | erythropterin                                                                                                                       | 255 181 16  | #ffcc00 | #ff5010       | Anthocharini   | Pierinae       | Pieridae   | Ditrysia    | Lepidoptera | Feb-16 |
| Anthocharis cardamines   | 1.9825 | 0.6286 | 0.2793 |        | 2.0396                              | 1.3379 | 1.4535 | 1.2335 | 2.3985 | 0.6424 | 0.4325 | 0.9441 | Orange        | pterins                                  | erythropterin                                                                                                                       | 255 175 16  | #ff9900 | #ffa110       | Anthocharini   | Pierinae       | Pieridae   | Ditrysia    | Lepidoptera | Feb-16 |
| Anthocharis cardamines   | 2.1584 | 0.7218 | 0.2853 |        | 2.0966                              | 1.351  | 1.5005 | 1.3304 | 1.66   | 0.6242 | 0.3884 | 0.9499 | Orange        | pterins                                  | erythropterin                                                                                                                       | 255 172 16  | #ff9900 | #ffac10       | Anthocharini   | Pierinae       | Pieridae   | Ditrysia    | Lepidoptera | Feb-16 |
| Anthocharis cardamines   | 2.1164 | 0.6399 | 0.2931 |        | 2.5326                              | 1.651  | 1.3899 | 1.4406 | 2.2877 | 0.6823 | 0.4751 | 0.9805 | White         | pterins                                  | leucopterin                                                                                                                         | 242 244 182 | #ffccff | #f2f4b6       | Anthocharini   | Pierinae       | Pieridae   | Ditrysia    | Lepidoptera | Feb-16 |
| Anthocharis cardamines   | 1.9831 | 0.6243 | 0.2933 |        | 2.4305                              | 1.8213 | 1.5348 | 1.3212 | 2.0391 | 0.6298 | 0.3706 | 1.0267 | White         | pterins                                  | leucopterin                                                                                                                         | 243 245 189 | #ffccff | #3f5bd        | Anthocharini   | Pierinae       | Pieridae   | Ditrysia    | Lepidoptera | Feb-16 |
| Anthocharis cardamines   | 2.5544 | 0.6854 | 0.2864 |        | 2.4599                              | 1.5572 | 1.2566 | 1.2172 | 2.4187 | 0.7643 | 0.3165 | 1.0018 | Grey          | N/A                                      | N/A                                                                                                                                 | 171 159 24  | #669900 | #ab9f1b       | Anthocharini   | Pierinae       | Pieridae   | Ditrysia    | Lepidoptera | Feb-16 |
| Anthocharis cardamines   | 2.3605 | 0.552  | 0.2366 |        | 2.5367                              | 1.1801 | 1.1826 | 1.1036 | 1.3117 | 0.4951 | 0.3346 | 0.8014 | Grey          | N/A                                      | N/A                                                                                                                                 | 116 107 23  | #666600 | #746b17       | Anthocharini   | Pierinae       | Pieridae   | Ditrysia    | Lepidoptera | Feb-16 |
| Anthocharis cardamines   | 1.9922 | 0.6253 | 0.3017 |        | 2.7216                              | 1.5376 | 1.295  | 1.4699 | 1.8098 | 0.5791 | 0.4501 | 0.944  | Grey          | N/A                                      | N/A                                                                                                                                 | 94 87 20    | #666600 | #5e5714       | Anthocharini   | Pierinae       | Pieridae   | Ditrysia    | Lepidoptera | Feb-16 |
| Pieris rapae             | 2.367  | 0.4444 | 0.2077 |        | 1.3733                              | 0.5001 | 1.4954 | 1.3562 | 0.9662 | 0.6299 | 0.2382 | 0.2562 | Brown         | melanins                                 | melanins                                                                                                                            | 67 60 12    | #333300 | #433c0c       | Eudamini       | Pierinae       | Pieridae   | Ditrysia    | Lepidoptera | Feb-16 |
| Pieris rapae             | 2.2156 | 0.4712 | 0.2238 |        | 1.6633                              | 0.5932 | 1.5459 | 1.5008 | 1.1649 | 0.396  | 0.2674 | 0.2758 | Brown         | melanins                                 | melanins                                                                                                                            | 75 67 12    | #333300 | #4b430c       | Eudamini       | Pierinae       | Pieridae   | Ditrysia    | Lepidoptera | Feb-16 |
| Pieris rapae             | 1.9999 | 0.4536 | 0.2531 |        | 1.6949                              | 0.6993 | 1.4178 | 1.6024 | 4.6168 | 0.6411 | 0.5833 | 0.2833 | Brown         | melanins                                 | melanins                                                                                                                            | 80 70 11    | #663300 | #50460b       | Eudamini       | Pierinae       | Pieridae   | Ditrysia    | Lepidoptera | Feb-16 |
| Pieris rapae             | 2.4251 | 0.3676 | 0.2152 |        | 1.6104                              | 0.6835 | 1.4334 | 1.5154 | 1.5799 | 0.5333 | 0.2677 | 0.3696 | Yellow        | 1: pterins. 2: uric acid. 3: carotenoids | 1: leucopterin, xanthopterin. 2: uric acid. 3: tourene, β-cryptoxanthin, canthaxanthin, lutein epoxide, neoxanthin and mutatochrome | 238 253 251 | #ffffff | #eefdfb       | Eudamini       | Pierinae       | Pieridae   | Ditrysia    | Lepidoptera | Feb-16 |
| Pieris rapae             | 2.5353 | 0.4203 | 0.2452 |        | 1.5985                              | 0.586  | 1.3387 | 1.461  | 1.1059 | 0.5111 | 0.3271 | 0.2686 | Yellow        | 1: pterins. 2: uric acid. 3: carotenoids | 1: leucopterin, xanthopterin. 2: uric acid. 3: tourene, β-cryptoxanthin, canthaxanthin, lutein epoxide, neoxanthin and mutatochrome | 235 251 175 | #ff9999 | #ebfbaf       | Eudamini       | Pierinae       | Pieridae   | Ditrysia    | Lepidoptera | Feb-16 |
| Pieris rapae             | 2.827  | 0.3457 | 0.195  |        | 1.6475                              | 0.6191 | 1.6979 | 1.49   | 1.1259 | 0.449  | 0.204  | 0.2466 | Yellow        | 1: pterins. 2: uric acid. 3: carotenoids | 1: leucopterin, xanthopterin. 2: uric acid. 3: tourene, β-cryptoxanthin, canthaxanthin, lutein epoxide, neoxanthin and mutatochrome | 230 248 170 | #ff9999 | #e6f8aa       | Eudamini       | Pierinae       | Pieridae   | Ditrysia    | Lepidoptera | Feb-16 |
| Colotis regina           | 1.5099 | 0.3941 | 0.3334 |        | 1.6001                              | 1.6615 | 0.9021 | 0.8265 | 1.8964 | 0.4452 | 0.1908 | 0.2879 | Purple        | pterins                                  | leucopterin and erythropterin                                                                                                       | 219 221 206 | #cccccc | #dbddce       | Pierini        | Pierinae       | Pieridae   | Ditrysia    | Lepidoptera | Nov-17 |
| Colotis regina           | 1.3446 | 0.4046 | 0.3309 |        | 1.6056                              | 1.7269 | 0.8253 | 0.7654 | 1.4131 | 0.4018 | 0.1838 | 0.2693 | Purple        | pterins                                  | leucopterin and erythropterin                                                                                                       | 231 232 211 | #ffccff | #e7e8d3       | Pierini        | Pierinae       | Pieridae   | Ditrysia    | Lepidoptera | Nov-17 |
| Colotis regina           | 1.4538 | 0.382  | 0.3149 |        | 1.5772                              | 1.7559 | 0.8347 | 0.914  | 1.648  | 0.4152 | 0.1895 | 0.2808 | Purple        | pterins                                  | leucopterin and erythropterin                                                                                                       | 223 224 207 | #cccccc | #dfe0cf       | Pierini        | Pierinae       | Pieridae   | Ditrysia    | Lepidoptera | Nov-17 |
| Colotis regina           | 1.4162 | 0.369  | 0.2928 |        | 1.651                               | 1.1486 | 0.7928 | 0.6455 | 1.9347 | 0.4358 | 0.1846 | 0.2597 | White         | 1: flavonoids. 2: structural             | 1: anthoxanthin. 2: structural                                                                                                      | 127 82 93   | #666666 | #7f525d       | Pierini        | Pierinae       | Pieridae   | Ditrysia    | Lepidoptera | Nov-17 |
| Colotis regina           | 1.457  | 0.4113 | 0.3029 |        | 1.6523                              | 1.0723 | 0.9156 | 0.6775 | 2.0876 | 0.425  | 0.1879 | 0.244  | White         | 1: flavonoids. 2: structural             | 1: anthoxanthin. 2: structural                                                                                                      | 112 52 67   | #663333 | #703443       | Pierini        | Pierinae       | Pieridae   | Ditrysia    | Lepidoptera | Nov-17 |
| Colotis regina           | 1.4608 | 0.3759 | 0.2826 |        | 1.5702                              | 1.0015 | 0.7913 | 0.7643 | 1.4713 | 0.4285 | 0.1849 | 0.4069 | White         | 1: flavonoids. 2: structural             | 1: anthoxanthin. 2: structural                                                                                                      | 127 90 99   | #666666 | #7f5a63       | Pierini        | Pierinae       | Pieridae   | Ditrysia    | Lepidoptera | Nov-17 |

| species                      | P      | S             | Cl     | K      | concentration (µg/cm <sup>2</sup> ) |               |        | Fe            | Ni     | Cu     | Zn     | visual colour | coloration mechanism           | specific coloration                                                                                                                                                        | RGB values  | websafe | hex     | tribe      | subfamily    | family      | suborder | order       | run    |
|------------------------------|--------|---------------|--------|--------|-------------------------------------|---------------|--------|---------------|--------|--------|--------|---------------|--------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------|---------|---------|------------|--------------|-------------|----------|-------------|--------|
| <i>Abraxas grossulariata</i> | 2.1279 | 0.6108        | 0.321  | 1.8236 | 2.9913                              | 2.2849        | 1.0856 | 4.5335        | 0.4281 | 0.7243 | 0.7965 | Brown         | melanins                       | melanins                                                                                                                                                                   | 123 101 51  | #666633 | #7b6533 | Abraxini   | Ennominae    | Geometridae | Ditrysia | Lepidoptera | Feb-16 |
| <i>Abraxas grossulariata</i> | 1.9845 | 0.8808        | 0.3069 | 1.7969 | 2.2933                              | 1.3641        | 1.2696 | 4.7035        | 0.5496 | 0.4206 | 0.6933 | Brown         | melanins                       | melanins                                                                                                                                                                   | 102 81 35   | #666633 | #665123 | Abraxini   | Ennominae    | Geometridae | Ditrysia | Lepidoptera | Feb-16 |
| <i>Abraxas grossulariata</i> | 2.2515 | 0.6873        | 0.2962 | 2.2367 | 4.4523                              | 1.6254        | 1.4042 | 5.0275        | 0.5958 | 0.8163 | 0.905  | Brown         | melanins                       | melanins                                                                                                                                                                   | 141 122 70  | #996633 | #8d7a46 | Abraxini   | Ennominae    | Geometridae | Ditrysia | Lepidoptera | Feb-16 |
| <i>Abraxas grossulariata</i> | 2.0568 | 0.6209        | 0.2872 | 1.6643 | 2.1379                              | 1.7389        | 1.1274 | <b>8.32</b>   | 0.5904 | 9.6055 | 0.6292 | Yellow        | carotenoids                    | lycopene, β-cryptoxanthin, zeaxanthin, canthaxanthin, capsanthone, lutein epoxide, neoxanthin, mutatochrome and mutatoxanthin                                              | 223 187 51  | #cccc33 | #dfbb33 | Abraxini   | Ennominae    | Geometridae | Ditrysia | Lepidoptera | Feb-16 |
| <i>Abraxas grossulariata</i> | 2.0059 | 0.6767        | 0.3364 | 1.8681 | 2.5992                              | 1.4737        | 1.4041 | <b>6.7517</b> | 0.5784 | 0.4574 | 0.7131 | Yellow        | carotenoids                    | lycopene, β-cryptoxanthin, zeaxanthin, canthaxanthin, capsanthone, lutein epoxide, neoxanthin, mutatochrome and mutatoxanthin                                              | 239 206 51  | #ffcc33 | #efce33 | Abraxini   | Ennominae    | Geometridae | Ditrysia | Lepidoptera | Feb-16 |
| <i>Abraxas grossulariata</i> | 1.9352 | 0.5756        | 0.2562 | 1.7766 | 1.4639                              | 2.4195        | 1.2511 | 4.2411        | 0.5707 | 0.7147 | 0.6707 | White         | N/A                            | N/A                                                                                                                                                                        | 236 240 191 | #ffffcc | #ecf0bf | Abraxini   | Ennominae    | Geometridae | Ditrysia | Lepidoptera | Feb-16 |
| <i>Abraxas grossulariata</i> | 1.982  | 0.5465        | 0.264  | 1.6495 | 2.1189                              | 9.1574        | 1.3369 | 4.4205        | 0.5648 | 0.7819 | 0.6853 | White         | N/A                            | N/A                                                                                                                                                                        | 241 246 200 | #ffffcc | #f1f6c8 | Abraxini   | Ennominae    | Geometridae | Ditrysia | Lepidoptera | Feb-16 |
| <i>Abraxas grossulariata</i> | 2.0117 | 0.5387        | 0.2383 | 1.6494 | 2.3256                              | 1.5667        | 1.3027 | 4.9055        | 0.5156 | 0.8103 | 0.9104 | White         | N/A                            | N/A                                                                                                                                                                        | 232 237 196 | #ffffcc | #e8edc4 | Abraxini   | Ennominae    | Geometridae | Ditrysia | Lepidoptera | Feb-16 |
| <i>Bicyclus anynana</i>      | 2.4084 | 0.4138        | 0.2039 | 1.559  | 0.4533                              | 1.3446        | 1.1466 | 1.0049        | 0.5332 | 0.2061 | 0.3053 | Brown         | ommochromes                    | xanthommatin                                                                                                                                                               | 74 52 1     | #333300 | #4a3401 | Elymniini  | Satyrinae    | Nymphalidae | Ditrysia | Lepidoptera | Feb-16 |
| <i>Bicyclus anynana</i>      | 2.6636 | 0.5008        | 0.2264 | 1.4658 | 0.4497                              | 1.3295        | 1.5429 | 1.0585        | 0.6997 | 0.2378 | 0.255  | Brown         | ommochromes                    | xanthommatin                                                                                                                                                               | 75 53 3     | #333300 | #4b3503 | Elymniini  | Satyrinae    | Nymphalidae | Ditrysia | Lepidoptera | Feb-16 |
| <i>Bicyclus anynana</i>      | 2.6559 | 0.4147        | 0.2419 | 1.4804 | 0.4852                              | <b>1.5443</b> | 1.3161 | 0.9905        | 0.4725 | 0.2294 | 0.2402 | Brown         | ommochromes                    | xanthommatin                                                                                                                                                               | 187 170 75  | #cc9933 | #bbaa4b | Elymniini  | Satyrinae    | Nymphalidae | Ditrysia | Lepidoptera | Feb-16 |
| <i>Bicyclus anynana</i>      | 1.8118 | 0.3786        | 0.2466 | 1.4097 | 0.68                                | <b>1.6187</b> | 1.4731 | 1.4485        | 0.5021 | 0.1971 | 0.2999 | Brown         | ommochromes                    | xanthommatin                                                                                                                                                               | 172 151 57  | #999933 | #ac9739 | Elymniini  | Satyrinae    | Nymphalidae | Ditrysia | Lepidoptera | Feb-16 |
| <i>Bicyclus anynana</i>      | 2.9533 | 0.4048        | 0.2498 | 1.4459 | 0.5487                              | <b>1.5464</b> | 1.3905 | 1.1348        | 0.3691 | 0.2154 | 0.2494 | Brown         | ommochromes                    | xanthommatin                                                                                                                                                               | 156 133 38  | #999933 | #9c8526 | Elymniini  | Satyrinae    | Nymphalidae | Ditrysia | Lepidoptera | Feb-16 |
| <i>Bicyclus anynana</i>      | 2.4577 | 0.4538        | 0.2394 | 1.4169 | 0.4028                              | <b>1.727</b>  | 1.3518 | 1.7032        | 0.5352 | 0.2324 | 0.216  | Brown         | ommochromes                    | xanthommatin                                                                                                                                                               | 140 115 17  | #999933 | #8c7311 | Elymniini  | Satyrinae    | Nymphalidae | Ditrysia | Lepidoptera | Feb-16 |
| <i>Bicyclus anynana</i>      | 1.9775 | 0.3354        | 0.2371 | 1.4218 | 0.5943                              | 1.3196        | 1.4532 | 1.3701        | 0.5191 | 0.2069 | 0.3279 | Yellow        | ommochromes                    | 3-OHK                                                                                                                                                                      | 195 165 14  | #cc9900 | #c3a50e | Elymniini  | Satyrinae    | Nymphalidae | Ditrysia | Lepidoptera | Feb-16 |
| <i>Bicyclus anynana</i>      | 2.5233 | 0.4004        | 0.2529 | 1.5099 | 0.5392                              | 1.421         | 1.5257 | 1.3549        | 0.455  | 0.2255 | 0.2897 | Yellow        | ommochromes                    | 3-OHK                                                                                                                                                                      | 201 171 20  | #cc9900 | #c9ab14 | Elymniini  | Satyrinae    | Nymphalidae | Ditrysia | Lepidoptera | Feb-16 |
| <i>Bicyclus anynana</i>      | 2.5189 | 0.4385        | 0.2343 | 1.4475 | 0.4492                              | 1.1833        | 1.4883 | 0.9036        | 0.5485 | 0.1925 | 0.2889 | White         | N/A                            | N/A                                                                                                                                                                        | 233 250 224 | #ffffcc | #e9fae0 | Elymniini  | Satyrinae    | Nymphalidae | Ditrysia | Lepidoptera | Feb-16 |
| <i>Melanargia galathea</i>   | 2.1056 | 0.7017        | 0.2767 | 2.2218 | <b>2.4188</b>                       | <b>1.4897</b> | 1.424  | <b>2.0638</b> | 0.5165 | 0.3234 | 0.4865 | Brown         | ommochromes                    | xanthommatin                                                                                                                                                               | 63 41 9     | #333300 | #3f2909 | Satyrini   | Satyrinae    | Nymphalidae | Ditrysia | Lepidoptera | Feb-16 |
| <i>Melanargia galathea</i>   | 1.8527 | 0.6089        | 0.2782 | 2.2002 | <b>2.608</b>                        | <b>1.479</b>  | 1.1669 | <b>2.8248</b> | 0.594  | 0.3549 | 0.5607 | Brown         | ommochromes                    | xanthommatin                                                                                                                                                               | 48 30 4     | #333300 | #301e04 | Satyrini   | Satyrinae    | Nymphalidae | Ditrysia | Lepidoptera | Feb-16 |
| <i>Melanargia galathea</i>   | 2.254  | 0.8617        | 0.4142 | 3.2546 | <b>3.7887</b>                       | <b>1.5604</b> | 1.3902 | <b>2.1946</b> | 0.6176 | 0.6184 | 0.9162 | Brown         | ommochromes                    | xanthommatin                                                                                                                                                               | 54 38 11    | #333300 | #36260b | Satyrini   | Satyrinae    | Nymphalidae | Ditrysia | Lepidoptera | Feb-16 |
| <i>Melanargia galathea</i>   | 1.9038 | 0.7741        | 0.2859 | 2.3587 | 1.765                               | 1.3671        | 1.2396 | 1.4863        | 0.5918 | 0.3031 | 0.4862 | Yellow        | flavonoids                     | Anthoxanthin                                                                                                                                                               | 250 238 148 | #ffff99 | #faee94 | Satyrini   | Satyrinae    | Nymphalidae | Ditrysia | Lepidoptera | Feb-16 |
| <i>Melanargia galathea</i>   | 2.0417 | 0.7002        | 0.3091 | 2.3974 | 1.9341                              | 1.3603        | 1.3338 | 1.8549        | 0.5373 | 0.393  | 0.5542 | Yellow        | flavonoids                     | Anthoxanthin                                                                                                                                                               | 225 217 144 | #cccc99 | #e1d990 | Satyrini   | Satyrinae    | Nymphalidae | Ditrysia | Lepidoptera | Feb-16 |
| <i>Melanargia galathea</i>   | 1.9695 | 0.6724        | 0.2908 | 2.2214 | 1.7852                              | 1.4415        | 1.4972 | 2.6849        | 0.6583 | 0.4078 | 0.5666 | Yellow        | flavonoids                     | Anthoxanthin                                                                                                                                                               | 223 217 150 | #cccc99 | #dfd996 | Satyrini   | Satyrinae    | Nymphalidae | Ditrysia | Lepidoptera | Feb-16 |
| <i>Argynnis adippe</i>       | 2.6847 | 0.544         | 0.4819 | 2.2044 | 1.7413                              | 1.5877        | 1.413  | 2.7345        | 0.5806 | 0.3912 | 0.5774 | Black         | melanins                       | melanins                                                                                                                                                                   | 32 15 4     | #330000 | #200f04 | Argynniini | Heliconiinae | Nymphalidae | Ditrysia | Lepidoptera | Feb-16 |
| <i>Argynnis adippe</i>       | 2.6895 | 0.5531        | 0.4339 | 2.2037 | 1.8908                              | 1.3748        | 1.4612 | 2.1834        | 0.5436 | 0.3585 | 0.5285 | Black         | melanins                       | melanins                                                                                                                                                                   | 33 16 5     | #330000 | #211005 | Argynniini | Heliconiinae | Nymphalidae | Ditrysia | Lepidoptera | Feb-16 |
| <i>Argynnis adippe</i>       | 3.1326 | 0.5616        | 0.6867 | 2.4956 | 1.9944                              | 1.7098        | 1.9379 | 2.0218        | 0.6111 | 0.362  | 0.6101 | Black         | melanins                       | melanins                                                                                                                                                                   | 36 21 7     | #330000 | #241507 | Argynniini | Heliconiinae | Nymphalidae | Ditrysia | Lepidoptera | Feb-16 |
| <i>Argynnis adippe</i>       | 3.279  | <b>0.6476</b> | 0.5383 | 2.229  | <b>4.752</b>                        | 1.5057        | 1.7197 | 2.3615        | 0.5555 | 0.3483 | 0.5974 | Orange        | 1: ommochromes. 2: carotenoids | 1: dihydroxanthommatin. 2: lycopene, α-carotene, β-carotene, β-cryptoxanthin, lutein, α-doradexanthin, adonirubin, astaxanthin, lutein epoxide, neoxanthin and flavochrome | 214 137 1   | #cc9900 | #d68901 | Argynniini | Heliconiinae | Nymphalidae | Ditrysia | Lepidoptera | Feb-16 |
| <i>Argynnis adippe</i>       | 2.9777 | <b>0.6206</b> | 0.4665 | 2.0264 | <b>4.1141</b>                       | 1.413         | 1.3931 | 2.1011        | 0.5548 | 0.3654 | 0.5224 | Orange        | 1: ommochromes. 2: carotenoids | 1: dihydroxanthommatin. 2: lycopene, α-carotene, β-carotene, β-cryptoxanthin, lutein, α-doradexanthin, adonirubin, astaxanthin, lutein epoxide, neoxanthin and flavochrome | 209 134 1   | #cc9900 | #d18601 | Argynniini | Heliconiinae | Nymphalidae | Ditrysia | Lepidoptera | Feb-16 |
| <i>Argynnis adippe</i>       | 3.0691 | <b>0.6644</b> | 0.5338 | 2.0229 | <b>4.4234</b>                       | 1.624         | 1.4111 | 2.3224        | 0.5699 | 0.3623 | 0.5398 | Orange        | 1: ommochromes. 2: carotenoids | 1: dihydroxanthommatin. 2: lycopene, α-carotene, β-carotene, β-cryptoxanthin, lutein, α-doradexanthin, adonirubin, astaxanthin, lutein epoxide, neoxanthin and flavochrome | 220 144 3   | #cc9900 | #dc9003 | Argynniini | Heliconiinae | Nymphalidae | Ditrysia | Lepidoptera | Feb-16 |

| species                      | P      | S             | Cl     | K      | concentration (µg/cm <sup>2</sup> ) |               |        | Fe      | Ni     | Cu            | Zn             | visual colour | coloration mechanism           | specific coloration                                                                                                                                          | RGB values  | websafe | hex     | tribe         | subfamily      | family       | suborder | order       | run    |
|------------------------------|--------|---------------|--------|--------|-------------------------------------|---------------|--------|---------|--------|---------------|----------------|---------------|--------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------|---------|---------|---------------|----------------|--------------|----------|-------------|--------|
|                              |        |               |        |        | Ca                                  | Ti            | Mn     |         |        |               |                |               |                                |                                                                                                                                                              |             |         |         |               |                |              |          |             |        |
| <i>Aglais urticae</i>        | 0.286  | 0.0551        | 0.0287 | 0.1007 | 1.3369                              | 0.522         | 0.2642 | 1.5698  | 0.1334 | 0.0624        | 0.3108         | Black         | melanins                       | melanins                                                                                                                                                     | 49 39 41    | #333333 | #31272a | Nymphalini    | Nymphalinae    | Nymphalidae  | Ditrysia | Lepidoptera | Jun-17 |
| <i>Aglais urticae</i>        | 0.2366 | 0.0552        | 0.0318 | 0.1002 | 1.0001                              | 0.4502        | 0.2489 | 0.8637  | 0.1225 | 0.0549        | 0.1274         | Black         | melanins                       | melanins                                                                                                                                                     | 34 29 31    | #333333 | #221d1f | Nymphalini    | Nymphalinae    | Nymphalidae  | Ditrysia | Lepidoptera | Jun-17 |
| <i>Aglais urticae</i>        | 0.3754 | 0.0653        | 0.0263 | 0.1254 | 3.3083                              | 1.0207        | 0.4382 | 0.1564  | 0.1274 | 0.0549        | 0.1274         | Black         | N/A                            | N/A                                                                                                                                                          | 94 80 73    | #666633 | #5e5049 | Nymphalini    | Nymphalinae    | Nymphalidae  | Ditrysia | Lepidoptera | Jun-17 |
| <i>Aglais urticae</i>        | 0.3312 | 0.0638        | 0.0231 | 0.1082 | <b>2.6582</b>                       | <b>0.8167</b> | 0.3892 | 2.9163  | 0.1454 | 0.0671        | 0.3948         | Brown         | N/A                            | N/A                                                                                                                                                          | 100 83 72   | #666633 | #645248 | Nymphalini    | Nymphalinae    | Nymphalidae  | Ditrysia | Lepidoptera | Jun-17 |
| <i>Aglais urticae</i>        | 0.2403 | 0.0656        | 0.0279 | 0.1011 | 0.9815                              | 0.3551        | 0.2421 | 0.6338  | 0.1194 | 0.0578        | 0.0847         | Orange        | 1: ommochromes. 2: carotenoids | 1: xanthommatin, rhodommatin and ommatin D. 2: lycopene, β-carotene, zeaxanthin, lutein, adonirubin, astaxanthin, lutein epoxide, neoxanthin and flavochrome | 228 103 35  | #cc6633 | #e46723 | Nymphalini    | Nymphalinae    | Nymphalidae  | Ditrysia | Lepidoptera | Jun-17 |
| <i>Aglais urticae</i>        | 0.2178 | 0.0534        | 0.0262 | 0.1003 | 0.6294                              | 0.3581        | 0.2329 | 0.8724  | 0.1219 | 0.0516        | <b>0.073</b>   | Yellow        | N/A                            | N/A                                                                                                                                                          | 240 189 105 | #f0bd69 | #ffc666 | Nymphalini    | Nymphalinae    | Nymphalidae  | Ditrysia | Lepidoptera | Jun-17 |
| <i>Aglais urticae</i>        | 0.2621 | 0.0605        | 0.0303 | 0.0993 | <b>1.1244</b>                       | 0.4972        | 0.2625 | 1.4115  | 0.1279 | 0.0586        | 0.2316         | White         | N/A                            | N/A                                                                                                                                                          | 204 210 217 | #cccccc | #ccd2d9 | Nymphalini    | Nymphalinae    | Nymphalidae  | Ditrysia | Lepidoptera | Jun-17 |
| <i>Aglais urticae</i>        | 0.2515 | 0.0617        | 0.0302 | 0.1016 | <b>1.3815</b>                       | 0.5911        | 0.3092 | 1.9269  | 0.1325 | 0.0655        | 0.2262         | White         | N/A                            | N/A                                                                                                                                                          | 204 204 202 | #cccccc | #ccccc  | Nymphalini    | Nymphalinae    | Nymphalidae  | Ditrysia | Lepidoptera | Jun-17 |
| <i>Aglais urticae</i>        | 0.2709 | 0.0644        | 0.0252 | 0.0973 | <b>1.9619</b>                       | 0.5181        | 0.2938 | 1.3197  | 0.1375 | 0.0619        | 0.2143         | Blue          | N/A                            | N/A                                                                                                                                                          | 81 86 113   | #666666 | #515671 | Nymphalini    | Nymphalinae    | Nymphalidae  | Ditrysia | Lepidoptera | Jun-17 |
| <i>Aglais urticae</i>        | 0.1961 | 0.0475        | 0.0233 | 0.0874 | 0.7271                              | 0.3247        | 0.2086 | 0.7787  | 0.1139 | 0.0506        | <b>0.0729</b>  | Black         | melanins                       | melanins                                                                                                                                                     | 34 26 24    | #333300 | #221a18 | Nymphalini    | Nymphalinae    | Nymphalidae  | Ditrysia | Lepidoptera | Jun-17 |
| <i>Aglais urticae</i>        | 0.1816 | 0.061         | 0.0243 | 0.0958 | 0.9188                              | 0.3336        | 0.1965 | 0.4995  | 0.1127 | 0.0507        | <b>0.0704</b>  | Black         | melanins                       | melanins                                                                                                                                                     | 25 21 23    | #000000 | #191517 | Nymphalini    | Nymphalinae    | Nymphalidae  | Ditrysia | Lepidoptera | Jun-17 |
| <i>Aglais urticae</i>        | 0.1902 | 0.0414        | 0.0219 | 0.0846 | 0.3314                              | 0.2855        | 0.1911 | 0.3641  | 0.109  | 0.0479        | <b>0.0617</b>  | Brown         | N/A                            | N/A                                                                                                                                                          | 60 41 33    | #333333 | #3c2921 | Nymphalini    | Nymphalinae    | Nymphalidae  | Ditrysia | Lepidoptera | Jun-17 |
| <i>Aglais urticae</i>        | 0.1908 | 0.0383        | 0.0188 | 0.0807 | 0.3356                              | 0.3162        | 0.1999 | 0.4836  | 0.1067 | 0.051         | 0.0657         | Brown         | N/A                            | N/A                                                                                                                                                          | 68 53 46    | #333333 | #44352e | Nymphalini    | Nymphalinae    | Nymphalidae  | Ditrysia | Lepidoptera | Jun-17 |
| <i>Aglais urticae</i>        | 0.1769 | 0.0427        | 0.0181 | 0.0771 | 0.4247                              | 0.2551        | 0.192  | 0.363   | 0.1074 | 0.045         | 0.0555         | Orange        | 1: ommochromes. 2: carotenoids | 1: xanthommatin, rhodommatin and ommatin D. 2: lycopene, β-carotene, zeaxanthin, lutein, adonirubin, astaxanthin, lutein epoxide, neoxanthin and flavochrome | 201 97 36   | #cc6633 | #c96124 | Nymphalini    | Nymphalinae    | Nymphalidae  | Ditrysia | Lepidoptera | Jun-17 |
| <i>Aglais urticae</i>        | 0.2008 | 0.0496        | 0.0228 | 0.0926 | 0.5203                              | 0.3068        | 0.1906 | 0.4138  | 0.1049 | 0.0501        | 0.0637         | Orange        | 1: ommochromes. 2: carotenoids | 1: xanthommatin, rhodommatin and ommatin D. 2: lycopene, β-carotene, zeaxanthin, lutein, adonirubin, astaxanthin, lutein epoxide, neoxanthin and flavochrome | 194 92 32   | #cc6633 | #c25c20 | Nymphalini    | Nymphalinae    | Nymphalidae  | Ditrysia | Lepidoptera | Jun-17 |
| <i>Aglais urticae</i>        | 0.1779 | 0.0386        | 0.0173 | 0.0785 | 0.3213                              | 0.2697        | 0.1858 | 0.476   | 0.1107 | 0.0483        | 0.064          | Yellow        | N/A                            | N/A                                                                                                                                                          | 179 157 109 | #cc9966 | #b39d6d | Nymphalini    | Nymphalinae    | Nymphalidae  | Ditrysia | Lepidoptera | Jun-17 |
| <i>Aglais urticae</i>        | 0.1751 | 0.035         | 0.0137 | 0.076  | 0.2932                              | 0.2755        | 0.1751 | 0.3416  | 0.111  | 0.0488        | 0.0618         | Blue          | N/A                            | N/A                                                                                                                                                          | 90 111 155  | #666699 | #5a6f9b | Nymphalini    | Nymphalinae    | Nymphalidae  | Ditrysia | Lepidoptera | Jun-17 |
| <i>Cupido comyntas</i>       | 2.9725 | 0.3378        | 0.2743 | 1.5609 | 0.7294                              | 1.3581        | 1.4408 | 1.1682  | 0.468  | 0.192         | 0.4278         | Brown         | flavonoids                     | quercetin and kaempferol 3-glycoside                                                                                                                         | 69 58 18    | #333300 | #453a12 | Polyommataini | Polyommatainae | Lycaenidae   | Ditrysia | Lepidoptera | Feb-16 |
| <i>Cupido comyntas</i>       | 2.2911 | 0.4035        | 0.2535 | 1.4618 | 0.628                               | <b>1.6218</b> | 1.5921 | 0.9458  | 0.5186 | 0.2251        | 0.2998         | Orange        | carotenoids                    | torulene, β-cryptoxanthin, zeaxanthin, q-doradexanthin, adonirubin, astaxanthin, lutein epoxide, violaxanthin and auochrome                                  | 206 162 24  | #cc9900 | #cea218 | Polyommataini | Polyommatainae | Lycaenidae   | Ditrysia | Lepidoptera | Feb-16 |
| <i>Cupido comyntas</i>       | 2.3941 | 0.4573        | 0.2187 | 1.4239 | 0.6006                              | <b>1.7808</b> | 1.2318 | 0.9505  | 0.5578 | 0.2272        | 0.7642         | Orange        | carotenoids                    | torulene, β-cryptoxanthin, zeaxanthin, q-doradexanthin, adonirubin, astaxanthin, lutein epoxide, violaxanthin and auochrome                                  | 208 167 23  | #cc9900 | #d0a717 | Polyommataini | Polyommatainae | Lycaenidae   | Ditrysia | Lepidoptera | Feb-16 |
| <i>Cupido comyntas</i>       | 2.5374 | 0.3487        | 0.2329 | 1.3439 | 0.5099                              | 1.2017        | 1.44   | 1.2247  | 0.4339 | 0.1975        | 0.2106         | White         | structural                     | N/A                                                                                                                                                          | 201 220 176 | #cccccc | #c9dcb0 | Polyommataini | Polyommatainae | Lycaenidae   | Ditrysia | Lepidoptera | Feb-16 |
| <i>Cupido comyntas</i>       | 2.5222 | 0.4156        | 0.2383 | 1.3572 | 0.4981                              | 1.5758        | 1.3371 | 1.0078  | 0.56   | 0.2132        | 0.2771         | White         | structural                     | N/A                                                                                                                                                          | 205 225 183 | #cccccc | #cde1b7 | Polyommataini | Polyommatainae | Lycaenidae   | Ditrysia | Lepidoptera | Feb-16 |
| <i>Epargyreus clarus</i>     | 1.4646 | 0.4064        | 0.2432 | 1.4658 | 0.6953                              | 1.3284        | 1.4604 | 1.2943  | 0.5124 | 0.4215        | 0.3924         | Brown         | flavonoids                     | anthoxanthin                                                                                                                                                 | 111 86 34   | #666633 | #6f5622 | Eudamini      | Pyrginae       | Hesperiidae  | Ditrysia | Lepidoptera | Feb-16 |
| <i>Epargyreus clarus</i>     | 1.8652 | 0.3881        | 0.2691 | 1.4061 | 0.6045                              | 1.292         | 1.4799 | 2.1532  | 0.5542 | 0.402         | 0.4031         | Brown         | flavonoids                     | anthoxanthin                                                                                                                                                 | 103 83 29   | #666633 | #67531d | Eudamini      | Pyrginae       | Hesperiidae  | Ditrysia | Lepidoptera | Feb-16 |
| <i>Epargyreus clarus</i>     | 1.8765 | 0.3649        | 0.2532 | 1.4189 | 0.6676                              | 1.543         | 1.422  | 1.3154  | 0.6141 | 0.8121        | 0.4605         | Brown         | flavonoids                     | anthoxanthin                                                                                                                                                 | 80 65 30    | #663333 | #50411e | Eudamini      | Pyrginae       | Hesperiidae  | Ditrysia | Lepidoptera | Feb-16 |
| <i>Epargyreus clarus</i>     | 1.8553 | 0.4953        | 0.2537 | 1.729  | 1.0347                              | 1.4261        | 1.3936 | 1.3069  | 0.511  | <b>0.7816</b> | <b>0.6826</b>  | Silver        | N/A                            | N/A                                                                                                                                                          | 198 213 177 | #cccc99 | #c6d5d1 | Eudamini      | Pyrginae       | Hesperiidae  | Ditrysia | Lepidoptera | Feb-16 |
| <i>Epargyreus clarus</i>     | 1.4674 | 0.5174        | 0.2548 | 1.7037 | 0.9163                              | 1.0962        | 1.5552 | 1.3447  | 0.5966 | <b>0.5449</b> | <b>0.564</b>   | Silver        | N/A                            | N/A                                                                                                                                                          | 205 205 222 | #cccccc | #cdcdde | Eudamini      | Pyrginae       | Hesperiidae  | Ditrysia | Lepidoptera | Feb-16 |
| <i>Epargyreus clarus</i>     | 1.8655 | 0.3976        | 0.2827 | 1.6738 | 0.7376                              | 1.3037        | 1.7165 | 1.0748  | 0.5214 | <b>1.9746</b> | <b>0.6591</b>  | Silver        | N/A                            | N/A                                                                                                                                                          | 199 215 178 | #cccc99 | #c7d7b2 | Eudamini      | Pyrginae       | Hesperiidae  | Ditrysia | Lepidoptera | Feb-16 |
| <i>Epargyreus clarus</i>     | 1.7705 | 0.4549        | 0.3117 | 1.535  | 0.7209                              | 1.3563        | 1.7087 | 1.5801  | 0.5578 | 0.2584        | 0.3562         | Brown         | N/A                            | N/A                                                                                                                                                          | 115 93 22   | #666600 | #735d1b | Eudamini      | Pyrginae       | Hesperiidae  | Ditrysia | Lepidoptera | Feb-16 |
| <i>Epargyreus clarus</i>     | 1.6789 | 0.4179        | 0.2844 | 1.4547 | 0.8397                              | 1.284         | 1.3784 | 1.7231  | 0.567  | 0.2547        | 0.2588         | Brown         | N/A                            | N/A                                                                                                                                                          | 118 88 14   | #666600 | #76580e | Eudamini      | Pyrginae       | Hesperiidae  | Ditrysia | Lepidoptera | Feb-16 |
| <i>Epargyreus clarus</i>     | 1.7178 | 0.4431        | 0.285  | 1.4913 | 0.6762                              | 1.4264        | 1.4672 | 1.1647  | 0.4826 | 0.2424        | 0.2741         | Brown         | N/A                            | N/A                                                                                                                                                          | 109 84 11   | #666600 | #6d540b | Eudamini      | Pyrginae       | Hesperiidae  | Ditrysia | Lepidoptera | Feb-16 |
| <i>Epargyreus clarus</i>     | 1.8274 | 0.4824        | 0.2951 | 1.5388 | 0.6518                              | 1.3645        | 1.9356 | 1.8406  | 0.571  | 0.2481        | 0.3237         | Yellow        | N/A                            | N/A                                                                                                                                                          | 164 143 18  | #999900 | #a48f12 | Eudamini      | Pyrginae       | Hesperiidae  | Ditrysia | Lepidoptera | Feb-16 |
| <i>Epargyreus clarus</i>     | 1.5294 | 0.4383        | 0.303  | 1.5195 | 0.8038                              | 1.6366        | 1.8069 | 1.7267  | 0.5129 | 0.2388        | 0.3757         | Yellow        | N/A                            | N/A                                                                                                                                                          | 200 185 64  | #cccc33 | #c6b940 | Eudamini      | Pyrginae       | Hesperiidae  | Ditrysia | Lepidoptera | Feb-16 |
| <i>Epargyreus clarus</i>     | 1.7585 | 0.4059        | 0.3256 | 1.5336 | 0.9466                              | 1.3915        | 2.2137 | 1.8106  | 0.5569 | 0.2475        | 0.3392         | Yellow        | N/A                            | N/A                                                                                                                                                          | 182 150 6   | #cc9900 | #b69606 | Eudamini      | Pyrginae       | Hesperiidae  | Ditrysia | Lepidoptera | Feb-16 |
| <i>Parnassius apollonius</i> | 3.5756 | 0.9636        | 0.595  | 2.8075 | 5.6508                              | 2.2882        | 1.9469 | 9.1244  | 1.0938 | 0.5681        | 35.107         | Black         | melanins                       | melanins                                                                                                                                                     | 23 17 12    | #000000 | #17110c | Parnasiini    | Parnasiinae    | Papilionidae | Ditrysia | Lepidoptera | Feb-16 |
| <i>Parnassius apollonius</i> | 2.8575 | 0.8439        | 0.5002 | 2.8908 | 4.9781                              | 3.3757        | 1.9257 | 18.1872 | 0.7693 | 0.5446        | 24.1147        | Black         | melanins                       | melanins                                                                                                                                                     | 21 18 14    | #000000 | #15120e | Parnasiini    | Parnasiinae    | Papilionidae | Ditrysia | Lepidoptera | Feb-16 |
| <i>Parnassius apollonius</i> | 3.1508 | 0.8832        | 0.609  | 2.2086 | 4.398                               | 2.7883        | 2.2725 | 8.5948  | 1.2469 | 0.6641        | 45.0871        | Black         | melanins                       | melanins                                                                                                                                                     | 21 17 11    | #000000 | #15110b | Parnasiini    | Parnasiinae    | Papilionidae | Ditrysia | Lepidoptera | Feb-16 |
| <i>Parnassius apollonius</i> | 2.9284 | 0.7584        | 0.5443 | 2.0533 | 4.3633                              | 2.3612        | 2.2169 | 11.9659 | 1.0202 | 0.54          | <b>33.7284</b> | Yellow        | ommochromes                    | papiliochrome II                                                                                                                                             | 253 250 204 | #ffcc   | #dfacc  | Parnasiini    | Parnasiinae    | Papilionidae | Ditrysia | Lepidoptera | Feb-16 |
| <i>Parnassius apollonius</i> | 3.4212 | 0.8546        | 0.6306 | 1.9738 | 4.9934                              | 2.9215        | 2.6107 | 15.7248 | 1.3506 | 0.8556        | <b>58.1438</b> | Yellow        | ommochromes                    | papiliochrome II                                                                                                                                             | 249 244 198 | #ffcc   | #9f4c6  | Parnasiini    | Parnasiinae    | Papilionidae | Ditrysia | Lepidoptera | Feb-16 |
| <i>Parnassius apollonius</i> | 3.5543 | 1.0071        | 0.6175 | 2.2332 | 8.0893                              | 3.7635        | 2.7685 | 23.3625 | 1.3148 | 0.8041        | <b>49.627</b>  | Yellow        | ommochromes                    | papiliochrome II                                                                                                                                             | 251 247 103 | #ffcc   | #b767   | Parnasiini    | Parnasiinae    | Papilionidae | Ditrysia | Lepidoptera | Feb-16 |
| <i>Parnassius apollonius</i> | 2.8982 | <b>1.7027</b> | 0.7879 | 2.3145 | 4.4924                              | 2.4465        | 1.9961 | 5.3155  | 0.9523 | 0.5884        | 41.9923        | Red           | melanins                       | melanins                                                                                                                                                     | 242 95 37   | #ff6633 | #f25252 | Parnasiini    | Parnasiinae    | Papilionidae | Ditrysia | Lepidoptera | Feb-16 |
| <i>Parnassius apollonius</i> | 3.1582 | <b>1.5976</b> | 0.7172 | 2.219  | 14.1522                             | 2.2193        | 2.6615 | 10.6274 | 1.2874 | 0.7204        | 47.2107        | Red           | melanins                       | melanins                                                                                                                                                     | 241 96 46   | #ff6633 | #f1602e | Parnasiini    | Parnasiinae    | Papilionidae | Ditrysia | Lepidoptera | Feb-16 |

| species                      | P      | S      | Cl     | K      | concentration (µg/cm <sup>2</sup> ) |        |         |         | Fe     | Ni     | Cu     | Zn     | visual colour | coloration mechanism          | specific coloration                                                       | RGB values   | websafe | hex     | tribe       | subfamily    | family       | suborder  | order       | run    |
|------------------------------|--------|--------|--------|--------|-------------------------------------|--------|---------|---------|--------|--------|--------|--------|---------------|-------------------------------|---------------------------------------------------------------------------|--------------|---------|---------|-------------|--------------|--------------|-----------|-------------|--------|
|                              |        |        |        |        | Ca                                  | Ti     | Mn      |         |        |        |        |        |               |                               |                                                                           |              |         |         |             |              |              |           |             |        |
| <i>Papilio glaucus</i>       | 2.6006 | 0.4626 | 0.2391 | 1.5238 | 1.0391                              | 2.2821 | 1.6313  | 1.324   | 0.7146 | 0.2861 | 0.3469 | 0.3469 | Black         | melanins                      | melanins                                                                  | 38 30 5      | #333300 | #261e05 | Papilionini | Papilioninae | Papilionidae | Ditrysia  | Lepidoptera | Feb-16 |
| <i>Papilio glaucus</i>       | 2.7318 | 0.5028 | 0.244  | 1.5246 | 1.0696                              | 2.4339 | 1.7563  | 1.7279  | 0.7127 | 0.2995 | 0.3609 | 0.3609 | Black         | melanins                      | melanins                                                                  | 38 29 5      | #333300 | #241d05 | Papilionini | Papilioninae | Papilionidae | Ditrysia  | Lepidoptera | Feb-16 |
| <i>Papilio glaucus</i>       | 2.7318 | 0.483  | 0.2707 | 1.4859 | 0.8069                              | 2.4972 | 1.8619  | 1.3629  | 0.8681 | 0.2811 | 0.3541 | 0.3541 | Orange        | melanins                      | melanins                                                                  | 201 131 0    | #c0cc33 | #d0e054 | Papilionini | Papilioninae | Papilionidae | Ditrysia  | Lepidoptera | Feb-16 |
| <i>Papilio glaucus</i>       | 2.7911 | 0.4039 | 0.238  | 1.4433 | 0.7319                              | 2.1986 | 1.6949  | 1.3892  | 0.7019 | 0.2484 | 0.3473 | 0.3473 | Yellow        | ommochromes                   | papiliochrome II                                                          | 225 209 59   | #cccc33 | #d1d13b | Papilionini | Papilioninae | Papilionidae | Ditrysia  | Lepidoptera | Feb-16 |
| <i>Papilio glaucus</i>       | 2.8281 | 0.4149 | 0.2182 | 1.4661 | 0.7328                              | 2.5596 | 1.7935  | 1.5571  | 0.7085 | 0.2998 | 0.3802 | 0.3802 | Yellow        | ommochromes                   | papiliochrome II                                                          | 221 206 41   | #cccc33 | #d0e029 | Papilionini | Papilioninae | Papilionidae | Ditrysia  | Lepidoptera | Feb-16 |
| <i>Papilio glaucus</i>       | 2.5471 | 0.4457 | 0.2506 | 1.5624 | 0.8971                              | 2.5398 | 1.7877  | 1.5646  | 0.6141 | 0.2604 | 0.3287 | 0.3287 | Blue          | 1: ommochromes. 2: structural | 1: papiliochrome II. 2: structural                                        | 89 136 147   | #669999 | #598893 | Papilionini | Papilioninae | Papilionidae | Ditrysia  | Lepidoptera | Feb-16 |
| <i>Papilio glaucus</i>       | 2.893  | 0.4326 | 0.232  | 1.4228 | 0.9049                              | 2.3254 | 1.5452  | 1.3634  | 0.7228 | 0.2918 | 0.378  | 0.378  | Blue          | 1: ommochromes. 2: structural | 1: papiliochrome II. 2: structural                                        | 102 139 147  | #669999 | #668b93 | Papilionini | Papilioninae | Papilionidae | Ditrysia  | Lepidoptera | Feb-16 |
| <i>Papilio nireus</i>        | 1.5698 | 0.2777 | 0.2769 | 1.5223 | 1.1514                              | 1.0031 | 0.7948  | 1.5102  | 0.5251 | 0.2077 | 0.2773 | 0.2773 | Black         | melanins                      | melanins                                                                  | 41 30 17     | #333300 | #291e11 | Papilionini | Papilioninae | Papilionidae | Ditrysia  | Lepidoptera | Nov-17 |
| <i>Papilio nireus</i>        | 1.534  | 0.4156 | 0.2617 | 1.4242 | 1.2372                              | 1.0344 | 0.8053  | 2.037   | 0.4828 | 0.1957 | 0.3826 | 0.3826 | Black         | melanins                      | melanins                                                                  | 41 30 19     | #333300 | #291e13 | Papilionini | Papilioninae | Papilionidae | Ditrysia  | Lepidoptera | Nov-17 |
| <i>Papilio nireus</i>        | 1.5904 | 0.4709 | 0.2899 | 1.4971 | 1.2052                              | 0.9589 | 0.8591  | 1.408   | 0.4385 | 0.1948 | 0.3163 | 0.3163 | Black         | melanins                      | melanins                                                                  | 42 29 18     | #333300 | #2a1d12 | Papilionini | Papilioninae | Papilionidae | Ditrysia  | Lepidoptera | Nov-17 |
| <i>Papilio nireus</i>        | 1.6031 | 0.862  | 0.3553 | 1.8844 | 2.1266                              | 1.1006 | 0.9733  | 3.0371  | 0.4918 | 0.225  | 0.4008 | 0.4008 | Green         | ommochromes                   | papiliochrome II                                                          | 173 199 127  | #99cc66 | #adc77f | Papilionini | Papilioninae | Papilionidae | Ditrysia  | Lepidoptera | Nov-17 |
| <i>Papilio nireus</i>        | 1.6584 | 0.8249 | 0.3165 | 1.635  | 1.8404                              | 1.0792 | 0.7261  | 1.3146  | 0.4862 | 0.2237 | 0.3814 | 0.3814 | Green         | ommochromes                   | papiliochrome II                                                          | 180 206 128  | #cccc99 | #b4ce80 | Papilionini | Papilioninae | Papilionidae | Ditrysia  | Lepidoptera | Nov-17 |
| <i>Papilio nireus</i>        | 1.734  | 0.8275 | 0.3577 | 1.8195 | 1.9617                              | 0.9702 | 0.8066  | 1.792   | 0.4608 | 0.2183 | 0.3831 | 0.3831 | Green         | ommochromes                   | papiliochrome II                                                          | 184 207 122  | #cccc66 | #b8c77a | Papilionini | Papilioninae | Papilionidae | Ditrysia  | Lepidoptera | Nov-17 |
| <i>Zygaena filipendulae</i>  | 3.0164 | 0.934  | 0.7168 | 4.6913 | 10.6763                             | 1.982  | 2.3052  | 7.4857  | 0.6862 | 0.6561 | 1.1648 | 1.1648 | Brown         | N/A                           | N/A                                                                       | 118 90 8     | #666600 | #765a08 | Zygaenini   | Zygaeninae   | Zygaenidae   | Ditrysia  | Lepidoptera | Feb-16 |
| <i>Zygaena filipendulae</i>  | 3.1983 | 0.926  | 0.6034 | 3.9305 | 12.3791                             | 2.5046 | 2.759   | 8.6182  | 0.6808 | 0.6268 | 1.099  | 1.099  | Brown         | N/A                           | N/A                                                                       | 104 85 15    | #666600 | #66550f | Zygaenini   | Zygaeninae   | Zygaenidae   | Ditrysia  | Lepidoptera | Feb-16 |
| <i>Zygaena filipendulae</i>  | 2.8143 | 1.013  | 0.6742 | 4.0671 | 7.6861                              | 2.3199 | 2.0494  | 5.5458  | 0.6374 | 0.6261 | 1.2586 | 1.2586 | Brown         | N/A                           | N/A                                                                       | 117 97 12    | #666600 | #75610c | Zygaenini   | Zygaeninae   | Zygaenidae   | Ditrysia  | Lepidoptera | Feb-16 |
| <i>Zygaena filipendulae</i>  | 4.0031 | 2.3153 | 0.8871 | 4.6895 | 24.1306                             | 3.0714 | 2.9007  | 14.7144 | 0.7098 | 0.7208 | 1.3452 | 1.3452 | Red           | carotenoids                   | zeaxanthin, lutein, astaxanthin and mutatochrome                          | 165 13 4     | #990000 | #a50d04 | Zygaenini   | Zygaeninae   | Zygaenidae   | Ditrysia  | Lepidoptera | Feb-16 |
| <i>Zygaena filipendulae</i>  | 4.1141 | 1.9927 | 0.7721 | 4.1357 | 20.7157                             | 7.1963 | 2.8094  | 16.8571 | 0.683  | 1.1418 | 1.5053 | 1.5053 | Red           | carotenoids                   | zeaxanthin, lutein, astaxanthin and mutatochrome                          | 165 16 4     | #990000 | #a51004 | Zygaenini   | Zygaeninae   | Zygaenidae   | Ditrysia  | Lepidoptera | Feb-16 |
| <i>Zygaena filipendulae</i>  | 3.3901 | 1.7315 | 0.6397 | 3.3002 | 12.6184                             | 1.7195 | 1.7988  | 3.0529  | 0.6127 | 0.6498 | 0.9986 | 0.9986 | Red           | carotenoids                   | Y-carotene, rubixanthin, zeaxanthin, lutein, astaxanthin and mutatochrome | 173 17 3     | #990000 | #ad1103 | Zygaenini   | Zygaeninae   | Zygaenidae   | Ditrysia  | Lepidoptera | Feb-16 |
| <i>Erasmia pulchella</i>     | 1.5727 | 0.4538 | 0.2921 | 1.3926 | 4.8517                              | 1.0195 | 1.0429  | 2.0915  | 0.4316 | 0.3622 | 0.4818 | 0.4818 | Brown         | flavonoids                    | anthoxanthin                                                              | 16 11 7      | #000000 | #100b07 | N/A         | Chalcosinae  | Zygaenidae   | Ditrysia  | Lepidoptera | Nov-17 |
| <i>Erasmia pulchella</i>     | 1.4597 | 0.4199 | 0.2412 | 1.3628 | 3.6412                              | 0.9709 | 1.0733  | 2.4242  | 0.4194 | 0.3622 | 0.4818 | 0.4818 | Brown         | flavonoids                    | anthoxanthin                                                              | 10 11 13     | #000000 | #00b008 | N/A         | Chalcosinae  | Zygaenidae   | Ditrysia  | Lepidoptera | Nov-17 |
| <i>Erasmia pulchella</i>     | 1.3958 | 0.3907 | 0.1787 | 1.3104 | 3.2582                              | 0.9286 | 0.8569  | 1.5148  | 0.4139 | 0.2193 | 0.3506 | 0.3506 | Brown         | flavonoids                    | anthoxanthin                                                              | 15 11 8      | #000000 | #0f0b08 | N/A         | Chalcosinae  | Zygaenidae   | Ditrysia  | Lepidoptera | Nov-17 |
| <i>Erasmia pulchella</i>     | 1.3851 | 0.3517 | 0.2178 | 1.2164 | 1.323                               | 1.0785 | 0.756   | 1.4055  | 0.4553 | 0.2063 | 0.3278 | 0.3278 | White         | N/A                           | N/A                                                                       | 164 146 68   | #999933 | #a49244 | N/A         | Chalcosinae  | Zygaenidae   | Ditrysia  | Lepidoptera | Nov-17 |
| <i>Erasmia pulchella</i>     | 1.3751 | 0.3737 | 0.2217 | 1.241  | 1.5163                              | 1.0092 | 0.7262  | 1.39    | 0.4333 | 0.2137 | 0.3421 | 0.3421 | White         | N/A                           | N/A                                                                       | 166 143 60   | #999933 | #a6f83c | N/A         | Chalcosinae  | Zygaenidae   | Ditrysia  | Lepidoptera | Nov-17 |
| <i>Erasmia pulchella</i>     | 1.6307 | 0.4274 | 0.2064 | 1.3736 | 6.1831                              | 1.1146 | 0.9884  | 1.8916  | 0.4491 | 0.3585 | 0.501  | 0.501  | Orange        | N/A                           | N/A                                                                       | 126 47 4     | #993300 | #802f04 | N/A         | Chalcosinae  | Zygaenidae   | Ditrysia  | Lepidoptera | Nov-17 |
| <i>Erasmia pulchella</i>     | 1.5792 | 0.3927 | 0.1921 | 1.3938 | 5.4364                              | 1.0375 | 0.9035  | 1.3752  | 0.441  | 0.2498 | 0.414  | 0.414  | Orange        | N/A                           | N/A                                                                       | 126 47 3     | #663300 | #7e2f03 | N/A         | Chalcosinae  | Zygaenidae   | Ditrysia  | Lepidoptera | Nov-17 |
| <i>Erasmia pulchella</i>     | 1.9432 | 0.4955 | 0.2575 | 1.9225 | 7.3102                              | 1.2334 | 1.237   | 2.2952  | 0.4914 | 0.3695 | 0.8597 | 0.8597 | Orange        | N/A                           | N/A                                                                       | 130 43 4     | #993300 | #822b04 | N/A         | Chalcosinae  | Zygaenidae   | Ditrysia  | Lepidoptera | Nov-17 |
| <i>Erasmia pulchella</i>     | 1.3909 | 0.3907 | 0.179  | 1.3033 | 3.3198                              | 0.9836 | 0.8767  | 1.1053  | 0.4226 | 0.2147 | 0.3571 | 0.3571 | Blue          | N/A                           | N/A                                                                       | 47 133 110   | #339966 | #2f855e | N/A         | Chalcosinae  | Zygaenidae   | Ditrysia  | Lepidoptera | Nov-17 |
| <i>Erasmia pulchella</i>     | 1.418  | 0.4873 | 0.1846 | 1.3896 | 3.4412                              | 0.998  | 0.7863  | 1.2412  | 0.4614 | 0.2347 | 0.3472 | 0.3472 | Blue          | N/A                           | N/A                                                                       | 46 132 114   | #339966 | #2f855e | N/A         | Chalcosinae  | Zygaenidae   | Ditrysia  | Lepidoptera | Nov-17 |
| <i>Erasmia pulchella</i>     | 1.7984 | 0.4157 | 0.19   | 1.3523 | 6.7465                              | 1.012  | 0.8415  | 1.7471  | 0.4189 | 0.3586 | 0.4811 | 0.4811 | Blue          | N/A                           | N/A                                                                       | 54 80 56     | #336633 | #365038 | N/A         | Chalcosinae  | Zygaenidae   | Ditrysia  | Lepidoptera | Nov-17 |
| <i>Chalcosia coliadoidea</i> | 2.0728 | 0.8077 | 0.4418 | 1.5565 | 3.7424                              | 1.1271 | 0.9132  | 5.7911  | 0.546  | 0.4887 | 0.5138 | 0.5138 | Brown         | flavonoids                    | anthoxanthin                                                              | 46 30 13     | #333300 | #2e1e0d | N/A         | Chalcosinae  | Zygaenidae   | Ditrysia  | Lepidoptera | Nov-17 |
| <i>Chalcosia coliadoidea</i> | 2.1123 | 0.7421 | 0.3799 | 1.6478 | 3.7932                              | 1.0621 | 1.1986  | 1.5181  | 0.5241 | 0.426  | 0.5952 | 0.5952 | Brown         | flavonoids                    | anthoxanthin                                                              | 68 55 35     | #333333 | #443723 | N/A         | Chalcosinae  | Zygaenidae   | Ditrysia  | Lepidoptera | Nov-17 |
| <i>Chalcosia coliadoidea</i> | 1.9653 | 0.6165 | 0.374  | 2.0411 | 5.2226                              | 1.3826 | 1.2968  | 3.5954  | 0.6    | 0.3979 | 0.8674 | 0.8674 | Brown         | flavonoids                    | anthoxanthin                                                              | 55 51 32     | #333333 | #373320 | N/A         | Chalcosinae  | Zygaenidae   | Ditrysia  | Lepidoptera | Nov-17 |
| <i>Chalcosia coliadoidea</i> | 1.9325 | 0.7284 | 0.3954 | 1.9059 | 2.5771                              | 1.0768 | 1.1582  | 7.3611  | 0.6075 | 0.3103 | 0.4756 | 0.4756 | White         | N/A                           | N/A                                                                       | 255 221 113  | #ffcc66 | #ffdd71 | N/A         | Chalcosinae  | Zygaenidae   | Ditrysia  | Lepidoptera | Nov-17 |
| <i>Chalcosia coliadoidea</i> | 1.8309 | 0.7193 | 0.3653 | 1.7907 | 1.9942                              | 1.0214 | 0.9329  | 5.5958  | 0.5649 | 0.357  | 0.5188 | 0.5188 | White         | N/A                           | N/A                                                                       | 254 222 116  | #ffcc66 | #fe6e74 | N/A         | Chalcosinae  | Zygaenidae   | Ditrysia  | Lepidoptera | Nov-17 |
| <i>Chalcosia coliadoidea</i> | 1.8325 | 0.5461 | 0.3243 | 1.5074 | 2.044                               | 1.1335 | 0.9367  | 3.0516  | 0.5871 | 0.2661 | 0.4511 | 0.4511 | White         | N/A                           | N/A                                                                       | 255 241 147  | #ffcc66 | #ff9193 | N/A         | Chalcosinae  | Zygaenidae   | Ditrysia  | Lepidoptera | Nov-17 |
| <i>Chalcosia coliadoidea</i> | 2.2044 | 1.0869 | 0.4908 | 1.8724 | 5.0214                              | 1.0096 | 1.2209  | 2.7093  | 0.574  | 0.5218 | 0.7083 | 0.7083 | Blue          | N/A                           | N/A                                                                       | 61 97 84     | #669999 | #598893 | N/A         | Chalcosinae  | Zygaenidae   | Ditrysia  | Lepidoptera | Nov-17 |
| <i>Chalcosia coliadoidea</i> | 2.1161 | 1.1304 | 0.4369 | 2.0186 | 4.7429                              | 1.26   | 1.122   | 6.9533  | 0.388  | 0.5268 | 0.6803 | 0.6803 | Blue          | N/A                           | N/A                                                                       | 54 92 80     | #669999 | #598893 | N/A         | Chalcosinae  | Zygaenidae   | Ditrysia  | Lepidoptera | Nov-17 |
| <i>Goliathus goliathus</i>   | 4.3583 | 1.0801 | 0.5029 | 4.3609 | 5.943                               | 3.2188 | 10.1704 | 7.1935  | 1.4092 | 1.2604 | 1.9055 | 1.9055 | Brown         | melanins                      | melanins                                                                  | 18 17 18     | #000000 | #121112 | Goliathini  | Cetoniinae   | Scarabaeidae | Polyphaga | Coleoptera  | Feb-16 |
| <i>Goliathus goliathus</i>   | 3.6547 | 0.9202 | 0.5144 | 4.3027 | 6.2831                              | 3.8029 | 10.3649 | 5.8525  | 1.3733 | 1.1155 | 1.8509 | 1.8509 | Brown         | melanins                      | melanins                                                                  | 17 17 18     | #000001 | #111112 | Goliathini  | Cetoniinae   | Scarabaeidae | Polyphaga | Coleoptera  | Feb-16 |
| <i>Goliathus goliathus</i>   | 5.3873 | 0.9532 | 0.5156 | 4.436  | 6.9455                              | 3.4389 | 11.9146 | 10.09   | 1.6629 | 1.3283 | 4.0144 | 4.0144 | Brown         | melanins                      | melanins                                                                  | 17 17 18     | #000002 | #111112 | Goliathini  | Cetoniinae   | Scarabaeidae | Polyphaga | Coleoptera  | Feb-16 |
| <i>Goliathus goliathus</i>   | 4.4577 | 0.981  | 0.6415 | 4.9567 | 9.3593                              | 5.2666 | 10.874  | 30.3872 | 1.9427 | 1.3777 | 2.5638 | 2.5638 | White         | 1: structural. 2: wax         | 1: structural. 2: wax                                                     | 168 184 110  | #99cc66 | #a8b86e | Goliathini  | Cetoniinae   | Scarabaeidae | Polyphaga | Coleoptera  | Feb-16 |
| <i>Goliathus goliathus</i>   | 4.4523 | 1.1941 | 0.6696 | 4.921  | 10.9756                             | 5.0181 | 11.4733 | 26.4128 | 1.5726 | 1.1909 | 2.4059 | 2.4059 | White         | 1: structural. 2: wax         | 1: structural. 2: wax                                                     | 131 154 118  | #999966 | #939a76 | Goliathini  | Cetoniinae   | Scarabaeidae | Polyphaga | Coleoptera  | Feb-16 |
| <i>Goliathus goliathus</i>   | 3.9994 | 0.9906 | 0.6467 | 4.8285 | 8.6198                              | 4.1478 | 24.3879 | 26.0164 | 1.5509 | 1.2482 | 2.0599 | 2.0599 | White         | 1: structural. 2: wax         | 1: structural. 2: wax                                                     | 154 176 1223 | #999966 | #9a0b7a | Goliathini  | Cetoniinae   | Scarabaeidae | Polyphaga | Coleoptera  | Feb-16 |
| <i>Aphodius sp.</i>          | 2.2452 | 0.4475 | 0.4073 | 2.4098 | 4.3946                              | 2.673  | 13.5768 | 18.6785 | 0.571  | 0.3442 | 0.8838 | 0.8838 | Brown         | melanins                      | melanins                                                                  | 45 37 5      | #333300 | #2d2505 | Aphodini    | Aphodiinae   | Scarabaeidae | Polyphaga | Coleoptera  | Feb-16 |
| <i>Aphodius sp.</i>          | 2.1459 | 0.5506 | 0.4437 | 2.3229 | 4.0407                              | 2.5657 | 13.6082 | 17.0928 | 0.6387 | 0.3653 | 0.7345 | 0.7345 |               |                               |                                                                           |              |         |         |             |              |              |           |             |        |

| species                  | P             | S             | CI     | K      | Ca             | concentration (µg/cm <sup>2</sup> ) | Fe             | Ni             | Cu     | Zn     | visual colour | coloration mechanism | specific coloration | RGB values  | websafe     | hex     | tribe        | subfamily     | family        | suborder      | order      | run        |        |
|--------------------------|---------------|---------------|--------|--------|----------------|-------------------------------------|----------------|----------------|--------|--------|---------------|----------------------|---------------------|-------------|-------------|---------|--------------|---------------|---------------|---------------|------------|------------|--------|
|                          |               |               |        |        |                | TI                                  | Mn             |                |        |        |               |                      |                     |             |             |         |              |               |               |               |            |            |        |
| <i>Harmonia axyridis</i> | <b>2.7612</b> | <b>0.7869</b> | 0.639  | 3.9284 | <b>5.8162</b>  | 1.9007                              | <b>10.0414</b> | <b>8.5861</b>  | 0.7898 | 0.5462 | <b>3.3367</b> | Black                | melanins            | melanins    | 12 13 16    | #000000 | #0cd010      | Coccinellini  | Coccinellinae | Coccinellidae | Polyphaga  | Coleoptera | Feb-16 |
| <i>Harmonia axyridis</i> | <b>2.4551</b> | 0.6112        | 0.5031 | 3.0903 | 3.1214         | 1.7872                              | 5.3655         | 5.9079         | 0.7103 | 0.4248 | 2.0185        | Red                  | carotenoids         | carotenoids | 183 147 1   | #c59900 | #079301      | Coccinellini  | Coccinellinae | Coccinellidae | Polyphaga  | Coleoptera | Feb-16 |
| <i>Harmonia axyridis</i> | <b>3.3028</b> | 0.721         | 0.4428 | 2.9236 | <b>6.3108</b>  | 1.8279                              | <b>6.4047</b>  | <b>10.153</b>  | 0.8474 | 0.5661 | <b>4.8561</b> | Black                | melanins            | melanins    | 9 12 11     | #000000 | #00000b      | Coccinellini  | Coccinellinae | Coccinellidae | Polyphaga  | Coleoptera | Feb-16 |
| <i>Harmonia axyridis</i> | <b>2.4733</b> | 0.7075        | 0.3728 | 2.9208 | <b>3.7113</b>  | 1.8091                              | 5.269          | 9.0825         | 0.7123 | 0.5596 | <b>3.5248</b> | Red                  | carotenoids         | carotenoids | 175 140 1   | #999900 | #a8b0d1      | Coccinellini  | Coccinellinae | Coccinellidae | Polyphaga  | Coleoptera | Feb-16 |
| <i>Harmonia axyridis</i> | <b>2.8078</b> | 0.6844        | 0.4428 | 4.0153 | <b>6.5294</b>  | 1.7033                              | 5.8461         | 6.3128         | 0.726  | 0.706  | <b>4.4474</b> | Black                | melanins            | melanins    | 9 11 13     | #000000 | #00000d      | Coccinellini  | Coccinellinae | Coccinellidae | Polyphaga  | Coleoptera | Feb-16 |
| <i>Harmonia axyridis</i> | 2.7896        | 0.6305        | 0.4147 | 3.3501 | 3.6174         | 1.7819                              | 4.807          | 7.2148         | 0.6396 | 0.5916 | 2.9154        | Black                | carotenoids         | carotenoids | 197 146 0   | #c59900 | #c59200      | Coccinellini  | Coccinellinae | Coccinellidae | Polyphaga  | Coleoptera | Feb-16 |
| <i>Harmonia axyridis</i> | 3.3125        | <b>0.8908</b> | 0.5738 | 6.1911 | <b>8.3794</b>  | 2.1933                              | <b>11.3472</b> | 16.9483        | 0.6731 | 0.5117 | 1.4537        | Red                  | melanins            | melanins    | 9 9 9       | #000000 | #009009      | Coccinellini  | Coccinellinae | Coccinellidae | Polyphaga  | Coleoptera | Feb-16 |
| <i>Harmonia axyridis</i> | 3.299         | 0.7394        | 0.5536 | 6.7308 | 7.3132         | 1.8191                              | 8.4654         | 18.3005        | 0.6038 | 0.4369 | 1.2709        | Red                  | carotenoids         | carotenoids | 141 72 0    | #993300 | #8d400d      | Coccinellini  | Coccinellinae | Coccinellidae | Polyphaga  | Coleoptera | Feb-16 |
| <i>Harmonia axyridis</i> | <b>3.9707</b> | 0.7452        | 0.4412 | 3.7339 | <b>4.8997</b>  | 2.0445                              | <b>7.5387</b>  | 5.3322         | 0.7448 | 0.5519 | <b>3.4362</b> | Black                | melanins            | melanins    | 11 10 8     | #000000 | #000408      | Coccinellini  | Coccinellinae | Coccinellidae | Polyphaga  | Coleoptera | Feb-16 |
| <i>Harmonia axyridis</i> | 3.2432        | 0.7167        | 0.4477 | 3.7878 | 3.655          | 2.1562                              | 5.9163         | 0.6989         | 0.4988 | 2.5064 | Red           | carotenoids          | carotenoids         | 131 73 0    | #993300     | #933400 | Coccinellini | Coccinellinae | Coccinellidae | Polyphaga     | Coleoptera | Feb-16     |        |
| <i>Harmonia axyridis</i> | 2.4744        | <b>0.7242</b> | 0.4442 | 4.4842 | <b>6.0086</b>  | 1.7293                              | <b>5.1218</b>  | <b>12.7059</b> | 0.6622 | 0.5604 | <b>3.0745</b> | Red                  | melanins            | melanins    | 161 116 0   | #000000 | #020607      | Coccinellini  | Coccinellinae | Coccinellidae | Polyphaga  | Coleoptera | Feb-16 |
| <i>Harmonia axyridis</i> | 3.6988        | 0.5237        | 0.4625 | 4.0202 | 3.9135         | 2.2771                              | 3.4254         | 6.4036         | 0.6156 | 0.5419 | 2.5915        | Red                  | carotenoids         | carotenoids | 149 90 0    | #996600 | #955a00      | Coccinellini  | Coccinellinae | Coccinellidae | Polyphaga  | Coleoptera | Feb-16 |
| <i>Harmonia axyridis</i> | 2.8889        | <b>1.0875</b> | 0.6329 | 5.8685 | <b>10.0778</b> | 2.527                               | <b>5.7074</b>  | <b>14.5557</b> | 0.8994 | 0.7995 | <b>3.7302</b> | Black                | melanins            | melanins    | 6 8 8       | #000000 | #060808      | Coccinellini  | Coccinellinae | Coccinellidae | Polyphaga  | Coleoptera | Feb-16 |
| <i>Harmonia axyridis</i> | 3.4518        | 0.9249        | 0.6175 | 6.5241 | 7.254          | 2.5685                              | 3.886          | 12.2571        | 0.8473 | 0.6841 | 2.1778        | Red                  | carotenoids         | carotenoids | 152 95 0    | #996600 | #985f00      | Coccinellini  | Coccinellinae | Coccinellidae | Polyphaga  | Coleoptera | Feb-16 |
| <i>Harmonia axyridis</i> | 3.9724        | <b>1.0076</b> | 0.5024 | 5.167  | <b>6.6647</b>  | 2.2138                              | <b>4.1675</b>  | <b>8.5444</b>  | 0.7414 | 0.5619 | <b>3.6503</b> | Black                | melanins            | melanins    | 9 10 10     | #000000 | #090a0a      | Coccinellini  | Coccinellinae | Coccinellidae | Polyphaga  | Coleoptera | Feb-16 |
| <i>Harmonia axyridis</i> | 5.4512        | 0.7055        | 0.3888 | 3.4865 | 3.6227         | 2.1978                              | 3.1728         | 7.9273         | 0.6806 | 0.4817 | 1.5142        | Red                  | carotenoids         | carotenoids | 141 98 0    | #996600 | #846200      | Coccinellini  | Coccinellinae | Coccinellidae | Polyphaga  | Coleoptera | Feb-16 |
| <i>Harmonia axyridis</i> | <b>3.7241</b> | <b>0.8703</b> | 0.4855 | 4.833  | <b>8.3067</b>  | 2.47                                | <b>4.9131</b>  | <b>7.1234</b>  | 0.708  | 0.5624 | <b>2.3889</b> | Black                | melanins            | melanins    | 6 7 8       | #000000 | #060708      | Coccinellini  | Coccinellinae | Coccinellidae | Polyphaga  | Coleoptera | Feb-16 |
| <i>Harmonia axyridis</i> | 2.4744        | 0.6758        | 0.3947 | 4.4964 | 4.7116         | 1.7993                              | 2.8382         | 5.4781         | 0.8342 | 0.5346 | 0.997         | Red                  | carotenoids         | carotenoids | 161 116 0   | #996600 | #a17400      | Coccinellini  | Coccinellinae | Coccinellidae | Polyphaga  | Coleoptera | Feb-16 |
| <i>Harmonia axyridis</i> | <b>3.6419</b> | 0.8034        | 0.5617 | 5.9119 | 5.8954         | 2.1557                              | <b>5.3135</b>  | <b>7.8699</b>  | 0.7016 | 0.5503 | 2.1444        | Black                | melanins            | melanins    | 9 10 11     | #000000 | #000a0b      | Coccinellini  | Coccinellinae | Coccinellidae | Polyphaga  | Coleoptera | Feb-16 |
| <i>Harmonia axyridis</i> | 3.3852        | 0.8167        | 0.6063 | 6.5012 | 5.3901         | 1.8413                              | 3.5677         | 5.9052         | 0.6297 | 0.5848 | 2.5195        | Red                  | carotenoids         | carotenoids | 128 88 0    | #996600 | #005800      | Coccinellini  | Coccinellinae | Coccinellidae | Polyphaga  | Coleoptera | Feb-16 |
| <i>Harmonia axyridis</i> | <b>3.0087</b> | <b>0.7143</b> | 0.4712 | 4.4098 | <b>6.9479</b>  | 1.9033                              | 2.7104         | <b>3.358</b>   | 0.6963 | 0.4689 | 0.7954        | Black                | melanins            | melanins    | 10 11 12    | #000000 | #0a0b0c      | Coccinellini  | Coccinellinae | Coccinellidae | Polyphaga  | Coleoptera | Feb-16 |
| <i>Harmonia axyridis</i> | 2.404         | 0.6082        | 0.3912 | 3.4161 | 2.5437         | 1.8399                              | 2.2118         | 1.6511         | 0.6073 | 0.3446 | 0.4506        | Red                  | carotenoids         | carotenoids | 150 104 0   | #996600 | #966800      | Coccinellini  | Coccinellinae | Coccinellidae | Polyphaga  | Coleoptera | Feb-16 |
| <i>Harmonia axyridis</i> | 3.4506        | 0.708         | 0.4359 | 4.8176 | 5.9886         | 1.853                               | <b>3.055</b>   | 2.9863         | 0.6769 | 0.5357 | 0.5853        | Black                | melanins            | melanins    | 15 17 24    | #000000 | #0f1118      | Coccinellini  | Coccinellinae | Coccinellidae | Polyphaga  | Coleoptera | Feb-16 |
| <i>Harmonia axyridis</i> | 3.4919        | 0.6835        | 0.3961 | 7.8455 | 5.9094         | 1.9108                              | 1.8008         | 4.4252         | 0.6083 | 0.6088 | 0.5543        | Red                  | carotenoids         | carotenoids | 145 123 0   | #996600 | #917b00      | Coccinellini  | Coccinellinae | Coccinellidae | Polyphaga  | Coleoptera | Feb-16 |
| <i>Harmonia axyridis</i> | <b>2.6719</b> | <b>0.6719</b> | 0.1945 | 4.403  | <b>6.1139</b>  | 1.9597                              | 3.9249         | <b>6.1139</b>  | 0.7303 | 0.5929 | <b>3.6133</b> | Black                | melanins            | melanins    | 132 131 33  | #996600 | #917b00      | Coccinellini  | Coccinellinae | Coccinellidae | Polyphaga  | Coleoptera | Feb-16 |
| <i>Harmonia axyridis</i> | 2.2943        | 0.5316        | 0.4599 | 3.7669 | 3.5244         | 1.6187                              | 4.2865         | 5.2805         | 0.6693 | 0.5078 | 2.2566        | Red                  | carotenoids         | carotenoids | 135 101 19  | #996600 | #876513      | Coccinellini  | Coccinellinae | Coccinellidae | Polyphaga  | Coleoptera | Feb-16 |
| <i>Harmonia axyridis</i> | <b>3.8972</b> | <b>0.9789</b> | 0.5571 | 8.5135 | <b>6.9414</b>  | 1.985                               | <b>4.8883</b>  | <b>12.7543</b> | 0.7976 | 1.1167 | <b>9.8344</b> | Black                | melanins            | melanins    | 9 8 7       | #000000 | #090807      | Coccinellini  | Coccinellinae | Coccinellidae | Polyphaga  | Coleoptera | Feb-16 |
| <i>Harmonia axyridis</i> | 3.1371        | 0.8376        | 0.5232 | 7.4427 | 5.5996         | 1.8921                              | 3.7175         | 5.7229         | 0.6945 | 0.6217 | 4.3206        | Red                  | carotenoids         | carotenoids | 126 94 0    | #666600 | #7e5e00      | Coccinellini  | Coccinellinae | Coccinellidae | Polyphaga  | Coleoptera | Feb-16 |
| <i>Harmonia axyridis</i> | 2.154         | <b>0.6554</b> | 0.3957 | 3.6484 | <b>5.4571</b>  | 2.147                               | <b>11.9084</b> | <b>8.8028</b>  | 0.6231 | 0.7259 | <b>2.7138</b> | Black                | melanins            | melanins    | 10 12 12    | #000000 | #0a0c0c      | Coccinellini  | Coccinellinae | Coccinellidae | Polyphaga  | Coleoptera | Feb-16 |
| <i>Harmonia axyridis</i> | 2.3803        | 0.5518        | 0.4461 | 3.3085 | 3.0809         | 1.8097                              | 5.6499         | 4.9075         | 0.6833 | 0.533  | 1.897         | Red                  | carotenoids         | carotenoids | 149 109 7   | #996600 | #956d07      | Coccinellini  | Coccinellinae | Coccinellidae | Polyphaga  | Coleoptera | Feb-16 |
| <i>Harmonia axyridis</i> | 2.4424        | <b>0.7716</b> | 0.4327 | 4.2996 | <b>6.1692</b>  | 2.3702                              | <b>11.2293</b> | 6.1108         | 0.86   | 1.3502 | 2.3327        | Black                | melanins            | melanins    | 8 8 8       | #000000 | #080808      | Coccinellini  | Coccinellinae | Coccinellidae | Polyphaga  | Coleoptera | Feb-16 |
| <i>Harmonia axyridis</i> | 2.7038        | 0.6761        | 0.4463 | 4.0257 | 2.0533         | 1.4635                              | 4.4406         | 6.0735         | 0.4731 | 0.7493 | 0.7493        | Red                  | carotenoids         | carotenoids | 4 737 125 3 | #996600 | #071700      | Coccinellini  | Coccinellinae | Coccinellidae | Polyphaga  | Coleoptera | Feb-16 |
| <i>Harmonia axyridis</i> | <b>2.9667</b> | <b>0.843</b>  | 0.4405 | 4.2704 | <b>6.2684</b>  | 1.8994                              | <b>4.9333</b>  | <b>7.8972</b>  | 0.8604 | 0.7502 | <b>6.9094</b> | Black                | melanins            | melanins    | 9 11 12     | #000000 | #000b0c      | Coccinellini  | Coccinellinae | Coccinellidae | Polyphaga  | Coleoptera | Feb-16 |
| <i>Harmonia axyridis</i> | 2.616         | 0.6335        | 0.4299 | 3.9911 | 4.354          | 1.7826                              | 3.8237         | 5.354          | 0.7373 | 0.5936 | 4.5363        | Red                  | carotenoids         | carotenoids | 153 113 13  | #996600 | #99710d      | Coccinellini  | Coccinellinae | Coccinellidae | Polyphaga  | Coleoptera | Feb-16 |
| <i>Harmonia axyridis</i> | 2.4849        | 0.6479        | 0.4261 | 3.5321 | <b>4.8733</b>  | 1.9723                              | <b>8.7128</b>  | 6.0093         | 0.642  | 0.6564 | <b>4.7715</b> | Black                | melanins            | melanins    | 13 12 11    | #000000 | #0d0c0b      | Coccinellini  | Coccinellinae | Coccinellidae | Polyphaga  | Coleoptera | Feb-16 |
| <i>Harmonia axyridis</i> | 2.5095        | 0.6024        | 0.375  | 3.5241 | 3.9774         | 1.997                               | 5.4745         | 6.1031         | 0.7426 | 0.5443 | 2.9654        | Red                  | carotenoids         | carotenoids | 165 119 0   | #996600 | #a57700      | Coccinellini  | Coccinellinae | Coccinellidae | Polyphaga  | Coleoptera | Feb-16 |
| <i>Harmonia axyridis</i> | <b>3.2823</b> | 0.7621        | 0.5113 | 5.0828 | <b>8.2932</b>  | 1.7295                              | <b>10.9335</b> | 6.07           | 0.7114 | 0.7445 | 3.686         | Black                | melanins            | melanins    | 10 11 12    | #000000 | #0a0b0c      | Coccinellini  | Coccinellinae | Coccinellidae | Polyphaga  | Coleoptera | Feb-16 |
| <i>Harmonia axyridis</i> | 2.9355        | 0.7624        | 0.4568 | 5.2513 | 6.2201         | 2.0425                              | 5.3394         | 6.1054         | 0.7619 | 0.73   | 3.4125        | Red                  | carotenoids         | carotenoids | 147 103 18  | #996600 | #936712      | Coccinellini  | Coccinellinae | Coccinellidae | Polyphaga  | Coleoptera | Feb-16 |
| <i>Harmonia axyridis</i> | <b>2.6968</b> | 0.697         | 0.5357 | 4.4648 | <b>6.4126</b>  | 1.8246                              | <b>5.6162</b>  | <b>7.0612</b>  | 0.7304 | 0.5119 | <b>4.8338</b> | Black                | melanins            | melanins    | 12 12 11    | #000000 | #000000      | Coccinellini  | Coccinellinae | Coccinellidae | Polyphaga  | Coleoptera | Feb-16 |
| <i>Harmonia axyridis</i> | 2.4988        | <b>0.8077</b> | 0.4707 | 4.1248 | <b>6.1968</b>  | 1.8198                              | <b>5.9968</b>  | 5.9968         | 0.4926 | 0.6368 | <b>3.4768</b> | Black                | carotenoids         | carotenoids | 137 87 0    | #000000 | #000000      | Coccinellini  | Coccinellinae | Coccinellidae | Polyphaga  | Coleoptera | Feb-16 |
| <i>Harmonia axyridis</i> | <b>3.4875</b> | <b>0.7731</b> | 0.5116 | 4.6665 | <b>7.477</b>   | 2.1594                              | <b>5.3447</b>  | <b>8.2588</b>  | 0.763  | 0.6739 | <b>4.3158</b> | Black                | melanins            | melanins    | 8 8 8       | #000000 | #080808      | Coccinellini  | Coccinellinae | Coccinellidae | Polyphaga  | Coleoptera | Feb-16 |
| <i>Harmonia axyridis</i> | 2.8721        | 0.6666        | 0.4594 | 5.1182 | 5.3666         | 1.986                               | 3.8282         | 7.0834         | 0.789  | 0.6381 | 3.1788        | Red                  | carotenoids         | carotenoids | 131 94 5    | #996600 | #835e05      | Coccinellini  | Coccinellinae | Coccinellidae | Polyphaga  | Coleoptera | Feb-16 |
| <i>Harmonia axyridis</i> | <b>3.4203</b> | <b>0.9072</b> | 0.5826 | 4.1469 | <b>6.801</b>   | 2.1712                              | <b>9.4193</b>  | <b>6.2008</b>  | 0.8448 | 0.6169 | <b>8.9295</b> | Black                | melanins            | melanins    | 6 8 7       | #000000 | #060807      | Coccinellini  | Coccinellinae | Coccinellidae | Polyphaga  | Coleoptera | Feb-16 |
| <i>Harmonia axyridis</i> | 2.8753        | 0.7106        | 0.4446 | 3.2074 | 3.2089         | 1.8694                              | 3.3075         | 4.5854         | 0.8224 | 0.5498 | 8.5949        | Red                  | carotenoids         | carotenoids | 141 127 0   | #996600 | #8d7f00      | Coccinellini  | Coccinellinae | Coccinellidae | Polyphaga  | Coleoptera | Feb-16 |
| <i>Harmonia axyridis</i> | <b>3.4631</b> | <b>0.6632</b> | 0.4682 | 3.4251 | <b>4.1549</b>  | 2.5979                              | <b>5.0021</b>  | 5.5165         | 0.6374 | 0.5187 | <b>3.3018</b> | Black                | melanins            | melanins    | 16 11 5     | #000000 | #100b05      | Coccinellini  | Coccinellinae | Coccinellidae | Polyphaga  | Coleoptera | Feb-16 |
| <i>Harmonia axyridis</i> | 2.4032        | 0.5847        | 0.4146 | 3.1009 | 2.841          | 1.8594                              | 3.0552         | 5.7643         | 0.7022 | 0.4428 | 2.488         | Red                  | carotenoids         | carotenoids | 149 137 20  | #999900 | #956914      | Coccinellini  | Coccinellinae | Coccinellidae | Polyphaga  | Coleoptera | Feb-16 |
| <i>Epilachna argus</i>   | 1.3           |               |        |        |                |                                     |                |                |        |        |               |                      |                     |             |             |         |              |               |               |               |            |            |        |



| species                             | P             | S             | Cl            | K             | Ca             | concentration (µg/cm <sup>2</sup> ) | Fe             | Ni             | Cu            | Zn            | visual colour | coloration mechanism | specific coloration             | RGB values                                             | websafe     | hex     | tribe        | subfamily      | family        | suborder      | order           | run        |        |
|-------------------------------------|---------------|---------------|---------------|---------------|----------------|-------------------------------------|----------------|----------------|---------------|---------------|---------------|----------------------|---------------------------------|--------------------------------------------------------|-------------|---------|--------------|----------------|---------------|---------------|-----------------|------------|--------|
| <i>Leptinotarsa decemlineata</i>    | 3.008         | 0.6674        | 0.8257        | 5.6394        | 10.4609        | 2.2931                              | <b>19.4644</b> | 16.2596        | 0.8102        | 0.7973        | 2.1398        | Black                | melanins                        | melanins                                               | 22 23 22    | #000000 | #161716      | Chrysomelini   | Chrysomelinae | Chrysomelidae | Polyphaga       | Coleoptera | Feb-16 |
| <i>Leptinotarsa decemlineata</i>    | 2.5168        | 0.5001        | 0.4939        | 3.5683        | 6.1899         | 1.8446                              | <b>14.1391</b> | 8.4736         | 0.7486        | 0.675         | 1.3294        | Black                | melanins                        | melanins                                               | 16 17 18    | #000000 | #101112      | Chrysomelini   | Chrysomelinae | Chrysomelidae | Polyphaga       | Coleoptera | Feb-16 |
| <i>Leptinotarsa decemlineata</i>    | 2.8631        | 0.7993        | 0.8427        | 5.5915        | 11.9921        | 2.3928                              | <b>19.8466</b> | 14.4439        | 0.9079        | 0.8393        | 2.4749        | Black                | melanins                        | melanins                                               | 17 17 17    | #000000 | #111111      | Chrysomelini   | Chrysomelinae | Chrysomelidae | Polyphaga       | Coleoptera | Feb-16 |
| <i>Leptinotarsa decemlineata</i>    | 3.4           | 0.7483        | 0.7021        | 4.3079        | 11.4455        | 2.1133                              | <b>4.8382</b>  | <b>23.3422</b> | 0.8401        | 0.7467        | 2.8629        | Yellow               | carotenoids                     | xanthophylls and keto-carotenes                        | 122 115 6   | #666600 | #7a7306      | Chrysomelini   | Chrysomelinae | Chrysomelidae | Polyphaga       | Coleoptera | Feb-16 |
| <i>Leptinotarsa decemlineata</i>    | 2.9483        | 0.6877        | 0.6601        | 4.049         | 10.7676        | 1.8302                              | <b>22.0437</b> | 0.8051         | 0.7515        | 2.5447        | Yellow        | carotenoids          | xanthophylls and keto-carotenes | 118 110 5                                              | #666600     | #766e05 | Chrysomelini | Chrysomelinae  | Chrysomelidae | Polyphaga     | Coleoptera      | Feb-16     |        |
| <i>Leptinotarsa decemlineata</i>    | 2.6723        | 0.6466        | 0.6207        | 3.9998        | 9.2929         | 1.8351                              | 5.0665         | <b>17.5004</b> | 0.8053        | 0.7335        | 2.37          | Yellow               | carotenoids                     | xanthophylls and keto-carotenes                        | 120 110 5   | #666600 | #786e05      | Chrysomelini   | Chrysomelinae | Chrysomelidae | Polyphaga       | Coleoptera | Feb-16 |
| <i>Goniocitena decemnotata</i>      | 1.8051        | 0.5269        | 0.326         | 5.9523        | 6.5776         | 0.7803                              | <b>4.3532</b>  | 2.2176         | 0.3707        | 0.47          | 3.2463        | Black                | melanins                        | melanins                                               | 24 10 5     | #000000 | #180a05      | Chrysomelini   | Chrysomelinae | Chrysomelidae | Polyphaga       | Coleoptera | Nov-17 |
| <i>Goniocitena decemnotata</i>      | 1.767         | 0.5932        | 0.2937        | 5.8975        | 7.1517         | 0.8631                              | <b>3.8739</b>  | 2.4477         | 0.4728        | 0.5877        | 3.7963        | Black                | melanins                        | melanins                                               | 18 9 6      | #000000 | #120906      | Chrysomelini   | Chrysomelinae | Chrysomelidae | Polyphaga       | Coleoptera | Nov-17 |
| <i>Goniocitena decemnotata</i>      | 1.8952        | 0.7254        | 0.3734        | 6.7965        | 7.5815         | 0.6459                              | <b>4.0763</b>  | 2.8121         | 0.3551        | 0.613         | 4.3692        | Black                | melanins                        | melanins                                               | 18 9 6      | #000000 | #120906      | Chrysomelini   | Chrysomelinae | Chrysomelidae | Polyphaga       | Coleoptera | Nov-17 |
| <i>Goniocitena decemnotata</i>      | 2.0035        | 0.7153        | 0.3885        | 5.9725        | 6.5004         | 0.8401                              | 2.3966         | 2.3606         | 0.3782        | 0.6094        | 3.7123        | Yellow               | carotenoids                     | carotenoids                                            | 141 114 1   | #996600 | #8d7201      | Chrysomelini   | Chrysomelinae | Chrysomelidae | Polyphaga       | Coleoptera | Nov-17 |
| <i>Goniocitena decemnotata</i>      | 1.6746        | 0.6972        | 0.3934        | 5.7924        | 6.2041         | 0.8112                              | 2.3334         | 2.1096         | 0.3476        | 0.5506        | 2.9524        | Yellow               | carotenoids                     | carotenoids                                            | 121 94 1    | #666600 | #795e01      | Chrysomelini   | Chrysomelinae | Chrysomelidae | Polyphaga       | Coleoptera | Nov-17 |
| <i>Goniocitena decemnotata</i>      | 1.3133        | 0.3066        | 0.2555        | 3.375         | 3.478          | 0.8235                              | 1.946          | 1.7079         | 0.3129        | 0.4862        | 2.7236        | Yellow               | carotenoids                     | carotenoids                                            | 121 94 1    | #666600 | #795e01      | Chrysomelini   | Chrysomelinae | Chrysomelidae | Polyphaga       | Coleoptera | Nov-17 |
| <i>Phyllotritica quadrimaculata</i> | 1.3716        | 0.3671        | 0.1761        | 1.9746        | 2.7087         | 1.6675                              | 4.1433         | 6.5373         | 0.6704        | 0.6897        | 1.0312        | Black                | melanins                        | melanins                                               | 22 8 2      | #000000 | #160802      | Luperini       | Galerucinae   | Chrysomelidae | Polyphaga       | Coleoptera | Nov-17 |
| <i>Phyllotritica quadrimaculata</i> | <b>2.4778</b> | <b>0.5437</b> | <b>0.3408</b> | <b>5.165</b>  | <b>6.6838</b>  | <b>1.7067</b>                       | <b>8.7376</b>  | <b>7.7136</b>  | <b>0.9854</b> | <b>1.0312</b> | <b>2.0463</b> | Red                  | carotenoids                     | carotenoids                                            | 67 59 0     | #663300 | #573b00      | Luperini       | Galerucinae   | Chrysomelidae | Polyphaga       | Coleoptera | Nov-17 |
| <i>Nicrophorus investigator</i>     | 2.627         | 0.6808        | 0.8455        | 6.4623        | 6.037          | <b>2.2629</b>                       | <b>4.81</b>    | 3.8927         | 0.6719        | 0.4868        | 1.3121        | Black                | N/A                             | N/A                                                    | 9 10 11     | #000000 | #090a0b      | Nicrophorini   | Nicrophorinae | Silphidae     | Polyphaga       | Coleoptera | Feb-16 |
| <i>Nicrophorus investigator</i>     | 3.054         | 0.8567        | 0.8725        | 7.8807        | 6.3981         | <b>1.7983</b>                       | <b>4.7827</b>  | 3.5904         | 0.6273        | 0.4358        | 1.3776        | Black                | N/A                             | N/A                                                    | 9 10 11     | #000000 | #0a0a0a      | Nicrophorini   | Nicrophorinae | Silphidae     | Polyphaga       | Coleoptera | Feb-16 |
| <i>Nicrophorus investigator</i>     | 3.5674        | 0.7155        | 0.8869        | 7.0711        | 5.5468         | <b>2.1899</b>                       | <b>4.6248</b>  | 4.3426         | 0.8958        | 0.4206        | 1.4829        | Black                | N/A                             | N/A                                                    | 10 10 10    | #000000 | #0a0a0a      | Nicrophorini   | Nicrophorinae | Silphidae     | Polyphaga       | Coleoptera | Feb-16 |
| <i>Nicrophorus investigator</i>     | 2.6656        | 0.7731        | 1.0277        | 7.574         | 6.0384         | 1.6486                              | 3.4372         | 4.3631         | 0.7684        | 0.4075        | <b>1.8944</b> | Brown                | N/A                             | N/A                                                    | 99 46 10    | #663300 | #632e0a      | Nicrophorini   | Nicrophorinae | Silphidae     | Polyphaga       | Coleoptera | Feb-16 |
| <i>Nicrophorus investigator</i>     | 3.1807        | 0.9123        | 0.8396        | 6.9209        | 5.4696         | 1.2212                              | 3.1481         | 4.3652         | 0.7714        | 0.4684        | <b>1.7524</b> | Brown                | N/A                             | N/A                                                    | 107 50 11   | #663300 | #6b320b      | Nicrophorini   | Nicrophorinae | Silphidae     | Polyphaga       | Coleoptera | Feb-16 |
| <i>Nicrophorus investigator</i>     | 3.3494        | 0.8842        | 0.9788        | 7.391         | 6.1704         | 1.7692                              | 4.2269         | 3.9696         | 0.7516        | 0.446         | <b>1.6545</b> | Black                | N/A                             | N/A                                                    | 97 47 13    | #663300 | #612d0d      | Nicrophorini   | Nicrophorinae | Silphidae     | Polyphaga       | Coleoptera | Feb-16 |
| <i>Cicindela hybrida</i>            | 0.3136        | 0.0725        | 0.0485        | 0.2454        | <b>1.8445</b>  | 0.357                               | <b>0.8916</b>  | 0.914          | 0.1397        | 0.089         | 0.1511        | Black                | melanins                        | melanins                                               | 20 19 22    | #000000 | #141316      | Cicindelini    | Cicindelinae  | Carabidae     | Adephaga        | Coleoptera | Jun-17 |
| <i>Cicindela hybrida</i>            | 0.0654        | 0.0654        | 0.0654        | <b>1.732</b>  | <b>0.3578</b>  | 0.3281                              | <b>0.5558</b>  | 0.3281         | 0.0568        | 0.0568        | 0.1511        | Black                | melanins                        | melanins                                               | 20 19 22    | #000000 | #141316      | Cicindelini    | Cicindelinae  | Carabidae     | Adephaga        | Coleoptera | Jun-17 |
| <i>Cicindela hybrida</i>            | 0.3364        | <b>0.1503</b> | <b>0.0593</b> | <b>0.478</b>  | 1.2138         | 0.3658                              | 0.2866         | 0.8555         | 0.1375        | <b>0.1435</b> | 0.1501        | N/A                  | N/A                             | N/A                                                    | 230 221 106 | #ffc066 | #e6df6e      | Cicindelini    | Cicindelinae  | Carabidae     | Adephaga        | Coleoptera | Jun-17 |
| <i>Cicindela hybrida</i>            | 0.2919        | <b>0.1382</b> | <b>0.0595</b> | <b>0.4754</b> | 1.2346         | 0.3716                              | 0.3419         | 1.095          | 0.1422        | <b>0.1359</b> | 0.1654        | Yellow               | N/A                             | N/A                                                    | 230 223 110 | #ffc066 | #e6df6e      | Cicindelini    | Cicindelinae  | Carabidae     | Adephaga        | Coleoptera | Jun-17 |
| <i>Theresa petiveriana</i>          | 2.7402        | 0.6567        | 0.4371        | 2.4212        | 1.5647         | 1.354                               | 3.1664         | 0.6525         | 0.2902        | 1.0779        | 1.7979        | Black                | N/A                             | N/A                                                    | 31 36 42    | #333333 | #1f242a      | N/A            | Polyphaginae  | Polyphagidae  | Blattaria       | Blattodea  | Feb-16 |
| <i>Theresa petiveriana</i>          | 2.2224        | 0.6544        | 0.3927        | 2.3466        | <b>3.3289</b>  | 1.2513                              | 1.2967         | 2.4165         | 0.6272        | 0.324         | 0.9105        | Black                | N/A                             | N/A                                                    | 33 36 41    | #333333 | #212429      | N/A            | Polyphaginae  | Polyphagidae  | Blattaria       | Blattodea  | Feb-16 |
| <i>Theresa petiveriana</i>          | 2.6177        | 0.7476        | 0.3857        | 2.357         | <b>5.1508</b>  | 1.5638                              | 1.3477         | 2.7858         | 0.5589        | 0.3172        | 0.9361        | Black                | N/A                             | N/A                                                    | 30 34 40    | #333333 | #1e2228      | N/A            | Polyphaginae  | Polyphagidae  | Blattaria       | Blattodea  | Feb-16 |
| <i>Theresa petiveriana</i>          | 2.8202        | 0.6497        | 0.457         | 2.4782        | 2.9307         | <b>1.899</b>                        | 1.2191         | 4.2604         | 0.6527        | 0.3843        | 1.2982        | Yellow               | N/A                             | N/A                                                    | 61 60 48    | #333333 | #3d3c30      | N/A            | Polyphaginae  | Polyphagidae  | Blattaria       | Blattodea  | Feb-16 |
| <i>Theresa petiveriana</i>          | 2.0509        | 0.5442        | 0.4226        | 2.2284        | 2.9142         | <b>1.6036</b>                       | 1.0342         | 3.9017         | 0.7127        | 0.3364        | 1.2189        | Yellow               | N/A                             | N/A                                                    | 84 79 53    | #666633 | #544d35      | N/A            | Polyphaginae  | Polyphagidae  | Blattaria       | Blattodea  | Feb-16 |
| <i>Theresa petiveriana</i>          | 2.2976        | 0.6532        | 0.4299        | 2.1721        | 2.5637         | <b>1.5256</b>                       | 1.4309         | 2.6613         | 0.545         | 0.274         | 0.9958        | Yellow               | N/A                             | N/A                                                    | 102 94 56   | #666633 | #665e38      | N/A            | Polyphaginae  | Polyphagidae  | Blattaria       | Blattodea  | Feb-16 |
| <i>Cercopis vulnerata</i>           | <b>5.8801</b> | <b>1.6497</b> | <b>1.2766</b> | <b>7.9805</b> | <b>27.7479</b> | <b>2.2685</b>                       | <b>7.7854</b>  | <b>19.3899</b> | <b>0.9241</b> | <b>1.1963</b> | <b>2.6264</b> | Black                | N/A                             | N/A                                                    | 33 35 42    | #333333 | #21232a      | Cercopini      | Cercopinae    | Cercopidae    | Auchenorrhyncha | Hemiptera  | Feb-16 |
| <i>Cercopis vulnerata</i>           | <b>7.2969</b> | <b>2.8467</b> | <b>1.5506</b> | <b>7.9517</b> | <b>40.1811</b> | <b>3.2006</b>                       | <b>7.5142</b>  | <b>14.1556</b> | <b>1.068</b>  | <b>1.2025</b> | <b>2.7337</b> | Black                | N/A                             | N/A                                                    | 36 36 43    | #333333 | #24242b      | Cercopini      | Cercopinae    | Cercopidae    | Auchenorrhyncha | Hemiptera  | Feb-16 |
| <i>Cercopis vulnerata</i>           | <b>7.2518</b> | <b>2.2755</b> | <b>1.481</b>  | <b>7.574</b>  | <b>35.3432</b> | <b>2.362</b>                        | <b>5.1083</b>  | <b>10.1818</b> | <b>0.9212</b> | <b>0.6258</b> | <b>1.83</b>   | Black                | N/A                             | N/A                                                    | 35 36 42    | #333333 | #23242a      | Cercopini      | Cercopinae    | Cercopidae    | Auchenorrhyncha | Hemiptera  | Feb-16 |
| <i>Cercopis vulnerata</i>           | 4.7008        | 0.8857        | 0.26          | 3.5465        | 7.7092         | 1.9749                              | 3.083          | 4.6246         | 0.7283        | 0.4334        | 1.185         | Red                  | pterins                         | isoxanthopterin, violapterin and erythropterin         | 121 52 18   | #663300 | #793412      | Cercopini      | Cercopinae    | Cercopidae    | Auchenorrhyncha | Hemiptera  | Feb-16 |
| <i>Cercopis vulnerata</i>           | 3.8348        | 1.026         | 0.2452        | 4.4567        | 9.2361         | 1.4164                              | 4.7333         | 3.3073         | 0.446         | 0.5429        | 1.1111        | Red                  | pterins                         | isoxanthopterin, violapterin and erythropterin         | 128 53 16   | #993300 | #803510      | Cercopini      | Cercopinae    | Cercopidae    | Auchenorrhyncha | Hemiptera  | Feb-16 |
| <i>Cercopis vulnerata</i>           | 3.4796        | 0.9767        | 0.2413        | 3.7295        | 9.8037         | 2.7725                              | 3.5093         | 6.349          | 0.6731        | 0.8237        | 1.164         | Red                  | pterins                         | isoxanthopterin, violapterin and erythropterin         | 121 54 15   | #663300 | #79360f      | Cercopini      | Cercopinae    | Cercopidae    | Auchenorrhyncha | Hemiptera  | Feb-16 |
| <i>Graphosoma lineatum</i>          | <b>3.8024</b> | <b>0.8033</b> | 0.6834        | <b>5.9419</b> | <b>30.6633</b> | 2.8505                              | <b>6.4316</b>  | 18.6302        | 0.8679        | 0.8561        | 2.52          | Black                | melanins                        | melanins                                               | 12 14 14    | #000000 | #0c0e0e      | Graphosomatini | Podopinae     | Pentatomidae  | Heteroptera     | Hemiptera  | Feb-16 |
| <i>Graphosoma lineatum</i>          | <b>4.1962</b> | <b>0.8872</b> | 0.622         | <b>5.8938</b> | <b>35.7877</b> | 2.8359                              | <b>7.2936</b>  | 11.2339        | 0.8359        | 0.7597        | 2.2761        | Black                | melanins                        | melanins                                               | 13 15 15    | #000000 | #0d0f0f      | Graphosomatini | Podopinae     | Pentatomidae  | Heteroptera     | Hemiptera  | Feb-16 |
| <i>Graphosoma lineatum</i>          | <b>3.95</b>   | <b>0.83</b>   | 0.6793        | <b>5.8492</b> | <b>30.9099</b> | 3.5001                              | <b>6.4012</b>  | 20.5153        | 0.9835        | 0.8012        | 2.0274        | Black                | melanins                        | melanins                                               | 13 15 14    | #000000 | #0d0f0e      | Graphosomatini | Podopinae     | Pentatomidae  | Heteroptera     | Hemiptera  | Feb-16 |
| <i>Graphosoma lineatum</i>          | 2.9151        | 0.6212        | 0.6316        | 2.5469        | 8.7572         | 3.8379                              | 3.4539         | 19.1519        | 1.281         | 0.7924        | 2.4413        | Red                  | 1: pterins. 2: AKH              | isoxanthopterin, violapterin and erythropterin. 2: AKH | 104 30 3    | #663300 | #681e03      | Graphosomatini | Podopinae     | Pentatomidae  | Heteroptera     | Hemiptera  | Feb-16 |
| <i>Graphosoma lineatum</i>          | 2.4314        | 0.7227        | 0.5421        | 2.6868        | 9.0721         | 2.716                               | 4.0317         | 18.1754        | 0.9116        | 0.7617        | 2.2775        | Red                  | 1: pterins. 2: AKH              | isoxanthopterin, violapterin and erythropterin. 2: AKH | 102 29 2    | #663300 | #681d02      | Graphosomatini | Podopinae     | Pentatomidae  | Heteroptera     | Hemiptera  | Feb-16 |
| <i>Graphosoma lineatum</i>          | 2.7723        | 0.7028        | 0.5523        | 2.9275        | 10.5565        | 4.4689                              | 4.6239         | 31.4884        | 0.8568        | 0.6497        | 1.9285        | Red                  | 1: pterins. 2: AKH              | isoxanthopterin, violapterin and erythropterin. 2: AKH | 103 29 2    | #663301 | #681d03      | Graphosomatini | Podopinae     | Pentatomidae  | Heteroptera     | Hemiptera  | Feb-16 |
| <i>Graphosoma lineatum</i>          | 3.9925        | 1.2856        | 0.9365        | <b>8.5651</b> | <b>11.3567</b> | 2.1706                              | 3.7131         | 8.7795         | 0.973         | 0.8058        | <b>5.107</b>  | Black                | melanins                        | melanins                                               | 27 30 29    | #333333 | #1b1e1d      | Graphosomatini | Podopinae     | Pentatomidae  | Heteroptera     | Hemiptera  | Feb-16 |
| <i>Graphosoma lineatum</i>          | 4.1731        | 1.2197        | 0.9445        | <b>8.7994</b> | <b>11.897</b>  | 2.249                               | 4.6269         | 9.6573         | 1.1401        | 1.5255        | <b>6.2631</b> | Black                | melanins                        | melanins                                               | 29 32 30    | #333333 | #1d201e      | Graphosomatini | Podopinae     | Pentatomidae  | Heteroptera     | Hemiptera  | Feb-16 |
| <i>Graphosoma lineatum</i>          | 3.9567        | 1.183         | 0.9652        | <b>8.5802</b> | <b>12.1197</b> | 2.2687                              | 4.8293         | 11.6139        | 1.1262        | 0.8639        | <b>5.7445</b> | Black                | melanins                        | melanins                                               | 28 31 30    | #333333 | #1c1f1e      | Graphosomatini | Podopinae     | Pentatomidae  | Heteroptera     | Hemiptera  | Feb-16 |
| <i>Graphosoma lineatum</i>          | 3.6619        | 1.1447        | 1.0326        | 6.7179        | 6.5555         | 2.1667                              | 3.4219         | 8.975          | 0.9741        | 0.7174        | 3.6118        | Orange               | N/A                             | N/A                                                    | 137 90 13   | #996600 | #895a0d      | Graphosomatini | Podopinae     | Pentatomidae  | Heteroptera     | Hemiptera  | Feb-16 |
| <i>Graphosoma lineatum</i>          | 3.7849        | 1.0813        | 1.0095        | 6.5241        | 10.1911        | 2.2093                              | 3.4918         | 9.9141         | 0.9612        |               |               |                      |                                 |                                                        |             |         |              |                |               |               |                 |            |        |

| species                        | P             | S             | CI            | K              | Ca             | Ti     | Mn             | Fe             | Ni            | Cu            | Zn             | visual colour | coloration mechanism       | specific coloration        | RGB values  | websafe | hex     | tribe         | subfamily      | family       | suborder        | order       | run    |
|--------------------------------|---------------|---------------|---------------|----------------|----------------|--------|----------------|----------------|---------------|---------------|----------------|---------------|----------------------------|----------------------------|-------------|---------|---------|---------------|----------------|--------------|-----------------|-------------|--------|
| <i>Evacanthus interruptus</i>  | 2.8189        | 0.5712        | 0.3424        | 2.2594         | 6.067          | 1.9587 | 2.8184         | 5.8804         | 0.6329        | 0.2861        | 1.5885         | Black         | melanins                   | melanins                   | 47 48 46    | #333333 | #2f302e | Evacanthini   | Evacanthinae   | Cicadellidae | Auchenorrhyncha | Hemiptera   | Feb-16 |
| <i>Evacanthus interruptus</i>  | 2.9754        | 0.6106        | 0.3855        | 2.6422         | 6.0216         | 2.2103 | 3.202          | 3.752          | 0.5876        | 0.298         | 1.7118         | Black         | melanins                   | melanins                   | 68 56 39    | #333333 | #443827 | Evacanthini   | Evacanthinae   | Cicadellidae | Auchenorrhyncha | Hemiptera   | Feb-16 |
| <i>Evacanthus interruptus</i>  | <b>3.0588</b> | 0.6088        | <b>0.4283</b> | 2.8088         | 5.983          | 2.0015 | 2.858          | 3.541          | 0.585         | 0.343         | 1.96           | Yellow        | N/A                        | N/A                        | 163 165 45  | #999933 | #3a8d23 | Evacanthini   | Evacanthinae   | Cicadellidae | Auchenorrhyncha | Hemiptera   | Feb-16 |
| <i>Evacanthus interruptus</i>  | <b>3.5157</b> | 0.7432        | <b>0.587</b>  | 3.5367         | 7.4473         | 2.8579 | 3.6339         | 4.8401         | 0.6991        | <b>0.3419</b> | 2.8083         | Yellow        | N/A                        | N/A                        | 168 177 35  | #999933 | #a8b123 | Evacanthini   | Evacanthinae   | Cicadellidae | Auchenorrhyncha | Hemiptera   | Feb-16 |
| <i>Deselvana fulvofasciata</i> | 1.7577        | 0.5764        | 0.3582        | 2.2113         | 8.8337         | 1.359  | <b>3.2885</b>  | 8.4397         | 0.5537        | 0.7777        | 1.1694         | Brown         | melanins                   | melanins                   | 112 62 32   | #663333 | #703e20 | Proconiini    | Cicadellinae   | Cicadellidae | Auchenorrhyncha | Hemiptera   | Nov-17 |
| <i>Deselvana fulvofasciata</i> | 2.9908        | 0.6081        | 0.4991        | 4.5041         | 18.8243        | 1.2836 | <b>5.7443</b>  | 7.8229         | 0.7031        | 2.6004        | 2.832          | Brown         | melanins                   | melanins                   | 81 33 17    | #663300 | #512111 | Proconiini    | Cicadellinae   | Cicadellidae | Auchenorrhyncha | Hemiptera   | Nov-17 |
| <i>Deselvana fulvofasciata</i> | 2.3152        | 0.5522        | 0.4344        | 3.6587         | 8.966          | 1.1585 | <b>4.9515</b>  | 5.2983         | 0.7314        | 0.884         | 1.2677         | Brown         | melanins                   | melanins                   | 73 26 7     | #333300 | #491a07 | Proconiini    | Cicadellinae   | Cicadellidae | Auchenorrhyncha | Hemiptera   | Nov-17 |
| <i>Deselvana fulvofasciata</i> | 2.7938        | 0.5967        | 0.495         | 3.4613         | <b>12.0959</b> | 1.1689 | 1.6915         | 6.1713         | 0.6082        | 0.7318        | 0.908          | Yellow        | N/A                        | N/A                        | 161 137 58  | #999933 | #1893a  | Proconiini    | Cicadellinae   | Cicadellidae | Auchenorrhyncha | Hemiptera   | Nov-17 |
| <i>Deselvana fulvofasciata</i> | 2.7407        | 0.6386        | 0.4863        | 3.1376         | <b>20.2035</b> | 1.2716 | 2.1733         | 6.0582         | 0.5837        | 1.7579        | 2.149          | Yellow        | N/A                        | N/A                        | 150 127 55  | #996633 | #967f37 | Proconiini    | Cicadellinae   | Cicadellidae | Auchenorrhyncha | Hemiptera   | Nov-17 |
| <i>Deselvana fulvofasciata</i> | 3.6547        | 0.7335        | 0.5996        | 5.8598         | <b>24.8962</b> | 1.2774 | 3.1937         | 4.2738         | 0.6088        | 0.4723        | 0.8643         | Yellow        | N/A                        | N/A                        | 152 123 14  | #996600 | #9870e  | Proconiini    | Cicadellinae   | Cicadellidae | Auchenorrhyncha | Hemiptera   | Nov-17 |
| <i>Cicciana obliqua</i>        | <b>2.8949</b> | <b>0.7284</b> | 0.4009        | <b>1.9046</b>  | <b>13.8218</b> | 1.6573 | <b>2.0636</b>  | 6.4157         | <b>0.9917</b> | <b>0.991</b>  | <b>47.0045</b> | Brown         | melanins                   | melanins                   | 95 35 39    | #333333 | #332327 | Proconiini    | Cicadellinae   | Cicadellidae | Auchenorrhyncha | Hemiptera   | Nov-17 |
| <i>Cicciana obliqua</i>        | <b>2.979</b>  | <b>0.7057</b> | 0.4052        | <b>2.0553</b>  | <b>14.6552</b> | 2.0837 | <b>1.9986</b>  | 7.0164         | <b>0.9935</b> | <b>1.0632</b> | <b>42.8435</b> | Brown         | melanins                   | melanins                   | 56 26 27    | #333333 | #381a1b | Proconiini    | Cicadellinae   | Cicadellidae | Auchenorrhyncha | Hemiptera   | Nov-17 |
| <i>Cicciana obliqua</i>        | <b>2.8325</b> | <b>0.6466</b> | 0.3768        | <b>2.0028</b>  | <b>13.9626</b> | 1.6836 | <b>2.0209</b>  | 6.9001         | <b>1.0359</b> | <b>1.0098</b> | <b>48.4197</b> | Brown         | melanins                   | melanins                   | 54 27 29    | #333333 | #361b1d | Proconiini    | Cicadellinae   | Cicadellidae | Auchenorrhyncha | Hemiptera   | Nov-17 |
| <i>Cicciana obliqua</i>        | 1.671         | 0.5011        | 0.3898        | 1.4373         | 5.352          | 1.3238 | 1.1227         | 5.5819         | 0.7089        | 0.7704        | 5.0321         | Yellow        | N/A                        | N/A                        | 172 141 68  | #999933 | #ac8d44 | Proconiini    | Cicadellinae   | Cicadellidae | Auchenorrhyncha | Hemiptera   | Nov-17 |
| <i>Cicciana obliqua</i>        | 1.6812        | 0.5286        | 0.3997        | 1.3375         | 5.8289         | 1.9554 | 1.1618         | 5.0482         | 0.6405        | 0.7999        | 5.7201         | Yellow        | N/A                        | N/A                        | 157 132 65  | #999933 | #9d8417 | Proconiini    | Cicadellinae   | Cicadellidae | Auchenorrhyncha | Hemiptera   | Nov-17 |
| <i>Cicciana obliqua</i>        | 1.838         | 0.5623        | 0.4014        | 1.3619         | 6.1616         | 1.3361 | 1.2562         | 9.6865         | 0.6579        | 0.792         | 4.9888         | Yellow        | N/A                        | N/A                        | 146 116 55  | #996633 | #927437 | Proconiini    | Cicadellinae   | Cicadellidae | Auchenorrhyncha | Hemiptera   | Nov-17 |
| <i>Panorpa sp.</i>             | 3.444         | <b>1.1734</b> | 0.7638        | <b>9.2969</b>  | <b>13.5521</b> | 2.4221 | <b>8.9664</b>  | <b>14.5691</b> | <b>1.0177</b> | <b>0.6101</b> | 1.7678         | Brown         | melanins                   | melanins                   | 127 105 46  | #666633 | #7f692e | Hybotini      | Panorpiinae    | Panorpidae   | N/A             | Mecoptera   | Feb-16 |
| <i>Panorpa sp.</i>             | 3.3259        | <b>1.2311</b> | 0.7235        | <b>10.6601</b> | <b>14.854</b>  | 1.6161 | <b>12.4033</b> | <b>15.3616</b> | <b>1.3873</b> | <b>0.7178</b> | 1.9424         | Brown         | melanins                   | melanins                   | 195 142 118 | #c0966  | #c35b76 | Hybotini      | Panorpiinae    | Panorpidae   | N/A             | Mecoptera   | Feb-16 |
| <i>Panorpa sp.</i>             | 4.0109        | <b>1.2438</b> | 0.9229        | <b>12.127</b>  | <b>14.4964</b> | 2.2101 | <b>8.5285</b>  | <b>9.5685</b>  | <b>0.7614</b> | <b>0.5992</b> | 1.7529         | Brown         | melanins                   | melanins                   | 95 67 9     | #663300 | #5f4309 | Hybotini      | Panorpiinae    | Panorpidae   | N/A             | Mecoptera   | Feb-16 |
| <i>Panorpa sp.</i>             | 3.7189        | 0.9451        | 0.6417        | 6.477          | 8.2586         | 1.9558 | 4.6464         | 8.2001         | 0.7888        | 0.4874        | 0.9896         | Brown         | pterins                    | sepiapterin                | 202 199 161 | #cccc99 | #cac7a1 | Hybotini      | Panorpiinae    | Panorpidae   | N/A             | Mecoptera   | Feb-16 |
| <i>Panorpa sp.</i>             | 3.7703        | 1.0134        | 0.7605        | 8.1348         | 8.2173         | 1.7427 | 6.2335         | 6.6989         | 0.7376        | 0.4086        | 1.9794         | Brown         | pterins                    | sepiapterin                | 198 199 162 | #cccc99 | #c6c7a2 | Hybotini      | Panorpiinae    | Panorpidae   | N/A             | Mecoptera   | Feb-16 |
| <i>Panorpa sp.</i>             | 3.3709        | 0.8991        | 0.6185        | 5.7809         | 9.9397         | 2.2178 | 4.0485         | 11.477         | 0.7642        | 0.4522        | 1.2329         | Brown         | pterins                    | sepiapterin                | 199 199 161 | #cccc99 | #c7c7a1 | Hybotini      | Panorpiinae    | Panorpidae   | N/A             | Mecoptera   | Feb-16 |
| <i>Asilus crabroniformis</i>   | 1.6972        | 0.455         | 0.3598        | 1.3571         | 2.1427         | 1.2447 | 2.4363         | <b>6.9359</b>  | 0.4582        | 0.2187        | 0.2762         | Brown         | melanins                   | melanins                   | 122 102 46  | #666633 | #7a6e2e | N/A           | Asilinae       | Asilidae     | Brachycera      | Diptera     | Feb-16 |
| <i>Asilus crabroniformis</i>   | 1.6451        | 0.459         | 0.4317        | 1.4238         | 1.7903         | 1.2985 | 1.5844         | <b>8.3748</b>  | 0.4848        | 0.2537        | 0.372          | Brown         | melanins                   | melanins                   | 114 95 38   | #666633 | #725226 | N/A           | Asilinae       | Asilidae     | Brachycera      | Diptera     | Feb-16 |
| <i>Asilus crabroniformis</i>   | 1.6889        | 0.458         | 0.3811        | 1.4508         | 1.5972         | 1.2985 | 1.5844         | 1.6288         | 0.4848        | 0.2537        | 0.372          | Brown         | melanins                   | melanins                   | 114 97 44   | #666633 | #725226 | N/A           | Asilinae       | Asilidae     | Brachycera      | Diptera     | Feb-16 |
| <i>Asilus crabroniformis</i>   | 1.6123        | 0.529         | 0.3825        | 1.7616         | 2.3098         | 1.3295 | 1.381          | 2.5784         | 0.4432        | 0.2104        | 0.343          | Brown         | N/A                        | N/A                        | 195 182 112 | #cccc99 | #c3b670 | N/A           | Asilinae       | Asilidae     | Brachycera      | Diptera     | Feb-16 |
| <i>Asilus crabroniformis</i>   | 1.6676        | 0.4292        | 0.334         | 1.4976         | 2.1255         | 1.6634 | 1.4153         | 6.4904         | 0.4481        | 0.2467        | 0.2912         | Brown         | N/A                        | N/A                        | 207 193 117 | #cccc99 | #cfc175 | N/A           | Asilinae       | Asilidae     | Brachycera      | Diptera     | Feb-16 |
| <i>Asilus crabroniformis</i>   | 2.1417        | 0.493         | 0.2901        | 1.8214         | 1.4395         | 1.5774 | 1.5484         | 3.3525         | 0.4648        | 0.241         | 0.2979         | Brown         | N/A                        | N/A                        | 198 187 116 | #cccc99 | #c6bb74 | N/A           | Asilinae       | Asilidae     | Brachycera      | Diptera     | Feb-16 |
| <i>Odynerus gracilis</i>       | 0.3459        | <b>0.1103</b> | <b>0.055</b>  | <b>0.3525</b>  | <b>0.6998</b>  | 0.3496 | <b>0.3626</b>  | <b>7.4054</b>  | <b>0.1958</b> | 0.158         | <b>0.2346</b>  | Brown         | N/A                        | N/A                        | 107 77 23   | #666600 | #6b4d17 | N/A           | Eumeninae      | Vespidae     | Apocrita        | Hymenoptera | Jun-17 |
| <i>Odynerus gracilis</i>       | 0.4319        | <b>0.1316</b> | <b>0.0722</b> | <b>0.3801</b>  | <b>1.0872</b>  | 0.2305 | <b>0.4866</b>  | <b>7.4533</b>  | <b>0.2173</b> | 0.2183        | <b>0.2725</b>  | Brown         | N/A                        | N/A                        | 107 77 25   | #666600 | #6b4d19 | N/A           | Eumeninae      | Vespidae     | Apocrita        | Hymenoptera | Jun-17 |
| <i>Odynerus gracilis</i>       | 0.224         | 0.0684        | 0.0298        | 0.1591         | 0.3624         | 0.3762 | 0.2289         | 1.5565         | 0.1436        | 0.1337        | 0.1821         | Brown         | N/A                        | N/A                        | 201 207 199 | #cccccc | #c9dcf7 | N/A           | Eumeninae      | Vespidae     | Apocrita        | Hymenoptera | Jun-17 |
| <i>Odynerus gracilis</i>       | 0.2565        | 0.051         | 0.0312        | 0.1655         | 0.3755         | 0.333  | 0.214          | 1.5809         | 0.1154        | 0.1361        | 0.1951         | Brown         | N/A                        | N/A                        | 207 216 216 | #cccccc | #c4d6b8 | N/A           | Eumeninae      | Vespidae     | Apocrita        | Hymenoptera | Jun-17 |
| <i>Odynerus gracilis</i>       | 0.2453        | 0.0506        | 0.0505        | 0.1518         | 0.3504         | 0.3729 | 0.2214         | 2.0424         | 0.145         | 0.1025        | 0.1911         | Brown         | N/A                        | N/A                        | 208 213 212 | #cccccc | #c0d554 | N/A           | Eumeninae      | Vespidae     | Apocrita        | Hymenoptera | Jun-17 |
| <i>Andrena carantonica</i>     | <b>0.3764</b> | <b>0.0863</b> | 0.0394        | <b>0.3936</b>  | <b>0.7265</b>  | 0.535  | 0.4177         | <b>4.5771</b>  | 0.1664        | <b>0.3521</b> | <b>0.3438</b>  | Brown         | melanins                   | melanins                   | 103 83 43   | #666633 | #67532b | N/A           | Andreninae     | Andrenidae   | Apocrita        | Hymenoptera | Jun-17 |
| <i>Andrena carantonica</i>     | <b>0.3431</b> | <b>0.0972</b> | 0.0338        | <b>0.4058</b>  | <b>0.9851</b>  | 0.3912 | 0.338          | <b>3.9049</b>  | 0.0599        | <b>0.316</b>  | <b>0.3245</b>  | Brown         | melanins                   | melanins                   | 103 85 42   | #666633 | #67552a | N/A           | Andreninae     | Andrenidae   | Apocrita        | Hymenoptera | Jun-17 |
| <i>Andrena carantonica</i>     | 0.2719        | 0.061         | 0.0317        | 0.2164         | 0.6461         | 0.423  | 0.3314         | 2.4301         | 0.147         | 0.1737        | 0.139          | Brown         | N/A                        | N/A                        | 172 172 146 | #999999 | #aac92  | N/A           | Andreninae     | Andrenidae   | Apocrita        | Hymenoptera | Jun-17 |
| <i>Andrena carantonica</i>     | 0.2572        | 0.061         | 0.029         | 0.1866         | 0.6142         | 0.5016 | 0.3238         | 2.5032         | 0.1329        | 0.1297        | 0.116          | Brown         | N/A                        | N/A                        | 162 158 120 | #999999 | #a29e78 | N/A           | Andreninae     | Andrenidae   | Apocrita        | Hymenoptera | Jun-17 |
| <i>Andrena carantonica</i>     | 0.2539        | 0.0625        | 0.0289        | 0.1852         | 0.5321         | 0.3731 | 0.2622         | 1.9982         | 0.1284        | 0.1496        | 0.1193         | Brown         | N/A                        | N/A                        | 178 180 150 | #999999 | #b2b496 | N/A           | Andreninae     | Andrenidae   | Apocrita        | Hymenoptera | Jun-17 |
| <i>Calopteryx virgo</i>        | 1.9107        | 0.432         | 0.2377        | 1.1362         | <b>4.585</b>   | 0.8037 | 0.7687         | 1.127          | 0.4353        | 0.2492        | 0.5263         | Brown         | 1: melanins. 2: structural | 1: melanins. 2: structural | 114 86 15   | #666600 | #72560f | Calopterygini | Calopterygidae | Zygoptera    | Odonata         | Nov-17      |        |
| <i>Calopteryx virgo</i>        | 1.8894        | 0.3343        | 0.2585        | 1.1911         | <b>5.186</b>   | 0.9346 | 1.0327         | 3.1081         | 0.465         | 0.1969        | 0.6919         | Brown         | 1: melanins. 2: structural | 1: melanins. 2: structural | 120 93 17   | #666600 | #785d11 | Calopterygini | Calopterygidae | Zygoptera    | Odonata         | Nov-17      |        |
| <i>Calopteryx virgo</i>        | 1.6108        | 0.4191        | 0.2371        | 1.2047         | <b>4.5337</b>  | 1.1285 | 0.7496         | 1.2448         | 0.3422        | 0.2343        | 0.4599         | Brown         | 1: melanins. 2: structural | 1: melanins. 2: structural | 132 107 27  | #996633 | #       |               |                |              |                 |             |        |

**Supplementary Table 5.2 (previous page). Database of chemistry of extant specimens analyzed using synchrotron rapid-scanning X-ray fluorescence.**

| fossil number      | collection                       | age         | locality         | maps | location of map    | # of ROIs            | pixel size (um)  | dwell time of XRF maps (ms) |
|--------------------|----------------------------------|-------------|------------------|------|--------------------|----------------------|------------------|-----------------------------|
| <b>Hemiptera</b>   |                                  |             |                  |      |                    |                      |                  |                             |
| RR1-12-07-10       | Seeking accession number at MNCN | Miocene     | Rubielos de Mora | 1    | Entire specimen    | 6                    | 0.25 by 0.25     | 50                          |
| RR1-12-07-44       | Seeking accession number at MNCN | Miocene     | Rubielos de Mora | 1    | Entire specimen    | 6                    | 0.25 by 0.25     | 50                          |
| YPM IP522483       | YPMNH                            | Eocene      | Green River      | 1    | Wing               | 8                    | 0.1 by 0.1       | 50                          |
| MCZ14431           | MCZ                              | Miocene     | Oeningen         | 1    | Portion of thorax  | 6                    | 0.025 by 0.025   | 50                          |
| MCZ14426           | MCZ                              | Miocene     | Oeningen         | 2    | Portion of thorax  | 9 (map 1); 6 (map 2) | 0.1 by 0.1       | 50                          |
| <b>Coleoptera</b>  |                                  |             |                  |      |                    |                      |                  |                             |
| YPM IP454454       | YPMNH                            | Eocene      | Green River      | 1    | Elytrum            | 3                    | 0.025 by 0.025   | 50                          |
| YPM IP390330       | YPMNH                            | Eocene      | Green River      | 1    | Entire specimen    | 3                    | 0.025 by 0.025   | 50                          |
| UCM_51311a         | MCZ                              | Eocene      | Green River      | 2    | Portion of elytrum | 6 (map 1); 6 (map 2) | 0.003 by 0.003   | 55                          |
| OCB6               | Seeking accession number at RAS  | Pleistocene | Old Crow River   | 1    | Portion of elytrum | 6                    | 0.003 by 0.003   | 50                          |
| <b>Neuroptera</b>  |                                  |             |                  |      |                    |                      |                  |                             |
| NIGPAS NND11021-1  | CNU                              | Jurassic    | Daohugou         | 1    | Portion of wing    | 12                   | 0.05 by 0.05     | 25                          |
| NIGPAS NND11021-2  | CNU                              | Jurassic    | Daohugou         | 1    | Portion of wing    | 12                   | 0.05 by 0.05     | 25                          |
| NIGPAS NND11010    | CNU                              | Jurassic    | Daohugou         | 1    | Portion of wing    | 6                    | 0.025 by 0.025   | 25                          |
| NIGPAS NND11023    | CNU                              | Jurassic    | Daohugou         | 1    | Portion of wing    | 12                   | 0.025 by 0.025   | 25                          |
| NIGPAS NND140539   | CNU                              | Jurassic    | Daohugou         | 1    | Portion of wing    | 12                   | 0.0325 by 0.0325 | 25                          |
| <b>Hymenoptera</b> |                                  |             |                  |      |                    |                      |                  |                             |
| UAF_GS7            | UAF                              | Miocene     | Grubstake        | 1    | Wing               | 6                    | 0.025 by 0.025   | 25                          |

**Supplementary Table 5.3. Details of fossil specimens analyzed using synchrotron rapid-scanning X-ray fluorescence.** MNCN, Museo Nacional de Ciencias Naturales, Madrid; YPMNH, Yale Peabody Museum of Natural History; MCZ, Harvard Museum of Comparative Zoology; RAS, Russian Academy of Sciences, Moscow; CNU, Capital Normal University, Beijing; UAF, University of Alaska, Fairbanks.

| specimen #   | concentration (µg/cm <sup>2</sup> ) |        |          |         |          |        |        | visual colour | websafe | hex     | family     | order     | age     | location |
|--------------|-------------------------------------|--------|----------|---------|----------|--------|--------|---------------|---------|---------|------------|-----------|---------|----------|
|              | S                                   | K      | Ca       | Ti      | Fe       | Cu     | Zn     |               |         |         |            |           |         |          |
| RR1-12-07-10 | 2.4222                              | 4.1935 | 641.0077 | 11.2583 | 164.0164 | 1.0165 | 0.5651 | Dark Brown    | #000000 | #000000 | Corixidae  | Hemiptera | Miocene | RBM      |
| RR1-12-07-10 | 2.7455                              | 5.0395 | 731.4726 | 19.1381 | 323.1815 | 1.2057 | 0.64   | Dark Brown    | #000000 | #000000 | Corixidae  | Hemiptera | Miocene | RBM      |
| RR1-12-07-10 | 1.9058                              | 3.1167 | 476.0089 | 8.8529  | 195.3102 | 1.2234 | 0.6789 | Dark Brown    | #000000 | #000000 | Corixidae  | Hemiptera | Miocene | RBM      |
| RR1-12-07-10 | 1.7223                              | 2.8095 | 429.9041 | 6.62    | 97.5444  | 0.5508 | 0.4532 | Light Brown   | #999999 | #a1a1a1 | Corixidae  | Hemiptera | Miocene | RBM      |
| RR1-12-07-10 | 1.7225                              | 2.8188 | 396.5781 | 10.0211 | 157.677  | 0.5541 | 0.4359 | Light Brown   | #999999 | #a1a1a1 | Corixidae  | Hemiptera | Miocene | RBM      |
| RR1-12-07-10 | 1.4944                              | 3.0528 | 370.535  | 11.1016 | 217.9584 | 0.6198 | 0.6638 | Light Brown   | #999999 | #a1a1a1 | Corixidae  | Hemiptera | Miocene | RBM      |
| RR1-12-07-10 | 0.7999                              | 2.9824 | 124.7242 | 26.9955 | 473.2328 | 0.2943 | 1.003  | Sediment      | #ff9900 | #ff8c00 | RBM sed    | RBM sed   | Miocene | RBM      |
| RR1-12-07-10 | 0.8318                              | 3.6342 | 101.8691 | 39.8798 | 626.254  | 0.3295 | 1.2699 | Sediment      | #ff9900 | #ff8c00 | RBM sed    | RBM sed   | Miocene | RBM      |
| RR1-12-07-44 | 1.3517                              | 4.445  | 267.0104 | 33.9263 | 543.0747 | 1.0471 | 2.1184 | Dark Brown    | #000000 | #000000 | Corixidae  | Hemiptera | Miocene | RBM      |
| RR1-12-07-44 | 1.331                               | 4.4417 | 245.3206 | 27.3373 | 503.3022 | 1.3683 | 2.3505 | Dark Brown    | #000000 | #000000 | Corixidae  | Hemiptera | Miocene | RBM      |
| RR1-12-07-44 | 1.4746                              | 4.4623 | 276.1384 | 40.6148 | 488.2651 | 0.9316 | 1.8481 | Dark Brown    | #000000 | #000000 | Corixidae  | Hemiptera | Miocene | RBM      |
| RR1-12-07-44 | 1.2485                              | 4.066  | 226.473  | 25.707  | 493.9073 | 0.6985 | 1.8889 | Light Brown   | #999999 | #a1a1a1 | Corixidae  | Hemiptera | Miocene | RBM      |
| RR1-12-07-44 | 1.3458                              | 4.3495 | 236.3792 | 30.5973 | 549.5027 | 0.8534 | 2.0344 | Light Brown   | #999999 | #a1a1a1 | Corixidae  | Hemiptera | Miocene | RBM      |
| RR1-12-07-44 | 1.2878                              | 4.1374 | 233.1733 | 28.9653 | 513.4612 | 0.6488 | 1.9758 | Light Brown   | #999999 | #a1a1a1 | Corixidae  | Hemiptera | Miocene | RBM      |
| RR1-12-07-44 | 0.7931                              | 3.712  | 110.7194 | 29.4815 | 511.8656 | 0.4009 | 1.5879 | Sediment      | #ff9900 | #ff8c00 | RBM sed    | RBM sed   | Miocene | RBM      |
| RR1-12-07-44 | 0.747                               | 3.6495 | 92.3704  | 30.5985 | 530.949  | 0.4097 | 1.5359 | Sediment      | #ff9900 | #ff8c00 | RBM sed    | RBM sed   | Miocene | RBM      |
| 522483       | 0.7436                              | 2.504  | 93.4398  | 29.258  | 649.5568 | 1.0643 | 1.6502 | Dark Brown    | #000000 | #000000 | Fulgoridae | Hemiptera | Eocene  | GRF      |
| 522483       | 0.8596                              | 2.9795 | 106.3761 | 37.1044 | 733.8536 | 1.1172 | 1.9906 | Dark Brown    | #000000 | #000000 | Fulgoridae | Hemiptera | Eocene  | GRF      |
| 522483       | 0.8239                              | 2.7897 | 105.6661 | 31.6126 | 695.4624 | 1.113  | 1.7737 | Dark Brown    | #000000 | #000000 | Fulgoridae | Hemiptera | Eocene  | GRF      |
| 522483       | 0.9627                              | 3.5261 | 123.9771 | 39.3736 | 826.7398 | 1.3774 | 2.0627 | Dark Brown    | #000000 | #000000 | Fulgoridae | Hemiptera | Eocene  | GRF      |
| 522483       | 0.9526                              | 3.2427 | 121.7301 | 40.4457 | 814.4717 | 0.7503 | 2.0787 | Light Brown   | #999999 | #a1a1a1 | Fulgoridae | Hemiptera | Eocene  | GRF      |
| 522483       | 0.8499                              | 2.9473 | 108.0373 | 33.0459 | 712.8988 | 0.7809 | 1.7768 | Light Brown   | #999999 | #a1a1a1 | Fulgoridae | Hemiptera | Eocene  | GRF      |
| 522483       | 0.8481                              | 2.9489 | 110.8855 | 34.1644 | 748.7714 | 0.6603 | 1.8027 | Light Brown   | #999999 | #a1a1a1 | Fulgoridae | Hemiptera | Eocene  | GRF      |
| 522483       | 0.729                               | 2.4717 | 93.5414  | 31.1548 | 648.8336 | 0.5455 | 1.7094 | Light Brown   | #999999 | #a1a1a1 | Fulgoridae | Hemiptera | Eocene  | GRF      |
| 522483       | 0.5959                              | 1.9794 | 74.8655  | 22.2055 | 524.2074 | 0.4275 | 1.3641 | Sediment      | #ff9900 | #ff8c00 | GRF sed    | GRF sed   | Eocene  | GRF      |
| 522483       | 0.6654                              | 2.3211 | 84.007   | 27.0579 | 482.8123 | 0.4755 | 1.4084 | Sediment      | #ff9900 | #ff8c00 | GRF sed    | GRF sed   | Eocene  | GRF      |
| 14431        | 3.1671                              | 4.3431 | 747.7009 | 27.3293 | 183.1279 | 1.1146 | 0.687  | Dark Brown    | #000000 | #000000 | N/A        | Hemiptera | Miocene | Oeningen |
| 14431        | 2.3934                              | 3.6357 | 650.0528 | 13.28   | 120.7814 | 0.9259 | 0.5332 | Dark Brown    | #000000 | #000000 | N/A        | Hemiptera | Miocene | Oeningen |
| 14431        | 2.8482                              | 3.7434 | 643.3816 | 20.8427 | 182.246  | 0.9037 | 0.6736 | Dark Brown    | #000000 | #000000 | N/A        | Hemiptera | Miocene | Oeningen |
| 14431        | 1.8484                              | 2.6991 | 416.8329 | 14.019  | 142.7138 | 0.5182 | 0.428  | Light Brown   | #999999 | #a1a1a1 | N/A        | Hemiptera | Miocene | Oeningen |
| 14431        | 1.7588                              | 2.3771 | 376.7246 | 10.8801 | 115.993  | 0.4678 | 0.4511 | Light Brown   | #999999 | #a1a1a1 | N/A        | Hemiptera | Miocene | Oeningen |
| 14431        | 1.6884                              | 2.7034 | 445.8042 | 10.1693 | 83.4172  | 0.4366 | 0.3446 | Light Brown   | #999999 | #a1a1a1 | N/A        | Hemiptera | Miocene | Oeningen |

| specimen # | concentration ( $\mu\text{g}/\text{cm}^2$ ) |         |          |         |          |        | visual colour | websafe | hex     | family  | order      | age         | location |
|------------|---------------------------------------------|---------|----------|---------|----------|--------|---------------|---------|---------|---------|------------|-------------|----------|
|            | S                                           | K       | Ca       | Ti      | Fe       | Cu     |               |         |         |         |            |             |          |
| 454454     | 1.037                                       | 2.9053  | 155.0362 | 25.1063 | 558.2977 | 1.4176 | Dark Brown    | #000000 | #000000 | N/A     | Coleoptera | Eocene      | GRF      |
| 454454     | 1.0163                                      | 2.7848  | 159.9737 | 22.4629 | 527.3278 | 1.5873 | Dark Brown    | #000000 | #000000 | N/A     | Coleoptera | Eocene      | GRF      |
| 454454     | 1.0808                                      | 3.0161  | 155.3005 | 26.2215 | 576.3452 | 1.9735 | Dark Brown    | #000000 | #000000 | N/A     | Coleoptera | Eocene      | GRF      |
| 454454     | 0.8915                                      | 2.8193  | 151.7213 | 22.4775 | 440.5268 | 0.4863 | Sediment      | #ff9900 | #ff8c00 | GRF sed | GRF sed    | Eocene      | GRF      |
| 454454     | 0.5835                                      | 1.9006  | 96.1053  | 15.0684 | 329.3097 | 0.3695 | Sediment      | #ff9900 | #ff8c00 | GRF sed | GRF sed    | Eocene      | GRF      |
| 454454     | 0.7437                                      | 2.247   | 124.3376 | 19.1402 | 400.2159 | 0.4108 | Sediment      | #ff9900 | #ff8c00 | GRF sed | GRF sed    | Eocene      | GRF      |
| 390330     | 0.9302                                      | 3.7283  | 92.2064  | 40.5012 | 882.9615 | 1.9625 | Dark Brown    | #000000 | #000000 | N/A     | Coleoptera | Eocene      | GRF      |
| 390330     | 1.1672                                      | 4.0036  | 108.2797 | 39.8632 | 900.4828 | 1.8726 | Dark Brown    | #000000 | #000000 | N/A     | Coleoptera | Eocene      | GRF      |
| 390330     | 0.6744                                      | 2.8742  | 73.3571  | 29.8758 | 689.6323 | 1.8197 | Dark Brown    | #000000 | #000000 | N/A     | Coleoptera | Eocene      | GRF      |
| 390330     | 0.6821                                      | 2.8469  | 68.0823  | 26.9049 | 762.0241 | 0.6665 | Sediment      | #ff9900 | #ff8c00 | GRF sed | GRF sed    | Eocene      | GRF      |
| 390330     | 0.8885                                      | 3.903   | 96.6156  | 33.8904 | 966.5067 | 0.8113 | Sediment      | #ff9900 | #ff8c00 | GRF sed | GRF sed    | Eocene      | GRF      |
| 390330     | 0.5999                                      | 1.9445  | 47.3249  | 20.19   | 665.9956 | 0.5863 | Sediment      | #ff9900 | #ff8c00 | GRF sed | GRF sed    | Eocene      | GRF      |
| UCM_51311a | 0.5469                                      | 9.7721  | 95.3982  | 41.8241 | 549.7165 | 5.3886 | Dark Brown    | #000000 | #000000 | N/A     | Coleoptera | Eocene      | GRF      |
| UCM_51311a | 1.6415                                      | 10.0884 | 70.1563  | 42.5988 | 508.0951 | 6.0282 | Dark Brown    | #000000 | #000000 | N/A     | Coleoptera | Eocene      | GRF      |
| UCM_51311a | 1.364                                       | 8.5934  | 57.1167  | 38.4634 | 417.6255 | 5.5293 | Dark Brown    | #000000 | #000000 | N/A     | Coleoptera | Eocene      | GRF      |
| UCM_51311a | 1.8331                                      | 14.661  | 81.4674  | 55.0373 | 541.1469 | 1.9088 | Light Brown   | #999999 | #a1a1a1 | N/A     | Coleoptera | Eocene      | GRF      |
| UCM_51311a | 1.5022                                      | 12.3009 | 62.9956  | 47.2071 | 460.6269 | 1.5785 | Light Brown   | #999999 | #a1a1a1 | N/A     | Coleoptera | Eocene      | GRF      |
| UCM_51311a | 1.6723                                      | 12.4682 | 75.5611  | 45.9484 | 508.7716 | 1.9268 | Light Brown   | #999999 | #a1a1a1 | N/A     | Coleoptera | Eocene      | GRF      |
| UCM_51311a | 1.2014                                      | 9.0174  | 51.9041  | 34.148  | 402.4169 | 0.6986 | Sediment      | #ff9900 | #ff8c00 | GRF sed | GRF sed    | Eocene      | GRF      |
| UCM_51311a | 1.5974                                      | 11.1609 | 78.2011  | 48.0793 | 531.3766 | 0.8153 | Sediment      | #ff9900 | #ff8c00 | GRF sed | GRF sed    | Eocene      | GRF      |
| UCM_51311a | 1.5368                                      | 10.9226 | 65.4737  | 42.4279 | 456.6494 | 5.8529 | Dark Brown    | #000000 | #000000 | N/A     | Coleoptera | Eocene      | GRF      |
| UCM_51311a | 1.3634                                      | 10.3036 | 60.5318  | 49.2916 | 452.9027 | 4.8278 | Dark Brown    | #000000 | #000000 | N/A     | Coleoptera | Eocene      | GRF      |
| UCM_51311a | 1.3493                                      | 13.1683 | 69.6937  | 45.2478 | 392.6846 | 5.2184 | Dark Brown    | #999999 | #a1a1a1 | N/A     | Coleoptera | Eocene      | GRF      |
| UCM_51311a | 1.4584                                      | 10.9341 | 65.0835  | 39.9081 | 461.9231 | 1.7714 | Light Brown   | #999999 | #a1a1a1 | N/A     | Coleoptera | Eocene      | GRF      |
| UCM_51311a | 1.5687                                      | 11.6844 | 69.1532  | 38.8537 | 517.9591 | 1.6573 | Light Brown   | #999999 | #a1a1a1 | N/A     | Coleoptera | Eocene      | GRF      |
| UCM_51311a | 1.529                                       | 11.9877 | 65.5079  | 41.2796 | 432.186  | 1.8623 | Light Brown   | #999999 | #a1a1a1 | N/A     | Coleoptera | Eocene      | GRF      |
| UCM_51311a | 0.9378                                      | 7.4948  | 40.6611  | 24.8506 | 294.1569 | 0.5938 | Sediment      | #ff9900 | #ff8c00 | GRF sed | GRF sed    | Eocene      | GRF      |
| UCM_51311a | 1.7606                                      | 11.503  | 84.7455  | 41.451  | 518.2508 | 0.7801 | Sediment      | #ff9900 | #ff8c00 | GRF sed | GRF sed    | Eocene      | GRF      |
| OCB6       | 0.7524                                      | 0.5762  | 26.1216  | 9.4415  | 122.7546 | 0.8476 | Dark Brown    | #000000 | #000000 | N/A     | Coleoptera | Pleistocene | OCR      |
| OCB6       | 0.7914                                      | 0.6378  | 25.4344  | 10.0708 | 123.7579 | 0.9824 | Dark Brown    | #000000 | #000000 | N/A     | Coleoptera | Pleistocene | OCR      |
| OCB6       | 0.7842                                      | 0.6162  | 23.9597  | 9.725   | 111.6646 | 0.7783 | Dark Brown    | #000000 | #000000 | N/A     | Coleoptera | Pleistocene | OCR      |
| OCB6       | 0.436                                       | 0.6783  | 7.6784   | 10.5147 | 51.7204  | 0.4793 | Light Brown   | #999999 | #a1a1a1 | N/A     | Coleoptera | Pleistocene | OCR      |
| OCB6       | 0.5944                                      | 0.9249  | 10.6212  | 17.7027 | 82.6356  | 0.5981 | Light Brown   | #999999 | #a1a1a1 | N/A     | Coleoptera | Pleistocene | OCR      |
| OCB6       | 0.5926                                      | 1.1936  | 10.789   | 13.7885 | 91.3242  | 0.6855 | Light Brown   | #999999 | #a1a1a1 | N/A     | Coleoptera | Pleistocene | OCR      |

| specimen # | concentration ( $\mu\text{g}/\text{cm}^2$ ) |        |          |         |          |        |        | visual colour    | websafe | hex     | family       | order        | age      | location |
|------------|---------------------------------------------|--------|----------|---------|----------|--------|--------|------------------|---------|---------|--------------|--------------|----------|----------|
|            | S                                           | K      | Ca       | Ti      | Fe       | Cu     | Zn     |                  |         |         |              |              |          |          |
| MCZ14426   | 7.5093                                      | 4.7651 | 847.7469 | 13.8462 | 79.4345  | 0.9681 | 0.9492 | Dark Brown       | #000000 | #000000 | N/A          | Hemiptera    | Miocene  | Oeningen |
| MCZ14426   | 6.3471                                      | 3.987  | 745.4491 | 9.2949  | 46.3354  | 0.7118 | 0.3283 | Dark Brown       | #000000 | #000000 | N/A          | Hemiptera    | Miocene  | Oeningen |
| MCZ14426   | 6.3323                                      | 3.83   | 715.2697 | 8.4081  | 34.1577  | 0.7005 | 0.4136 | Dark Brown       | #000000 | #000000 | N/A          | Hemiptera    | Miocene  | Oeningen |
| MCZ14426   | 5.7274                                      | 3.7715 | 703.6915 | 7.7034  | 43.917   | 0.3547 | 0.3822 | Light Brown      | #999999 | #a1a1a1 | N/A          | Hemiptera    | Miocene  | Oeningen |
| MCZ14426   | 4.8623                                      | 3.5419 | 628.7082 | 7.2024  | 46.5369  | 0.2573 | 0.2966 | Light Brown      | #999999 | #a1a1a1 | N/A          | Hemiptera    | Miocene  | Oeningen |
| MCZ14426   | 6.6014                                      | 4.2821 | 803.1441 | 6.8376  | 36.7792  | 0.3766 | 0.4878 | Light Brown      | #999999 | #a1a1a1 | N/A          | Hemiptera    | Miocene  | Oeningen |
| MCZ14426   | 5.9031                                      | 3.9729 | 695.5643 | 13.2788 | 65.3576  | 0.4356 | 0.4663 | Light-Dark Brown | #000000 | #000000 | N/A          | Hemiptera    | Miocene  | Oeningen |
| MCZ14426   | 5.7098                                      | 3.9395 | 686.9954 | 12.6048 | 79.9647  | 0.4648 | 0.3461 | Light-Dark Brown | #000000 | #000000 | N/A          | Hemiptera    | Miocene  | Oeningen |
| MCZ14426   | 6.2323                                      | 3.9875 | 731.8909 | 15.4096 | 103.7289 | 0.4427 | 0.3374 | Light-Dark Brown | #000000 | #000000 | N/A          | Hemiptera    | Miocene  | Oeningen |
| MCZ14426   | 6.0786                                      | 3.9162 | 740.4982 | 8.2673  | 43.5648  | 0.6841 | 0.401  | Dark Brown       | #000000 | #000000 | N/A          | Hemiptera    | Miocene  | Oeningen |
| MCZ14426   | 6.5121                                      | 3.9943 | 723.1535 | 10.0831 | 58.2275  | 0.7588 | 0.3866 | Dark Brown       | #000000 | #000000 | N/A          | Hemiptera    | Miocene  | Oeningen |
| MCZ14426   | 6.4419                                      | 3.9223 | 744.3874 | 10.261  | 45.9848  | 0.8117 | 0.4072 | Dark Brown       | #000000 | #000000 | N/A          | Hemiptera    | Miocene  | Oeningen |
| MCZ14426   | 5.414                                       | 3.5716 | 643.6276 | 5.9467  | 54.9683  | 0.287  | 0.3553 | Light Brown      | #999999 | #a1a1a1 | N/A          | Hemiptera    | Miocene  | Oeningen |
| MCZ14426   | 6.1208                                      | 4.0139 | 758.6609 | 5.9638  | 36.8749  | 0.3592 | 0.3133 | Light Brown      | #999999 | #a1a1a1 | N/A          | Hemiptera    | Miocene  | Oeningen |
| MCZ14426   | 5.7169                                      | 3.769  | 704.9369 | 5.3442  | 29.5932  | 0.3066 | 0.3071 | Light Brown      | #999999 | #a1a1a1 | N/A          | Hemiptera    | Miocene  | Oeningen |
| NND11021   | 1.0554                                      | 6.0929 | 16.2125  | 27.2965 | 254.5979 | 1.3255 | 1.1944 | Dark Brown       | #000000 | #000000 | N/A          | Neuroptera   | Jurassic | Daohugou |
| NND11021   | 1.1045                                      | 6.064  | 15.4375  | 31.0676 | 302.2112 | 1.3185 | 1.3374 | Dark Brown       | #000000 | #000000 | N/A          | Neuroptera   | Jurassic | Daohugou |
| NND11021   | 1.276                                       | 8.2731 | 20.0183  | 30.2699 | 272.0179 | 1.4683 | 1.3964 | Dark Brown       | #000000 | #000000 | N/A          | Neuroptera   | Jurassic | Daohugou |
| NND11021   | 1.1551                                      | 8.3515 | 18.0387  | 36.2664 | 301.5722 | 0.9925 | 1.2821 | Light-Dark Brown | #000000 | #000000 | N/A          | Neuroptera   | Jurassic | Daohugou |
| NND11021   | 1.1581                                      | 5.8015 | 14.9454  | 27.7185 | 326.2016 | 0.8547 | 1.1911 | Brown            | #999999 | #a1a1a1 | N/A          | Neuroptera   | Jurassic | Daohugou |
| NND11021   | 1.0211                                      | 5.5197 | 14.8603  | 26.0475 | 266.6172 | 0.8919 | 1.1596 | Brown            | #999999 | #a1a1a1 | N/A          | Neuroptera   | Jurassic | Daohugou |
| NND11021   | 1.3245                                      | 7.2497 | 20.1778  | 34.6881 | 325.8808 | 0.9919 | 1.294  | Brown            | #999999 | #a1a1a1 | N/A          | Neuroptera   | Jurassic | Daohugou |
| NND11021   | 1.1663                                      | 6.4568 | 16.1538  | 32.3116 | 332.2902 | 0.9971 | 1.6939 | Brown            | #999999 | #a1a1a1 | N/A          | Neuroptera   | Jurassic | Daohugou |
| NND11021   | 1.1551                                      | 5.2486 | 15.8504  | 28.9105 | 309.1903 | 0.8856 | 1.1871 | Brown            | #999999 | #a1a1a1 | N/A          | Neuroptera   | Jurassic | Daohugou |
| NND11021   | 1.0761                                      | 6.537  | 14.9543  | 26.2236 | 307.4412 | 0.7469 | 1.1231 | Light Brown      | #999999 | #a1a1a1 | N/A          | Neuroptera   | Jurassic | Daohugou |
| NND11021   | 0.9666                                      | 5.1125 | 14.4736  | 26.1462 | 290.5633 | 0.7804 | 1.1029 | Light Brown      | #999999 | #a1a1a1 | N/A          | Neuroptera   | Jurassic | Daohugou |
| NND11021   | 1.1801                                      | 6.448  | 15.6583  | 28.2219 | 267.421  | 0.8541 | 1.15   | Light Brown      | #999999 | #a1a1a1 | N/A          | Neuroptera   | Jurassic | Daohugou |
| NND11021   | 1.1476                                      | 6.9293 | 16.8055  | 29.7245 | 271.9064 | 0.7431 | 1.1902 | Sediment         | #ff9900 | #ff8c00 | Daohugou sed | Daohugou sed | Jurassic | Daohugou |
| NND11021   | 0.9548                                      | 6.0625 | 15       | 24.4284 | 237.169  | 0.7178 | 1.1555 | Sediment         | #ff9900 | #ff8c00 | Daohugou sed | Daohugou sed | Jurassic | Daohugou |

| specimen #  | concentration ( $\mu\text{g}/\text{cm}^2$ ) |         |         |         |          |        |        | visual colour    | websafe | hex     | family       | order        | age      | location |
|-------------|---------------------------------------------|---------|---------|---------|----------|--------|--------|------------------|---------|---------|--------------|--------------|----------|----------|
|             | S                                           | K       | Ca      | Ti      | Fe       | Cu     | Zn     |                  |         |         |              |              |          |          |
| NND11010    | 1.1136                                      | 9.6849  | 15.7368 | 32.1277 | 248.2907 | 1.154  | 1.114  | Dark Brown       | #000000 | #000000 | N/A          | Neuroptera   | Jurassic | Daohugou |
| NND11010    | 1.1921                                      | 10.1003 | 17.2485 | 34.9124 | 247.9541 | 1.5438 | 1.296  | Dark Brown       | #000000 | #000000 | N/A          | Neuroptera   | Jurassic | Daohugou |
| NND11010    | 1.1042                                      | 9.8342  | 16.5311 | 30.9972 | 243.6669 | 1.2546 | 1.172  | Dark Brown       | #000000 | #000000 | N/A          | Neuroptera   | Jurassic | Daohugou |
| NND11010    | 0.9582                                      | 6.9341  | 11.9566 | 37.8724 | 264.3662 | 0.5966 | 1.1424 | Light Brown      | #999999 | #a1a1a1 | N/A          | Neuroptera   | Jurassic | Daohugou |
| NND11010    | 1.0458                                      | 7.3836  | 12.6861 | 41.3757 | 303.1046 | 0.6845 | 1.1915 | Light Brown      | #999999 | #a1a1a1 | N/A          | Neuroptera   | Jurassic | Daohugou |
| NND11010    | 0.806                                       | 6.1361  | 11.2826 | 31.4608 | 203.4766 | 0.5605 | 1.0445 | Light Brown      | #999999 | #a1a1a1 | N/A          | Neuroptera   | Jurassic | Daohugou |
| NND11010    | 1.008                                       | 8.5759  | 13.0711 | 28.3723 | 227.5883 | 0.7383 | 1.134  | Sediment         | #ff9900 | #ff8c00 | Daohugou sed | Daohugou sed | Jurassic | Daohugou |
| NND11010    | 0.9603                                      | 9.0714  | 13.3466 | 30.7685 | 227.0087 | 0.7588 | 1.3578 | Sediment         | #ff9900 | #ff8c00 | Daohugou sed | Daohugou sed | Jurassic | Daohugou |
| NND11010    | 1.1268                                      | 9.4527  | 16.7576 | 31.7713 | 246.5222 | 0.7177 | 1.1122 | Sediment         | #ff9900 | #ff8c00 | Daohugou sed | Daohugou sed | Jurassic | Daohugou |
| NND11023    | 1.3079                                      | 5.9453  | 16.8845 | 31.6144 | 408.6371 | 1.2369 | 1.702  | Dark Brown       | #000000 | #000000 | N/A          | Neuroptera   | Jurassic | Daohugou |
| NND11023    | 1.2153                                      | 5.6475  | 15.9638 | 28.7722 | 404.9056 | 1.1088 | 1.676  | Dark Brown       | #000000 | #000000 | N/A          | Neuroptera   | Jurassic | Daohugou |
| NND11023    | 1.2681                                      | 5.9477  | 16.0137 | 29.3176 | 424.2358 | 1.1079 | 1.6807 | Dark Brown       | #000000 | #000000 | N/A          | Neuroptera   | Jurassic | Daohugou |
| NND11023    | 1.2237                                      | 5.7824  | 14.5248 | 27.1251 | 396.2514 | 0.9865 | 1.6666 | Light-Dark Brown | #000000 | #000000 | N/A          | Neuroptera   | Jurassic | Daohugou |
| NND11023    | 1.1418                                      | 5.6656  | 12.8579 | 28.1373 | 391.5322 | 0.7965 | 1.6387 | Light-Dark Brown | #000000 | #000000 | N/A          | Neuroptera   | Jurassic | Daohugou |
| NND11023    | 1.1288                                      | 5.8701  | 13.9402 | 26.3421 | 380.5885 | 0.9476 | 1.5895 | Light-Dark Brown | #000000 | #000000 | N/A          | Neuroptera   | Jurassic | Daohugou |
| NND11023    | 1.2645                                      | 6.2409  | 14.6463 | 31.7973 | 427.7134 | 0.864  | 1.7022 | Light Brown      | #999999 | #a1a1a1 | N/A          | Neuroptera   | Jurassic | Daohugou |
| NND11023    | 1.3859                                      | 6.9686  | 14.9182 | 29.8924 | 431.7134 | 0.8354 | 1.6328 | Light Brown      | #999999 | #a1a1a1 | N/A          | Neuroptera   | Jurassic | Daohugou |
| NND11023    | 1.403                                       | 6.566   | 15.7592 | 32.0366 | 429.413  | 0.9716 | 1.7524 | Light Brown      | #999999 | #a1a1a1 | N/A          | Neuroptera   | Jurassic | Daohugou |
| NND11023    | 1.1921                                      | 6.0441  | 13.8255 | 27.9364 | 412.474  | 0.7724 | 1.6052 | Light            | #999999 | #a1a1a1 | N/A          | Neuroptera   | Jurassic | Daohugou |
| NND11023    | 1.2801                                      | 5.092   | 13.3263 | 32.2877 | 434.3728 | 0.7064 | 1.596  | Light            | #999999 | #a1a1a1 | N/A          | Neuroptera   | Jurassic | Daohugou |
| NND11023    | 1.3478                                      | 5.3546  | 14.6311 | 31.4677 | 432.8355 | 0.767  | 1.5763 | Light            | #999999 | #a1a1a1 | N/A          | Neuroptera   | Jurassic | Daohugou |
| NND11023    | 1.165                                       | 4.4486  | 12.6348 | 26.4797 | 416.7632 | 0.5853 | 1.5118 | Sediment         | #ff9900 | #ff8c00 | Daohugou sed | Daohugou sed | Jurassic | Daohugou |
| NND11023    | 1.1327                                      | 4.608   | 12.8118 | 27.9353 | 437.0869 | 0.5983 | 1.5882 | Sediment         | #ff9900 | #ff8c00 | Daohugou sed | Daohugou sed | Jurassic | Daohugou |
| NND140539-1 | 1.0596                                      | 2.8258  | 9.4077  | 22.0893 | 548.1732 | 0.5912 | 1.1466 | Dark Brown       | #000000 | #000000 | N/A          | Neuroptera   | Jurassic | Daohugou |
| NND140539-1 | 0.9859                                      | 2.9517  | 10.0277 | 23.3969 | 495.2253 | 0.6575 | 1.1386 | Dark Brown       | #000000 | #000000 | N/A          | Neuroptera   | Jurassic | Daohugou |
| NND140539-1 | 0.9515                                      | 3.4211  | 11.0869 | 23.0983 | 148.194  | 0.6104 | 1.2471 | Dark Brown       | #000000 | #000000 | N/A          | Neuroptera   | Jurassic | Daohugou |
| NND140539-1 | 3.2053                                      | 3.2053  | 10.3037 | 22.3272 | 503.6325 | 0.5407 | 1.2655 | Light Brown      | #999999 | #a1a1a1 | N/A          | Neuroptera   | Jurassic | Daohugou |
| NND140539-1 | 1.0176                                      | 2.9685  | 9.3598  | 21.3604 | 512.4456 | 0.5041 | 1.1773 | Light Brown      | #999999 | #a1a1a1 | N/A          | Neuroptera   | Jurassic | Daohugou |
| NND140539-1 | 0.9582                                      | 2.8578  | 9.1171  | 21.1097 | 485.4236 | 0.4785 | 1.1736 | Light Brown      | #999999 | #a1a1a1 | N/A          | Neuroptera   | Jurassic | Daohugou |



| specimen #  | concentration ( $\mu\text{g}/\text{cm}^2$ ) |        |         |         |          |        |        | visual colour | websafe | hex     | family        | order         | age      | location  |
|-------------|---------------------------------------------|--------|---------|---------|----------|--------|--------|---------------|---------|---------|---------------|---------------|----------|-----------|
|             | S                                           | K      | Ca      | Ti      | Fe       | Cu     | Zn     |               |         |         |               |               |          |           |
| NND140539-2 | 1.4065                                      | 3.9144 | 9.3782  | 27.2005 | 503.9791 | 0.7813 | 1.5081 | Dark Brown    | #000000 | #000000 | N/A           | Neuroptera    | Jurassic | Daohugou  |
| NND140539-2 | 1.3466                                      | 4.3222 | 10.4154 | 28.3729 | 503.6089 | 0.8779 | 1.5525 | Dark Brown    | #000000 | #000000 | N/A           | Neuroptera    | Jurassic | Daohugou  |
| NND140539-2 | 1.417                                       | 3.8755 | 9.5815  | 27.0815 | 505.0512 | 0.8009 | 1.4564 | Dark Brown    | #000000 | #000000 | N/A           | Neuroptera    | Jurassic | Daohugou  |
| NND140539-2 | 1.3638                                      | 3.2341 | 8.3845  | 24.9762 | 490.2111 | 0.6642 | 1.4134 | Light Brown   | #999999 | #a1a1a1 | N/A           | Neuroptera    | Jurassic | Daohugou  |
| NND140539-2 | 1.3978                                      | 3.3659 | 8.3868  | 25.9351 | 497.7453 | 0.659  | 1.4104 | Light Brown   | #999999 | #a1a1a1 | N/A           | Neuroptera    | Jurassic | Daohugou  |
| NND140539-2 | 1.3552                                      | 3.4432 | 8.564   | 24.9917 | 509.0932 | 0.6769 | 1.4039 | Light Brown   | #999999 | #a1a1a1 | N/A           | Neuroptera    | Jurassic | Daohugou  |
| NND140539-2 | 1.1965                                      | 2.3533 | 8.0331  | 19.1438 | 555.9459 | 0.4474 | 0.8851 | Sediment      | #ff9900 | #ff8c00 | Daohugou sed  | Daohugou sed  | Jurassic | Daohugou  |
| NND140539-2 | 1.1611                                      | 2.1964 | 7.4191  | 19.2921 | 567.8513 | 0.4165 | 0.8379 | Sediment      | #ff9900 | #ff8c00 | Daohugou sed  | Daohugou sed  | Jurassic | Daohugou  |
| UAF_GS7     | 1.6108                                      | 0.8035 | 4.3795  | 22.5907 | 254.1166 | 0.7135 | 0.787  | Dark Brown    | #000000 | #000000 | N/A           | Hymenoptera   | Miocene  | Grubstake |
| UAF_GS7     | 1.6189                                      | 0.7952 | 4.3163  | 22.1497 | 208.648  | 0.6307 | 0.7068 | Dark Brown    | #000000 | #000000 | N/A           | Hymenoptera   | Miocene  | Grubstake |
| UAF_GS7     | 1.678                                       | 0.8779 | 5.1841  | 24.9059 | 323.1379 | 0.941  | 1.0311 | Dark Brown    | #000000 | #000000 | N/A           | Hymenoptera   | Miocene  | Grubstake |
| UAF_GS7     | 1.6269                                      | 0.7569 | 3.9059  | 21.0212 | 145.5693 | 0.4977 | 0.5698 | Light Brown   | #999999 | #a1a1a1 | N/A           | Hymenoptera   | Miocene  | Grubstake |
| UAF_GS7     | 1.6303                                      | 0.7717 | 4.1533  | 20.6341 | 142.2028 | 0.5017 | 0.5272 | Light Brown   | #999999 | #a1a1a1 | N/A           | Hymenoptera   | Miocene  | Grubstake |
| UAF_GS7     | 1.7093                                      | 0.7735 | 4.024   | 21.225  | 162.2584 | 0.5436 | 0.6441 | Light Brown   | #999999 | #a1a1a1 | N/A           | Hymenoptera   | Miocene  | Grubstake |
| UAF_GS7     | 1.6993                                      | 0.8499 | 4.7695  | 23.1872 | 226.9845 | 0.5485 | 0.7546 | Sediment      | #ff9900 | #ff8c00 | Grubstake sed | Grubstake sed | Miocene  | Grubstake |
| UAF_GS7     | 1.5324                                      | 0.6025 | 3.0603  | 13.6823 | 39.1267  | 0.2649 | 0.3175 | Sediment      | #ff9900 | #ff8c00 | Grubstake sed | Grubstake sed | Miocene  | Grubstake |

**Supplementary Table 5.4 (previous page). Database of chemistry of fossil specimens analyzed using synchrotron rapid-scanning X-ray fluorescence.**

| <b>Pigments</b> | <b>Total</b> | <b>P</b>  | <b>S</b>  | <b>Cl</b> | <b>K</b>  | <b>Ca</b> | <b>Ti</b> | <b>Mn</b> | <b>Fe</b> | <b>Ni</b> | <b>Cu</b> | <b>Zn</b> |
|-----------------|--------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| All pigments    | 3.02E-11     | 1.55E-02  | 1.37E-01  | 1.69E-02  | 4.60E-07  | 5.17E-04  | 3.75E-04  | 1.62E-03  | 1.25E-03  | 1.31E-05  | 1.40E-01  | 7.18E-02  |
| Carotenoids     | <2.20E-16    | 9.32E-09  | <2.20E-16 | 2.12E-13  | 1.03E-05  | <2.20E-16 | 4.45E-08  | <2.20E-16 | 9.06E-10  | <2.20E-16 | 2.54E-03  | 5.11E-04  |
| Flavonoids      | 3.04E-11     | 4.26E-05  | 2.06E-04  | 2.62E-04  | 2.90E-03  | 8.29E-05  | 1.03E-03  | 4.61E-02  | 7.72E-03  | 4.00E-02  | 4.67E-02  | 3.69E-03  |
| Melanin         | <2.20E-16    | <2.20E-16 | <2.20E-16 | <2.20E-16 | <2.20E-16 | <2.20E-16 | <2.20E-16 | <2.20E-16 | 1.31E-13  | 3.39E-16  | <2.20E-16 | <2.20E-16 |
| Ommochromes     | <2.20E-16    | 8.05E-10  | 1.84E-10  | 3.97E-11  | 5.35E-08  | 3.79E-08  | 3.73E-08  | 3.43E-11  | 5.78E-08  | 1.62E-05  | 2.09E-07  | 2.21E-10  |
| Pterin          | <2.20E-16    | 6.93E-09  | 1.02E-09  | 3.46E-07  | 1.44E-09  | 1.45E-15  | 9.12E-05  | 3.68E-08  | 4.94E-09  | 4.25E-05  | 1.48E-08  | 4.75E-07  |
| <b>Orders</b>   | <b>Total</b> | <b>P</b>  | <b>S</b>  | <b>Cl</b> | <b>K</b>  | <b>Ca</b> | <b>Ti</b> | <b>Mn</b> | <b>Fe</b> | <b>Ni</b> | <b>Cu</b> | <b>Zn</b> |
| All Orders      | <2.20E-16    | 1.45E-13  | 1.76E-10  | 4.10E-12  | <2.20E-16 | <2.20E-16 | 7.35E-05  | 1.66E-15  | 4.20E-11  | 1.93E-13  | 4.37E-05  | 6.37E-02  |
| Coleoptera      | <2.20E-16    | <2.20E-16 | <2.20E-16 | <2.20E-16 | 3.09E-13  | <2.20E-16 | <2.20E-16 | <2.20E-16 | 9.78E-12  | <2.20E-16 | <2.20E-16 | 1.44E-12  |
| Hemiptera       | <2.20E-16    | 1.52E-06  | 1.01E-03  | 1.34E-02  | 9.47E-04  | 1.48E-01  | 1.47E-02  | 1.12E-07  | 1.19E-03  | 4.68E-02  | 2.83E-09  | 7.49E-05  |
| Lepidoptera     | <2.20E-16    | <2.20E-16 | <2.20E-16 | <2.20E-16 | <2.20E-16 | <2.20E-16 | 4.52E-13  | <2.20E-16 | <2.20E-16 | 1.18E-06  | 4.70E-03  | <2.20E-16 |
| Odonata         | 9.73E-10     | 3.59E-10  | 4.04E-03  | 4.28E-04  | 8.95E-03  | 1.65E-05  | 2.70E-13  | 1.51E-02  | 5.44E-08  | 1.87E-02  | 9.37E-04  | 5.89E-05  |
| <b>Families</b> | <b>Total</b> | <b>P</b>  | <b>S</b>  | <b>Cl</b> | <b>K</b>  | <b>Ca</b> | <b>Ti</b> | <b>Mn</b> | <b>Fe</b> | <b>Ni</b> | <b>Cu</b> | <b>Zn</b> |
| Nymphalidae     | <2.20E-16    | <2.20E-16 | <2.20E-16 | <2.20E-16 | 5.38E-04  | 9.34E-12  | <2.20E-16 | <2.20E-16 | <2.20E-16 | 8.12E-02  | <2.20E-16 | <2.20E-16 |
| Zygaenidae      | 8.94E-10     | 1.14E-10  | 4.29E-06  | 1.08E-12  | 4.54E-14  | 4.05E-06  | 8.07E-04  | 1.37E-10  | 7.53E-05  | 8.70E-09  | 2.20E-07  | 6.96E-09  |

**Supplementary Table 5.5 (previous page). MANOVA  $p$ -value indicating the statistical significance of differences in elemental concentrations among pigments and taxonomic relations in extant insects.** Statistically significant results are indicated in bold font. Only elements responsible for the variation in the data for each pigment are represented.

|                      | Modern run A |        |       |         |             | Modern run B |        |       |         |             | Fossil without sediment |          |       |       |             | Fossil with sediment |          |       |       |             |
|----------------------|--------------|--------|-------|---------|-------------|--------------|--------|-------|---------|-------------|-------------------------|----------|-------|-------|-------------|----------------------|----------|-------|-------|-------------|
|                      | LDA          | Family | Order | Pigment | PCA         | LDA          | Family | Order | Pigment | PCA         | LDA                     | Locality | Order | Age   | PCA         | LDA                  | Locality | Order | Age   | PCA         |
| Log-transformed data | LD1 %        | 48.6   | 82.85 | 48.9    | PC1 % 61.1  | LD1 %        | 77.61  | 56.19 | 81.41   | PC1 % 69.33 | LD1 %                   | 69.99    | 81.13 | 66.97 | PC1 % 58.4  | LD1 %                | 66.04    | 72.68 | 67.53 | PC1 % 54.66 |
|                      | LD2 %        | 18.55  | 9.31  | 22.7    | PC2 % 9.1   | LD2 %        | 11.38  | 30.67 | 18.59   | PC2 % 15.07 | LD2 %                   | 22.96    | 13.17 | 20.49 | PC2 % 22.04 | LD2 %                | 27.31    | 17.84 | 20.22 | PC2 % 22.81 |
| Raw data             | LD1 %        | 35.81  | 63.66 | 42.72   | PC1 % 50.23 | LD1 %        | 62.15  | 66.39 | 91.58   | PC1 % 56.51 | LD1 %                   | 60.66    | 76.23 | 67.54 | PC1 % 58.35 | LD1 %                | 62.04    | 69.45 | 63.42 | PC1 % 55.32 |
|                      | LD2 %        | 14.22  | 27.97 | 25.97   | PC2 % 12.02 | LD2 %        | 18.56  | 21.84 | 8.42    | PC2 % 17.96 | LD2 %                   | 30.52    | 14.73 | 28.53 | PC2 % 20.72 | LD2 %                | 30.03    | 16.49 | 31.75 | PC2 % 21.66 |

**Supplementary Table 5.6 (previous page). Summary of Principal Component Analysis and Linear Discriminant Analysis results for raw and log-transformed concentration data for various elements, derived from synchrotron rapid-scanning X-ray fluorescence analysis.**

| <b>Fossil locality</b> | <b>Metal partitioning<br/>in dark region</b> | <b>Metal partitioning<br/>light region</b> |
|------------------------|----------------------------------------------|--------------------------------------------|
| Rubielos de Mora       | Ca, K, Fe                                    | Ca, K, Zn                                  |
| Oeningen               | Ca, Ti                                       | Ca, K                                      |
| Grubstake              | Ti, S, Zn                                    | Ti, S, Cu                                  |
| Old Crow River         | Cu                                           | Cu, Ti, S                                  |
| Daohugou               | K, Fe, Zn                                    | K, Fe, Cu                                  |
| Green River            | K, Fe, Cu                                    | K, Fe, Ca                                  |

**Supplementary Table 5.7. Elements summarising associations between cuticle regions of different tones in the fossils analyzed.**

| Family               | LDA     | PCA     | # species |
|----------------------|---------|---------|-----------|
| Asilidae             | 0.72    | 0.15    | 1         |
| Calopterigidae       | 4.45    | 1.15    | 2         |
| Cerambycidae         | 9.66    | 25.33   | 3         |
| Cercopidae           | 3.04    | 5.79    | 1         |
| Chrysomelidae        | 3.27    | 3.52    | 3         |
| Cicadelidae          | 11.31   | 5.52    | 3         |
| Coccinelidae         | 14.68   | 14.27   | 3         |
| Corydiidae           | 0.81    | 0.25    | 1         |
| Geometridae          | 2.06    | 1.62    | 1         |
| Hesperidae           | 0.7     | 1.05    | 1         |
| Libellulidae         | 0.91    | 3.2     | 1         |
| Lycaenidae           | 0.14    | 0.16    | 1         |
| Nymphalidae          | 5.93    | 3.64    | 3         |
| Panorpidae           | 3.16    | 0.9     | 2         |
| Papilionidae         | 11.87   | 8.03    | 3         |
| Pentatomidae         | 14.84   | 14.37   | 3         |
| Pieridae             | 3.93    | 4.29    | 3         |
| Scarabaeidae         | 3.2     | 6.66    | 4         |
| Silphidae            | 0.37    | 0.07    | 1         |
| Sphingidae           | 3.04    | 1.18    | 1         |
| Zygaenidae           | 10.9    | 5.89    | 3         |
| <b>r</b>             | 0.70799 | 0.58732 |           |
| <b>Pearson's r p</b> | 0.00023 | 0.00405 |           |
| <b>n</b>             | 22      | 22      |           |

**Supplementary Table 5.8. Pearson's r values for the correlation between sample size for different families and the area of chemospace occupied by that family on LDA and PCA plots.**



|                         |                       | <b>S</b> | <b>K</b> | <b>Ca</b> | <b>Ti</b> | <b>Fe</b> | <b>Cu</b> | <b>Zn</b> |
|-------------------------|-----------------------|----------|----------|-----------|-----------|-----------|-----------|-----------|
| <b>avg concn extant</b> | <b>µg/</b>            | 0.697    | 3.381    | 6.141     | 1.790     | 6.870     | 0.686     | 3.268     |
| <b>avg concn fossil</b> | <b>cm<sup>2</sup></b> | 1.772    | 4.869    | 167.044   | 25.950    | 364.464   | 1.034     | 1.220     |
| <b>extant/fossil</b>    | <b>r</b>              | -0.02    | 0.24     | 0.053     | 0.108     | -0.209    | 0.22      | -0.25     |
| <b>insects</b>          | <b>p</b>              | 0.843    | 0.01     | 0.537     | 0.205     | 0.013     | 0.01      | 0.003     |
|                         | <b>n</b>              | 140      | 140      | 140       | 140       | 140       | 140       | 140       |

**Supplementary Table 5.9. Pearson's r values for the correlation between concentrations of various elements in extant and fossil insect cuticles.**

**A5.3. R script:**

```

library(graphics)
library(nlme)
library(stats)
library(spatstat)
library(MASS)

# Call the data (all)
setwd
("/Users/aidin_13_/Desktop/thesis(laptop)/XRF/SSRL_june_done/R/csv/good/")
all <- read.csv ("all2.csv")
attach (all)
names (all)

# PCA for logged:
all.l <- log (all[,2:12])
pca <- prcomp (all.l, centre= TRUE, scale= TRUE)
attach (pca)
prop.pca = pca$sdev^2 / sum(pca$sdev^2) # proportions
print (prop.pca) # If using proportions for LDA (can't use percentages) then this is
the one for PCA

# Plot scores
pcascores <- pca$x
plot (pcascores[,1:2], pch=19, col= c("red", "grey", "green", "orange", "yellow",
"black", "blue", "pink", "brown", "salmon", "gold"), xlab= "PC1 - 61.1%", ylab=
"PC2 - 9.1%")

#Plot loadings

plot (pca, col= c("wheat", "slategrey", "yellowgreen", "steelblue2", "seagreen2",
"palevioletred1", "coral1", "lightpink", "dodgerblue", "purple1"), xlab= "Principal
component")

```

```
pcaloadings <- pca$rotation
```

```
plot(pcaloadings[,1:2], pch= 19, col= c("red", "grey", "green", "orange", "yellow",  
"black", "blue", "pink", "brown", "darkturquoise", "darkolivegreen4"), xlab= "PC1  
- 61.1%", ylab= "PC2 - 9.1%")
```

```
legend(locator(1), c("Fe", "Zn", "Cu", "Mn", "Ni", "Ti", "Ca", "K", "Cl", "S", "P"),  
pch= 19, col= c("red", "grey", "green", "orange", "yellow", "black", "blue",  
"pink", "brown", "darkturquoise", "darkolivegreen4"), cex= 2/3, bty= "n")
```

```
plot(pcaloadings[,3:4], pch= 19, col= c("red", "grey", "green", "orange", "yellow",  
"black", "blue", "pink", "brown", "darkturquoise", "darkolivegreen4"), xlab= "PC1  
- 8.1%", ylab= "PC2 - 5.6%")
```

```
legend(locator(1), c("Fe", "Zn", "Cu", "Mn", "Ni", "Ti", "Ca", "K", "Cl", "S", "P"),  
pch= 19, col= c("red", "grey", "green", "orange", "yellow", "black", "blue",  
"pink", "brown", "darkturquoise", "darkolivegreen4"), cex= 2/3, bty= "n")
```

```
plot(pcaloadings[,5:6], pch= 19, col= c("red", "grey", "green", "orange", "yellow",  
"black", "blue", "pink", "brown", "darkturquoise", "darkolivegreen4"), xlab= "PC1  
- 4.1%", ylab= "PC2 - 3.5%")
```

```
legend(locator(1), c("Fe", "Zn", "Cu", "Mn", "Ni", "Ti", "Ca", "K", "Cl", "S", "P"),  
pch= 19, col= c("red", "grey", "green", "orange", "yellow", "black", "blue",  
"pink", "brown", "darkturquoise", "darkolivegreen4"), cex= 2/3, bty= "n")
```

```
plot(pcaloadings[,7:8], pch= 19, col= c("red", "grey", "green", "orange", "yellow",  
"black", "blue", "pink", "brown", "darkturquoise", "darkolivegreen4"), xlab= "PC1  
- 2.5%", ylab= "PC2 - 2.0%")
```

```
legend(locator(1), c("Fe", "Zn", "Cu", "Mn", "Ni", "Ti", "Ca", "K", "Cl", "S", "P"),  
pch= 19, col= c("red", "grey", "green", "orange", "yellow", "black", "blue",  
"pink", "brown", "darkturquoise", "darkolivegreen4"), cex= 2/3, bty= "n")
```

```
plot(pcaloadings[,9:10], pch= 19, col= c("red", "grey", "green", "orange",  
"yellow", "black", "blue", "pink", "brown", "darkturquoise", "darkolivegreen4"),  
xlab= "PC1 - 1.6%", ylab= "PC2 - 1.4%")
```

```
legend(locator(1), c("Fe", "Zn", "Cu", "Mn", "Ni", "Ti", "Ca", "K", "Cl", "S", "P"),
```

```
pch= 19, col= c("red", "grey", "green", "orange", "yellow", "black", "blue",
"pink", "brown", "darkturquoise", "darkolivegreen4"), cex= 2/3, bty= "n")
```

```
plot (pcaloadings[,10:11], pch= 19, col= c("red", "grey", "green", "orange",
"yellow", "black", "blue", "pink", "brown", "darkturquoise", "darkolivegreen4"),
xlab= "PC1 - 1.4%", ylab= "PC2 - 1.2%")
```

```
legend (locator(1), c("Fe", "Zn", "Cu", "Mn", "Ni", "Ti", "Ca", "K", "Cl", "S", "P"),
pch= 19, col= c("red", "grey", "green", "orange", "yellow", "black", "blue",
"pink", "brown", "darkturquoise", "darkolivegreen4"), cex= 2/3, bty= "n")
```

```
# Change each group to vectors - To be able to use the convexhull formula
```

```
hex <- all$Hex
```

```
hex <- as.vector(hex)
```

```
webs <- all$Websafe
```

```
webs <- as.vector(webs)
```

```
col <- all$colour
```

```
col <- as.vector(col)
```

```
pigm <- all$pigment
```

```
pigm <- as.vector(pigm)
```

```
fams <- all$Family
```

```
fams <- as.vector(fams)
```

```
sp <- all$species
```

```
sp <- as.vector(sp)
```

```
rn <- all$Run
```

```
rn <- as.vector(rn)
```

```
mu <- all$Museum
```

```

mu <- as.vector(mu)

or <- all$Orders
or <- as.vector(or)

# To rename species with abbreviations
sp[which(sp == "Parnassius apollonius")] <- "P.a"
sp[which(sp == "Argynnis adippe")] <- "A.a"
sp[which(sp == "Melanargia galathea")] <- "M.g"
sp[which(sp == "Zygaena filipendulae")] <- "Z.f"
sp[which(sp == "Panorpa sp.")] <- "P.sp"
sp[which(sp == "Asilus crabroniformis")] <- "A.c"
sp[which(sp == "Therea petiveriana")] <- "T.p"
sp[which(sp == "Cercopis vulnerata")] <- "C.v"
sp[which(sp == "Graphosoma lineatum")] <- "G.l"
sp[which(sp == "Goliathus goliathus")] <- "G.g"
sp[which(sp == "Nicrophorus investigator")] <- "N.i"
sp[which(sp == "Harmonia axyridis")] <- "H.a"
sp[which(sp == "Papilio glaucus")] <- "P.g"
sp[which(sp == "Pieris rapae")] <- "P.r"
sp[which(sp == "Epargyreus clarus")] <- "E.c"
sp[which(sp == "Anthocharis cardamines")] <- "A.car"
sp[which(sp == "Abraxas grossulariata")] <- "A.g"
sp[which(sp == "Cupido comyntas")] <- "C.c"
sp[which(sp == "Aphodius sp.")] <- "A.sp"
sp[which(sp == "Evacanthus interruptus")] <- "E.i"
sp[which(sp == "Bicyclus anynana")] <- "B.a"
sp[which(sp == "Macroglossum stellatarum")] <- "M.s"
sp[which(sp == "Aphodius sticticus")] <- "A.s"
sp[which(sp == "Aphodius luridus")] <- "A.l"
sp[which(sp == "Leptinotarsa decemlineata")] <- "L.d"
sp[which(sp == "Calopteryx virgo")] <- "C.v"
sp[which(sp == "Calopteryx splendens")] <- "C.s"
sp[which(sp == "Chalcusia coliadoideas")] <- "C.co"

```

```

sp[which(sp == "Gonioctena decemnotata")] <-"G.d"
sp[which(sp == "Colotis regina")] <-"C.r"
sp[which(sp == "Sternotomis bohemanii")] <-"S.b"
sp[which(sp == "Brachyta interrogationes")] <-"B.i"
sp[which(sp == "Saperda populnea")] <-"S.p"
sp[which(sp == "Libellula quadrimaculata")] <-"L.q"
sp[which(sp == "Papilio nireus")] <-"P.n"
sp[which(sp == "Deselvana fulvofasciata")] <-"D.f"
sp[which(sp == "Erasmia pulchella")] <-"E.p"
sp[which(sp == "Cicciana obliqua")] <-"C.o"
sp[which(sp == "Epilachna argus")] <-"E.a"
sp[which(sp == "Aelia acuminata")] <-"A.a"
sp[which(sp == "Hippodamia variegata")] <-"H.v"
sp[which(sp == "Nezara robusta")] <-"N.r"
sp[which(sp == "Phyllobrotica quadrimaculata")] <-"P.q"

```

```

plot (x[,1], x[,2], col= hex, pch= c("Abraxas grossulariata" = 19, "Anthocharis
cardamines"= 19, "Parnassius apollonius"= 19, "Argynnis adippe"= 19,
"Melanargia galathea"= 19, "Zygaena filipendulae"= 19, "Panorpa sp."= 19,
"Asilus crabroniformis"= 19, "Therea petiveriana"= 19, "Cercopis vulnerata"= 19,
"Graphosoma lineatum"= 19, "Goliathus goliathus"= 19, "Nicrophorus
investigator"=19, "Harmonia axyridis"= 19, "Papilio glaucus"= 19, "Pieris rapae"=
19, "Epargyreus clarus"= 19, "Anthocharis cardamines"= 19, "Cupido
comyntas"= 19, "Aphodius sp."= 19, "Evacanthus interruptus"= 19, "Bicyclus
anyana"= 19, "Macroglossum stellatarum"= 19, "Aphodius sticticus"= 19,
"Aphodius luridus" = 19, "Leptinotarsa decemlineata"= 19, "Calopteryx virgo"=
19, "Calopteryx splendens"= 19, "Chalcidia coliadoides" = 19, "Gonioctena
decemnotata"= 19, "Colotis regina" = 19, "Sternotomis bohemanii"= 19,
"Brachyta interrogationes"= 19, "Saperda populnea"= 19, "Libellula
quadrimaculata"= 19, "Papilio nireus"= 19, "Deselvana fulvofasciata"= 19,
"Erasmia pulchella"=19, "Cicciana obliqua"= 19, "Epilachna argus"= 19, "Aelia
acuminata"= 19, "Hippodamia variegata"= 19, "Nezara robusta"= 19,
"Phyllobrotica quadrimaculata"= 19, xlab= "PC1 - 61.1%", ylab= "PC2 - 9.1%"))

```

```
plot (pca$x[,1:2], col=hex, pch= 19, xlab= "PC1 - 61.1%", ylab= "PC2 - 9.1%")
text (x[,1],x[,2], labels= sp, col= hex, cex= 0.5, pos= 3)
```

```
# Plot Runs
```

```
levels(Run)
```

```
# Plot Run 2016
```

```
levelPlot <- levels(Run)[1]
levelPlot # this will plot Run 2016
rowNums <- which(Run == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#ff330030")
```

```
# Plot Run 2017
```

```
levelPlot <- levels(Run)[2]
levelPlot # this will plot Run 2017
rowNums <- which(Run == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#fff9930")
```

```
# Plot Museums
```

```
levelPlot <- levels(Museum)[5]
levelPlot # this will plot OUMNH
rowNums <- which(Museum == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#70809050")
```

```

levelPlot <- levels(Museum)[2]
levelPlot # this will plot Collected Europe
rowNums <- which(Museum == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#8470ff50")

```

```

levelPlot <- levels(Museum)[4]
levelPlot # this will plot NHMO
rowNums <- which(Museum == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#ffbbff50")

```

```

levelPlot <- levels(Museum)[3]
levelPlot # this will plot Collected North America
rowNums <- which(Museum == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#54ff9f50")

```

```

levelPlot <- levels(Museum)[1]
levelPlot # this will plot Collected Africa
rowNums <- which(Museum == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#f0808050")

```

```

levelPlot <- levels(Museum)[6]
levelPlot # this will plot YPMNH
rowNums <- which(Museum == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#ffec8b50")

```



```

legend (locator(1), c("Collected Africa (n = 15)", "Collected Europe (n = 46)",
  "Collected North America (n = 24)", "NHMO (n = 32)", "OUMNH (n = 200)",
  "YPMNH (n = 6)"), pch=c(19, 19, 19, 19, 19, 19), col= c("#f0808050",
  "#8470ff50", "#54ff9f50", "#ffbbff50", "#70809050", "#ffec8b50"), cex= 1, bty=
  "n")

```

```

# Plot coloration mechanism

```

```

plot (pca$x[,1:2], col= hex, pch= 19, xlab= "PC1 - 61.1%", ylab= "PC2 - 9.1%")
text (x[,1],x[,2], labels= sp, col= hex, cex= 0.5, pos= 3)

```

```

levels(pigment)

```

```

# Plot Carotenoids

```

```

levelPlot <- levels(pigment)[1]
levelPlot # this will plot "Carotenoids"
rowNums <- which(pigment == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#ff330050")
area(hpts)

```

```

# Plot Flavonoids

```

```

levelPlot <- levels(pigment)[2]
levelPlot # this will plot "Flavonoid"
rowNums <- which(pigment == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#ffff9950")
area(hpts)

```

```

# Plot Melanin

```

```

levelPlot <- levels(pigment)[3]
levelPlot # this will plot "Melanin"
rowNums <- which(pigment == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#00000050")
area(hpts)

```

# Plot Ommochromes

```

levelPlot <- levels(pigment)[4]
levelPlot # this will plot "Ommochromes"
rowNums <- which(pigment == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#cc993350")
area(hpts)

```

# Plot Pterin

```

levelPlot <- levels(pigment)[5]
levelPlot # this will plot "Pterin"
rowNums <- which(pigment == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#ff990050")
area(hpts)

```

# Plot Structural

```

levelPlot <- levels(pigment)[6]
levelPlot # this will plot "Structural"
rowNums <- which(pigment == levelPlot)

```

```

hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#66999950")
area(hpts)

legend (locator(1), c("Carotenoids n = 51", "Flavonoids n = 19", "Melanin n = 95",
"Ommochromes n = 24", "Pterin n = 27", "Structural n = 5"), pch= c(19, 19, 19, 19,
19, 19), col= c("#ff330050", "#fff9950", "#00000050", "#cc993350",
"#ff990050", "#66999950"), cex= 1, bty= "n")

# Plot Families

plot (x[,1], x[,2], col=hex, pch=19, xlab="PC1 - 61.1%", ylab="PC2 - 9.1%")
text (x[,1],x[,2], labels=sp, col=hex, cex=0.5, pos=3)

levels(Family)

levelPlot <- levels(Family)[1]
levelPlot # this will plot "Asilidae"
rowNums <- which(Family == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#786e0550")
area(hpts)

levelPlot <- levels(Family)[2]
levelPlot # this will plot "Calopterygidae"
rowNums <- which(Family == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#cccccc50")
area(hpts)

levelPlot <- levels(Family)[3]

```

```

levelPlot # this will plot "Cerambycidae"
rowNums <- which(Family == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#66660050")
area(hpts)

```

```

levelPlot <- levels(Family)[4]
levelPlot # this will plot "Cercopidae"
rowNums <- which(Family == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#5BA9FE50")
area(hpts)

```

```

levelPlot <- levels(Family)[5]
levelPlot # this will plot "Chrysomelidae"
rowNums <- which(Family == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#66330050")
area(hpts)

```

```

levelPlot <- levels(Family)[6]
levelPlot # this will plot "Cicadellidae"
rowNums <- which(Family == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#8792F850")
area(hpts)

```

```

levelPlot <- levels(Family)[7]
levelPlot # this will plot "Coccinellidae"
rowNums <- which(Family == levelPlot)

```

```

hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#a4b9c450")
area(hpts)

```

```

levelPlot <- levels(Family)[8]
levelPlot # this will plot "Geometridae"
rowNums <- which(Family == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#fdcff850")
area(hpts)

```

```

levelPlot <- levels(Family)[9]
levelPlot # this will plot "Hesperiidae"
rowNums <- which(Family == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#fdc7da50")
area(hpts)

```

```

levelPlot <- levels(Family)[10]
levelPlot # this will plot "Libellulidae"
rowNums <- which(Family == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#99660050")
area(hpts)

```

```

levelPlot <- levels(Family)[11]
levelPlot # this will plot "Lycaenidae"
rowNums <- which(Family == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x

```

```

polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#a7e8ff50")
area(hpts)

```

```

levelPlot <- levels(Family)[12]
levelPlot # this will plot "Nymphalidae"
rowNums <- which(Family == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#dbdbdb50")
area(hpts)

```

```

levelPlot <- levels(Family)[13]
levelPlot # this will plot "Panorpidae"
rowNums <- which(Family == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#ffb19f50")
area(hpts)

```

```

levelPlot <- levels(Family)[14]
levelPlot # this will plot "Papilionidae"
rowNums <- which(Family == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#f8cbad50")
area(hpts)

```

```

levelPlot <- levels(Family)[15]
levelPlot # this will plot "Pentatomidae"
rowNums <- which(Family == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#b8ade950")
area(hpts)

```

```

levelPlot <- levels(Family)[16]
levelPlot # this will plot "Pieridae"
rowNums <- which(Family == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#ffe69950")
area(hpts)

```

```

levelPlot <- levels(Family)[17]
levelPlot # this will plot "Polyphagidae"
rowNums <- which(Family == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#dcffc550")
area(hpts)

```

```

levelPlot <- levels(Family)[18]
levelPlot # this will plot "Scarabaeidae"
rowNums <- which(Family == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#8ea68a50")
area(hpts)

```

```

levelPlot <- levels(Family)[19]
levelPlot # this will plot "Silphidae"
rowNums <- which(Family == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#af818150")
area(hpts)

```

```

levelPlot <- levels(Family)[20]

```

```

levelPlot # this will plot "Sphingidae"
rowNums <- which(Family == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#c5e0b450")
area(hpts)

```

```

levelPlot <- levels(Family)[21]
levelPlot # this will plot "Zygaenidae"
rowNums <- which(Family == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#b4c7e750")
area(hpts)

```

```

legend(locator(1), c("Asilidae n = 1", "Calopterygidae n = 2", "Cerambycidae n = 3", "Cercopidae n = 1", "Chrysomelidae n = 3", "Cicadellidae n = 3", "Coccinellidae n = 3", "Geometridae n = 1", "Hesperiidae n = 1", "Libellulidae n = 1", "Lycaenidae n = 1", "Nymphalidae n = 3", "Panorpidae n = 2", "Papilionidae n = 3", "Pentatomidae n = 3", "Pieridae n = 3", "Corydiidae n = 1", "Scarabaeidae n = 4", "Silphidae n = 1", "Sphingidae n = 1", "Zygaenidae n = 3"), pch= c(19, 19, 19, 19, 19, 19, 19, 19, 19, 19, 19, 19, 19, 19, 19, 19, 19, 19, 19), col= c("#786e0550", "#cccccc50", "#66660050", "#5BA9FE50", "#66330050", "#8792F850", "#a4b9c450", "#fdcff850", "#fdc7da50", "#99660050", "#a7e8ff50", "#dbdbdb50", "#ffb19f50", "#f8cbad50", "#b8ade950", "#ffe69950", "#dcffc550", "#8ea68a50", "#af818150", "#c5e0b450", "#b4c7e750"), cex= 3/5, bty= "n")

```

```

# Plot Orders

```

```

plot (x[,1], x[,2], col= hex, pch= 19, xlab= "PC1 - 61.1%", ylab= "PC2 - 9.1%")
text (x[,1],x[,2], labels= sp, col= hex, cex= 0.5, pos= 3)

```

```

levels(Order)

```



```
# Blattodea
```

```
levelPlot <- levels(Order)[1]
levelPlot
rowNums <- which(Order == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#ffe69950")
area(hpts)
```

```
# Coleoptera
```

```
levelPlot <- levels(Order)[2]
levelPlot
rowNums <- which(Order == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#eeclfl50")
area(hpts)
```

```
# Diptera
```

```
levelPlot <- levels(Order)[3]
levelPlot
rowNums <- which(Order == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#f676b050")
area(hpts)
```

```
# Hemiptera
```

```
levelPlot <- levels(Order)[4]
```

```

levelPlot
rowNums <- which(Order == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#d1c77d50")
area(hpts)

```

```
# Lepidoptera
```

```

levelPlot <- levels(Order)[5]
levelPlot
rowNums <- which(Order == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#9cf09850")
area(hpts)

```

```
# Mecoptera
```

```

levelPlot <- levels(Order)[6]
levelPlot
rowNums <- which(Order == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#ffb19f50")
area(hpts)

```

```
# Odonata
```

```

levelPlot <- levels(Order)[7]
levelPlot
rowNums <- which(Order == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x

```

```

polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#66660050")
area(hpts)

legend (locator(1), c("Blattodea n = 1", "Coleoptera n = 14", "Diptera n = 1",
  "Hemiptera n = 7", "Lepidoptera n = 16", "Mecoptera n = 2", "Odonata n = 3"),
  pch = c(19, 19, 19, 19, 19, 19, 19), col = c("#ffe69950", "#e6c1f150",
  "#f676b050", "#d1c77d50", "#9cf09850", "#ffb19f50", "#66660050"), bty= "n",
  cex= 3/4)

# Plot Subfamilies

plot (x[,1], x[,2], col= hex, pch= 19, xlab= "PC1 - 61.1%", ylab= "PC2 - 9.1%")
text (x[,1],x[,2], labels= sp, col= hex, cex= 0.5, pos= 3)

levels(Subfamily)

levelPlot <- levels(Subfamily)[1]
levelPlot # this will plot "Aphodiinae"
rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#786e0550")

levelPlot <- levels(Subfamily)[2]
levelPlot # this will plot "Asilinae"
rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#cccccc50")

levelPlot <- levels(Subfamily)[3]
levelPlot # this will plot "Calopterygina"
rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])

```

```
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#66660050")
```

```
levelPlot <- levels(Subfamily)[4]
levelPlot # this will plot "Cercopinae"
rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#5BA9FE50")
```

```
levelPlot <- levels(Subfamily)[5]
levelPlot # this will plot "Cetoniinae"
rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#66330050")
```

```
levelPlot <- levels(Subfamily)[6]
levelPlot # this will plot "Chalcosiinae"
rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#8792F850")
```

```
levelPlot <- levels(Subfamily)[7]
levelPlot # this will plot "Chrysomelinae"
rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#a4b9c450")
```

```
levelPlot <- levels(Subfamily)[8]
levelPlot # this will plot "Cicadellinae"
rowNums <- which(Subfamily == levelPlot)
```

```

hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#fdcff850")

levelPlot <- levels(Subfamily)[9]
levelPlot # this will plot "Coccinellinae"
rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#fdc7da50")

levelPlot <- levels(Subfamily)[10]
levelPlot # this will plot "Ennominae"
rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#99660050")

levelPlot <- levels(Subfamily)[11]
levelPlot # this will plot "Epilachninae"
rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#a7e8ff50")

levelPlot <- levels(Subfamily)[12]
levelPlot # this will plot "Evacanthinae"
rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#dbdbdb50")

levelPlot <- levels(Subfamily)[13]
levelPlot # this will plot "Galerucinae"

```

```

rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#fb19f50")

```

```

levelPlot <- levels(Subfamily)[14]
levelPlot # this will plot "Heliconiinae"
rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#f8cbad50")

```

```

levelPlot <- levels(Subfamily)[15]
levelPlot # this will plot "Lamiinae"
rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#b8ade950")

```

```

levelPlot <- levels(Subfamily)[16]
levelPlot # this will plot "Lepturinae"
rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#ffe69950")

```

```

levelPlot <- levels(Subfamily)[17]
levelPlot # this will plot "Libellulinae"
rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#dcffc550")

```

```

levelPlot <- levels(Subfamily)[18]

```

```

levelPlot # this will plot "Macroglossinae"
rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#8ea68a50")

```

```

levelPlot <- levels(Subfamily)[19]
levelPlot # this will plot "Nichrophorinae"
rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#af818150")

```

```

levelPlot <- levels(Subfamily)[20]
levelPlot # this will plot "Panorpinae"
rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#c5e0b450")

```

```

levelPlot <- levels(Subfamily)[21]
levelPlot # this will plot "Papilioninae"
rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#b4c7e750")

```

```

levelPlot <- levels(Subfamily)[22]
levelPlot # this will plot "Parnasiinae"
rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#ffffcc50")

```

```

levelPlot <- levels(Subfamily)[23]
levelPlot # this will plot "Pentatominae"
rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#66663350")

```

```

levelPlot <- levels(Subfamily)[24]
levelPlot # this will plot "Pierinae"
rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#ff993350")

```

```

levelPlot <- levels(Subfamily)[25]
levelPlot # this will plot "Podopinae"
rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#cccc9950")

```

```

levelPlot <- levels(Subfamily)[26]
levelPlot # this will plot "Polyommatainae"
rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#33330050")

```

```

levelPlot <- levels(Subfamily)[27]
levelPlot # this will plot "Polyphaginae"
rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#00000050")

```



```

levelPlot <- levels(Subfamily)[28]
levelPlot # this will plot "Pyrginae"
rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#66330150")

```

```

levelPlot <- levels(Subfamily)[29]
levelPlot # this will plot "Satyrinae"
rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#cccc6650")

```

```

levelPlot <- levels(Subfamily)[30]
levelPlot # this will plot "Zygaeninae"
rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#33333350")

```

```

legend(locator(1), c("Aphodiinae n = 3", "Asilinae n = 1", "Calopterygina n = 2",
"Cercopinae n = 1", "Cetoniinae n = 1", "Chalcosiinae n = 2", "Chrysomelinae n =
2", "Cicadellinae n = 2", "Coccinellinae n = 2", "Ennominae n = 1", "Epilachninae n
= 1", "Evacanthinae n = 1", "Galerucinae n = 1", "Heliconiinae n = 1", "Lamiinae n
= 2", "Lepturinae n = 1", "Libellulinae n = 1", "Macroglossinae n = 1",
"Nichrophorinae n = 1", "Panorpinae n = 2", "Papilioninae n = 2", "Parnasiinae n =
1", "Pentatominae n = 2", "Pierinae n = 3", "Podopinae n = 2", "Polyommatainae n =
1", "Polyphaginae n = 1", "Pyrginae n = 1", "Satyrinae n = 2", "Zygaeninae n = 1"),
pch = c(19, 19, 19, 19, 19, 19), col = c("#786e0550", "#cccccc50", "#66660050",
"#5BA9FE50", "#66330050", "#8792F850", "#a4b9c450", "#fdcff850",
"#fdc7da50", "#99660050", "#a7e8ff50", "#dbdbdb50", "#ffb19f50", "#f8cbad50",

```

```
"#b8ade950", "#ffe69950", "#dcffc550", "#8ea68a50", "#af818150", "#c5e0b450",
"#b4c7e750", "#ffffcc50", "#66663350", "#ff993350", "#cccc9950", "#33330050",
"#00000050", "#66330150", "#cccc6650", "#33333350"), bty= "n", cex= 3/6)
```

```
# LDA for logged: Museum
```

```
lda.m <- lda(Museum~ Fe +Zn + Cu + Mn + Ni + Ti + Ca + K + Cl + S + P, all.l)
attach(lda.m)
prop.lda.m = lda.m$svd^2/sum(lda.m$svd^2) # proportions
print(prop.lda.m)
```

```
# Predict LDA. It returns the classification and the posterior probabilities of the
new data based on the LD model:
```

```
plda.m <- predict (object = lda.m, newdata = all.l) # plot
```

```
plot (plda.m$x[,1], plda.m$x[,2], pch= 19, col = hex, xlab= "LD1 - 56.84%", ylab=
"LD2 - 25.18%") # make scatterplot
text (plda.m$x[,1],plda.m$x[,2], labels= sp, col= hex, cex= 0.5, pos= 3)
```

```
levelPlot <- levels(Museum)[5]
levelPlot # this will plot OUMNH
rowNums <- which(Museum == levelPlot)
hpts <- convexhull.xy(plda.m$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#70809050")
```

```
levelPlot <- levels(Museum)[2]
levelPlot # this will plot Collected Europe
rowNums <- which(Museum == levelPlot)
hpts <- convexhull.xy(plda.m$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#8470ff50")
```

```
levelPlot <- levels(Museum)[4]
```

```

levelPlot # this will plot NHMO
rowNums <- which(Museum == levelPlot)
hpts <- convexhull.xy(plda.m$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#ffbbff50")

levelPlot <- levels(Museum)[3]
levelPlot # this will plot Collected North America
rowNums <- which(Museum == levelPlot)
hpts <- convexhull.xy(plda.m$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#54ff9f50")

levelPlot <- levels(Museum)[1]
levelPlot # this will plot Collected Africa
rowNums <- which(Museum == levelPlot)
hpts <- convexhull.xy(plda.m$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#f0808050")

levelPlot <- levels(Museum)[6]
levelPlot # this will plot YPMNH
rowNums <- which(Museum == levelPlot)
hpts <- convexhull.xy(plda.m$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#ffec8b50")

legend (locator(1), c("Collected Africa (n = 15)", "Collected Europe (n = 46)",
"Collected North America (n = 24)", "NHMO (n = 32)", "OUMNH (n = 200)",
"YPMNH (n = 6)"), pch=c(19, 19, 19, 19, 19, 19), col= c("#f0808050",
"#8470ff50", "#54ff9f50", "#ffbbff50", "#70809050", "#ffec8b50"), cex= 1, bty=
"n")

# Plot Biplot for LDA Museum

```

```

heads.m <- coef(lda.m)
heads.m
biplot.m <- arrows(x0 = 0, y0 = 0, x1 = heads.m[,1], y1 = heads.m[,2], length =
  1/10, col = "red")
text (heads.m[,1:2], labels = row.names(heads.m))

# LDA for logged: Colortion mechanism

lda.p <- lda(pigment ~ Fe + Zn + Cu + Mn + Ni + Ti + Ca + K + Cl + S + P, all.l)
attach(lda.p)
prop.lda.p = lda.p$svd^2/sum(lda.p$svd^2) # proportions
print(prop.lda.p)

# Predict LDA. It returns the classification and the posterior probabilities of the
  new data based on the LD model:
plda.p <- predict (object = lda.p, newdata = all.l) # plot

plot (plda.p$x[,1], plda.p$x[,2], pch= 19, col= hex, xlab= "LD1 - 48.9%", ylab=
  "LD2 - 22.7%") # make scatterplot
text (plda.p$x[,1],plda.p$x[,2], labels= sp, col= hex, cex= 0.5, pos= 3)

levels(pigment)

# Plot Carotenoids

levelPlot <- levels(pigment)[1]
levelPlot # this will plot "Carotenoids"
rowNums <- which(pigment == levelPlot)
hpts <- convexhull.xy(plda.p$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#ff330050")

# Plot Flavonoids

```

```

levelPlot <- levels(pigment)[2]
levelPlot # this will plot "Flavonoid"
rowNums <- which(pigment == levelPlot)
hpts <- convexhull.xy(plda.p$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#fff9950")

# Plot Melanin

levelPlot <- levels(pigment)[3]
levelPlot # this will plot "Melanin"
rowNums <- which(pigment == levelPlot)
hpts <- convexhull.xy(plda.p$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#00000050")

# Plot Ommochromes

levelPlot <- levels(pigment)[4]
levelPlot # this will plot "Ommochromes"
rowNums <- which(pigment == levelPlot)
hpts <- convexhull.xy(plda.p$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#cc993350")

# Plot Pterin

levelPlot <- levels(pigment)[5]
levelPlot # this will plot "Pterin"
rowNums <- which(pigment == levelPlot)
hpts <- convexhull.xy(plda.p$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#ff990050")

```

```
# Plot Structural
```

```
levelPlot <- levels(pigment)[6]
levelPlot # this will plot "Structural"
rowNums <- which(pigment == levelPlot)
hpts <- convexhull.xy(plda.p$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#66999950")

legend (locator(1), c("Carotenoids n = 51", "Flavonoids n = 19", "Melanin n = 95",
  "Ommochromes n = 24", "Pterin n = 27", "Structural n = 5"), pch=c(19, 19, 19,
  19, 19, 19), col= c("#ff330050", "#fff9950", "#00000050", "#cc993350",
  "#ff990050", "#66999950"), cex= 1, bty= "n")

plot (plda.p$x[,1], plda.p$x[,2], pch= 19, col= hex, xlab= "LD1 - 48.9%", ylab=
  "LD2 - 22.7%") # make scatterplot
text (plda.p$x[,1],plda.p$x[,2], labels= sp, col= hex, cex= 0.5, pos= 3)
```

```
# Plot Biplot for LDA pigments
```

```
heads.p <- coef(lda.p)
heads.p
biplot.p <- arrows(x0 = 0, y0 = 0, x1 = heads.p [,1], y1 = heads.p [,2], length =
  1/10, col = "red")
text (heads.p[,1:2], labels = row.names(heads.p))
```

```
# LDA for logged: Order
```

```
lda.o <- lda(Order~ Fe +Zn + Cu + Mn + Ni + Ti + Ca + K + Cl + S + P, all.1)
attach(lda.o)
prop.llda.o = lda.o$svd^2/sum(lda.o$svd^2) # proportions
print(prop.llda.o)
```

```
# Predict LDA. It returns the classification and the posterior probabilities of the
```

```

new data based on the LD model:

plda.o <- predict (object = lda.o, newdata = all.l) # plot

summary (prop.lda.o)

# Plot loadings

plot (plda.o$x[,1], g=or)

plot (ldaloadings[,1:2], pch= 19, col= c("red", "grey", "green", "orange", "yellow",
    "black", "blue", "pink", "brown", "darkturquoise", "darkolivegreen4"), xlab= "PC1
    - 82.9%", ylab= "PC2 - 9.3%")
legend (locator(1), c("Fe", "Zn", "Cu", "Mn", "Ni", "Ti", "Ca", "K", "Cl", "S", "P"),
    pch= 19, col= c("red", "grey", "green", "orange", "yellow", "black", "blue", "pink",
    "brown", "darkturquoise", "darkolivegreen4"), cex= 2/3, bty= "n")

plot (pcaloadings[,3:4], pch= 19, col= c("red", "grey", "green", "orange", "yellow",
    "black", "blue", "pink", "brown", "darkturquoise", "darkolivegreen4"), xlab= "PC1
    - 4.0%", ylab= "PC2 - 2.4%")
legend (locator(1), c("Fe", "Zn", "Cu", "Mn", "Ni", "Ti", "Ca", "K", "Cl", "S", "P"),
    pch= 19, col= c("red", "grey", "green", "orange", "yellow", "black", "blue",
    "pink", "brown", "darkturquoise", "darkolivegreen4"), cex= 2/3, bty= "n")

plot (pcaloadings[,5:6], pch= 19, col= c("red", "grey", "green", "orange", "yellow",
    "black", "blue", "pink", "brown", "darkturquoise", "darkolivegreen4"), xlab= "PC1
    - 1.2%", ylab= "PC2 - 0.2%")
legend (locator(1), c("Fe", "Zn", "Cu", "Mn", "Ni", "Ti", "Ca", "K", "Cl", "S", "P"),
    pch= 19, col= c("red", "grey", "green", "orange", "yellow", "black", "blue", "pink",
    "brown", "darkturquoise", "darkolivegreen4"), cex= 2/3, bty= "n")

# Plot LDA Order

plot (plda.o$x[,1], plda.o$x[,2], pch= 19, col= hex, xlab= "LD1 - 82.85%", ylab=
    "LD2 - 9.31%") # make scatterplot

```

```
text (plda.o$x[,1],plda.o$x[,2], labels= sp, col= hex, cex= 0.5, pos= 3)
```

```
levels(Order)
```

```
# Blattodea
```

```
levelPlot <- levels(Order)[1]
```

```
levelPlot
```

```
rowNums <- which(Order == levelPlot)
```

```
hpts <- convexhull.xy(plda.o$x[rowNums,1:2])
```

```
hptsa <- hpts$bdry[[1]]$x
```

```
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#ffe69950")
```

```
area(hpts)
```

```
# Coleoptera
```

```
levelPlot <- levels(Order)[2]
```

```
levelPlot
```

```
rowNums <- which(Order == levelPlot)
```

```
hpts <- convexhull.xy(plda.o$x[rowNums,1:2])
```

```
hpts$bdry[[1]]$x
```

```
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#ee1f150")
```

```
area(hpts)
```

```
# Diptera
```

```
levelPlot <- levels(Order)[3]
```

```
levelPlot
```

```
rowNums <- which(Order == levelPlot)
```

```
hpts <- convexhull.xy(plda.o$x[rowNums,1:2])
```

```
hpts$bdry[[1]]$x
```

```
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#f676b050")
```

```
area(hpts)
```



```
# Hemiptera
```

```
levelPlot <- levels(Order)[4]
levelPlot
rowNums <- which(Order == levelPlot)
hpts <- convexhull.xy(plda.o$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#d1c77d50")
area(hpts)
```

```
# Lepidoptera
```

```
levelPlot <- levels(Order)[5]
levelPlot
rowNums <- which(Order == levelPlot)
hpts <- convexhull.xy(plda.o$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#9cf09850")
area(hpts)
```

```
# Mecoptera
```

```
levelPlot <- levels(Order)[6]
levelPlot
rowNums <- which(Order == levelPlot)
hpts <- convexhull.xy(plda.o$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#ffb19f50")
area(hpts)
```

```
# Odonata
```

```
levelPlot <- levels(Order)[7]
levelPlot
```

```

rowNums <- which(Order == levelPlot)
hpts <- convexhull.xy(plda.o$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#66660050")
area(hpts)

legend (locator(1), c("Blattodea n = 1", "Coleoptera n = 14", "Diptera n = 1",
  "Hemiptera n = 7", "Lepidoptera n = 16", "Mecoptera n = 2", "Odonata n = 3"),
  pch= c(19, 19, 19, 19, 19, 19,19), col= c("#ffe69950", "#e6c1f150", "#f676b050",
  "#d1c77d50", "#9cf09850", "#ffb19f50", "#66660050"), bty= "n", cex= 3/4)

# Plot Biplot for LDA Order

heads.o <- coef(lda.o)
heads.o
biplot.o <- arrows(x0 = 0, y0 = 0, x1 = heads.o [,1], y1 = heads.o [,2], length =
  1/10, col = "red")
text(heads.o[,1:2], labels = row.names(heads.o))

# LDA for logged: Subfamily

lda.s <- lda(Subfamily~ Fe +Zn + Cu + Mn + Ni + Ti + Ca + K + Cl + S + P, all.l)
attach(lda.s)
prop.lda.s= lda.s$s$svd^2/sum(lda.s$s$svd^2) # proportions
print(prop.lda.s)

# Predict LDA. It returns the classification and the posterior probabilities of the
  new data based on the LD model:
plda.s <- predict (object = lda.s, newdata = all.l) # plot

plot (plda.s$x[,1], plda.s$x[,2], pch= 19, col= hex, xlab= "LD1 - 44.42%", ylab=
  "LD2 - 15.38%") # make scatterplot
text (plda.s$x[,1],plda.s$x[,2], labels= sp, col= hex, cex= 0.5, pos= 3)

```

```
levels(Subfamily)
```

```
levelPlot <- levels(Subfamily)[1]
```

```
levelPlot # this will plot "Aphodiinae"
```

```
rowNums <- which(Subfamily == levelPlot)
```

```
hpts <- convexhull.xy(plda.s$x[rowNums,1:2])
```

```
hpts$bdry[[1]]$x
```

```
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#786e0550")
```

```
levelPlot <- levels(Subfamily)[2]
```

```
levelPlot # this will plot "Asilinae"
```

```
rowNums <- which(Subfamily == levelPlot)
```

```
hpts <- convexhull.xy(plda.s$x[rowNums,1:2])
```

```
hpts$bdry[[1]]$x
```

```
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#cccccc50")
```

```
levelPlot <- levels(Subfamily)[3]
```

```
levelPlot # this will plot "Calopterygina"
```

```
rowNums <- which(Subfamily == levelPlot)
```

```
hpts <- convexhull.xy(plda.s$x[rowNums,1:2])
```

```
hpts$bdry[[1]]$x
```

```
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#66660050")
```

```
levelPlot <- levels(Subfamily)[4]
```

```
levelPlot # this will plot "Cercopinae"
```

```
rowNums <- which(Subfamily == levelPlot)
```

```
hpts <- convexhull.xy(plda.s$x[rowNums,1:2])
```

```
hpts$bdry[[1]]$x
```

```
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#5BA9FE50")
```

```
levelPlot <- levels(Subfamily)[5]
```

```
levelPlot # this will plot "Cetoniinae"
```

```
rowNums <- which(Subfamily == levelPlot)
```

```
hpts <- convexhull.xy(plda.s$x[rowNums,1:2])
```

```
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#66330050")
```

```
levelPlot <- levels(Subfamily)[6]
levelPlot # this will plot "Chalcosiinae"
rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(plda.s$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#8792F850")
```

```
levelPlot <- levels(Subfamily)[7]
levelPlot # this will plot "Chrysomelinae"
rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(plda.s$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#a4b9c450")
```

```
levelPlot <- levels(Subfamily)[8]
levelPlot # this will plot "Cicadellinae"
rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(plda.s$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#fdcff850")
```

```
levelPlot <- levels(Subfamily)[9]
levelPlot # this will plot "Coccinellinae"
rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(plda.s$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#fdc7da50")
```

```
levelPlot <- levels(Subfamily)[10]
levelPlot # this will plot "Ennominae"
rowNums <- which(Subfamily == levelPlot)
```

```
hpts <- convexhull.xy(plda.s$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#99660050")
```

```
levelPlot <- levels(Subfamily)[11]
levelPlot # this will plot "Epilachninae"
rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(plda.s$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#a7e8ff50")
```

```
levelPlot <- levels(Subfamily)[12]
levelPlot # this will plot "Evacanthinae"
rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(plda.s$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#dbdbdb50")
```

```
levelPlot <- levels(Subfamily)[13]
levelPlot # this will plot "Galerucinae"
rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(plda.s$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#ffb19f50")
```

```
levelPlot <- levels(Subfamily)[14]
levelPlot # this will plot "Heliconiinae"
rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(plda.s$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#f8cbad50")
```

```
levelPlot <- levels(Subfamily)[15]
levelPlot # this will plot "Lamiinae"
```

```

rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(plda.s$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#b8ade950")

```

```

levelPlot <- levels(Subfamily)[16]
levelPlot # this will plot "Lepturinae"
rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(plda.s$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#ffe69950")

```

```

levelPlot <- levels(Subfamily)[17]
levelPlot # this will plot "Libellulinae"
rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(plda.s$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#dcffc550")

```

```

levelPlot <- levels(Subfamily)[18]
levelPlot # this will plot "Macroglossinae"
rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(plda.s$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#8ea68a50")

```

```

levelPlot <- levels(Subfamily)[19]
levelPlot # this will plot "Nichrophorinae"
rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(plda.s$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#af818150")

```

```

levelPlot <- levels(Subfamily)[20]

```

```

levelPlot # this will plot "Panorpinae"
rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(plda.s$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#c5e0b450")

```

```

levelPlot <- levels(Subfamily)[21]
levelPlot # this will plot "Papilioninae"
rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(plda.s$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#b4c7e750")

```

```

levelPlot <- levels(Subfamily)[22]
levelPlot # this will plot "Parnasiinae"
rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(plda.s$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#ffffcc50")

```

```

levelPlot <- levels(Subfamily)[23]
levelPlot # this will plot "Pentatominae"
rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(plda.s$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#66663350")

```

```

levelPlot <- levels(Subfamily)[24]
levelPlot # this will plot "Pierinae"
rowNums <- which(Subfamily == levelPlot)
hpts<- convexhull.xy(plda.s$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col="#ff993350")

```

```

levelPlot <- levels(Subfamily)[25]
levelPlot # this will plot "Podopinae"
rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(plda.s$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#cccc9950")

levelPlot <- levels(Subfamily)[26]
levelPlot # this will plot "Polyommatainae"
rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(plda.s$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#33330050")

levelPlot <- levels(Subfamily)[27]
levelPlot # this will plot "Polyphaginae"
rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(plda.s$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#00000050")

levelPlot <- levels(Subfamily)[28]
levelPlot # this will plot "Pyrginae"
rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(plda.s$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#66330150")

levelPlot <- levels(Subfamily)[29]
levelPlot # this will plot "Satyrinae"
rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(plda.s$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#cccc6650")

```



```

levelPlot <- levels(Subfamily)[30]
levelPlot # this will plot "Zygaeninae"
rowNums <- which(Subfamily == levelPlot)
hpts <- convexhull.xy(plda.s$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#33333350")

legend (locator(1), c("Aphodiinae n = 3", "Asilinae n = 1", "Calopterygina n = 2",
"Cercopinae n = 1", "Cetoniinae n = 1", "Chalcosiinae n = 2", "Chrysomelinae n =
2", "Cicadellinae n = 2", "Coccinellinae n =2", "Ennominae n = 1", "Epilachninae
n = 1", "Evacanthinae n = 1", "Galerucinae n = 1", "Heliconiinae n = 1",
"Lamiinae n = 2", "Lepturinae n = 1", "Libellulinae n = 1", "Macroglossinae n =
1", "Nichrophorinae n = 1", "Panorpinae n = 2", "Papilioninae n = 2", "Parnasiinae
n = 1", "Pentatominae n = 2", "Pierinae n = 3", "Podopinae n = 2",
"Polyommatainae n = 1", "Polyphaginae n = 1", "Pyrginae n = 1", "Satyrinae n =
2", "Zygaeninae n = 1"), pch=c(19, 19, 19, 19, 19, 19), col = c("#786e0550",
"#cccccc50", "#66660050", "#5BA9FE50", "#66330050", "#8792F850",
"#a4b9c450", "#fdcff850", "#fdc7da50", "#99660050", "#a7e8ff50",
"#dbdbdb50", "#ffb19f50", "#f8cbad50", "#b8ade950", "#ffe69950", "#dcffc550",
"#8ea68a50", "#af818150", "#c5e0b450", "#b4c7e750", "#ffffcc50",
"#66663350", "#ff993350", "#cccc9950", "#33330050", "#00000050",
"#66330150", "#cccc6650", "#33333350"), bty= "n", cex= 3/6)

plot (plda.s$x[,1], plda.s$x[,2], pch= 19, col= hex, xlab= "LD1 - 44.42%", ylab=
"LD2 - 15.38%") # make scatterplot
text (plda.s$x[,1],plda.s$x[,2], labels= sp, col= hex, cex= 0.5, pos= 3)

# Plot Biplot for LDA Subfamily

heads.s <- coef(lda.s)
heads.s
biplot.s <- arrows(x0 = 0, y0 = 0, x1 = heads.s [,1], y1 = heads.s [,2], length = 1/10,

```

```

col = "red")
text(heads.s[,1:2], labels = row.names(heads.s))

# LDA for logged: Family

lda.f <- lda(Family~ Fe +Zn + Cu + Mn + Ni + Ti + Ca + K + Cl + S + P, all.l)
attach(lda.f)
prop.lda.f = lda.f$svd^2/sum(lda.f$svd^2) # proportions
print(prop.lda.f)

# Predict LDA. It returns the classification and the posterior probabilities of the
new data based on the LD model:
plda.f <- predict (object = lda.f, newdata = all.l )# plot

plot (plda.f$x[,1], plda.f$x[,2], pch= 19, col= hex, xlab= "LD1 - 48.6%", ylab=
"LD2 - 18.55%") # make scatterplot
text (plda.f$x[,1],plda.f$x[,2], labels= sp, col= hex, cex= 0.5, pos= 3)

levels(Family)

levelPlot <- levels(Family)[1]
levelPlot # this will plot "Asilidae"
rowNums <- which(Family == levelPlot)
hpts <- convexhull.xy(plda.f$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#786e0550")
area(hpts)

levelPlot <- levels(Family)[2]
levelPlot # this will plot "Calopterygidae"
rowNums <- which(Family == levelPlot)
hpts <- convexhull.xy(plda.f$x[rowNums,1:2])
hpts$bdry[[1]]$x

```

```

polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#cccccc50")
area(hpts)

```

```

levelPlot <- levels(Family)[3]
levelPlot # this will plot "Cerambycidae"
rowNums <- which(Family == levelPlot)
hpts <- convexhull.xy(plda.f$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#66660050")
area(hpts)

```

```

levelPlot <- levels(Family)[4]
levelPlot # this will plot "Cercopidae"
rowNums <- which(Family == levelPlot)
hpts <- convexhull.xy(plda.f$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#5BA9FE50")
area(hpts)

```

```

levelPlot <- levels(Family)[5]
levelPlot # this will plot "Chrysomelidae"
rowNums <- which(Family == levelPlot)
hpts <- convexhull.xy(plda.f$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#66330050")
area(hpts)

```

```

levelPlot <- levels(Family)[6]
levelPlot # this will plot "Cicadellidae"
rowNums <- which(Family == levelPlot)
hpts <- convexhull.xy(plda.f$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#8792F850")
area(hpts)

```

```

levelPlot <- levels(Family)[7]
levelPlot # this will plot "Coccinellidae"
rowNums <- which(Family == levelPlot)
hpts <- convexhull.xy(plda.f$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#a4b9c450")
area(hpts)

```

```

levelPlot <- levels(Family)[8]
levelPlot # this will plot "Geometridae"
rowNums <- which(Family == levelPlot)
hpts <- convexhull.xy(plda.f$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#fdcff850")
area(hpts)

```

```

levelPlot <- levels(Family)[9]
levelPlot # this will plot "Hesperiidae"
rowNums <- which(Family == levelPlot)
hpts <- convexhull.xy(plda.f$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#fdc7da50")
area(hpts)

```

```

levelPlot <- levels(Family)[10]
levelPlot # this will plot "Libellulidae"
rowNums <- which(Family == levelPlot)
hpts <- convexhull.xy(plda.f$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#99660050")
area(hpts)

```

```

levelPlot <- levels(Family)[11]

```

```

levelPlot # this will plot "Lycaenidae"
rowNums <- which(Family == levelPlot)
hpts <- convexhull.xy(plda.f$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#a7e8ff50")
area(hpts)

```

```

levelPlot <- levels(Family)[12]
levelPlot # this will plot "Nymphalidae"
rowNums <- which(Family == levelPlot)
hpts <- convexhull.xy(plda.f$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#dbdbdb50")
area(hpts)

```

```

levelPlot <- levels(Family)[13]
levelPlot # this will plot "Panorpidae"
rowNums <- which(Family == levelPlot)
hpts <- convexhull.xy(plda.f$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#ffb19f50")
area(hpts)

```

```

levelPlot <- levels(Family)[14]
levelPlot # this will plot "Papilionidae"
rowNums <- which(Family == levelPlot)
hpts <- convexhull.xy(plda.f$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#f8cbad50")
area(hpts)

```

```

levelPlot <- levels(Family)[15]
levelPlot # this will plot "Pentatomidae"
rowNums <- which(Family == levelPlot)

```

```

hpts <- convexhull.xy(plda.f$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#b8ade950")
area(hpts)

```

```

levelPlot <- levels(Family)[16]
levelPlot # this will plot "Pieridae"
rowNums <- which(Family == levelPlot)
hpts <- convexhull.xy(plda.f$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#ffe69950")
area(hpts)

```

```

levelPlot <- levels(Family)[17]
levelPlot # this will plot "Polyphagidae"
rowNums <- which(Family == levelPlot)
hpts <- convexhull.xy(plda.f$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#dcffc550")
area(hpts)

```

```

levelPlot <- levels(Family)[18]
levelPlot # this will plot "Scarabaeidae"
rowNums <- which(Family == levelPlot)
hpts <- convexhull.xy(plda.f$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#8ea68a50")
area(hpts)

```

```

levelPlot <- levels(Family)[19]
levelPlot # this will plot "Silphidae"
rowNums <- which(Family == levelPlot)
hpts <- convexhull.xy(plda.f$x[rowNums,1:2])
hpts$bdry[[1]]$x

```

```

polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#af818150")
area(hpts)

```

```

levelPlot <- levels(Family)[20]
levelPlot # this will plot "Sphingidae"
rowNums <- which(Family == levelPlot)
hpts <- convexhull.xy(plda.f$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#c5e0b450")
area(hpts)

```

```

levelPlot <- levels(Family)[21]
levelPlot # this will plot "Zygaenidae"
rowNums <- which(Family == levelPlot)
hpts <- convexhull.xy(plda.f$x[rowNums,1:2])
hpts$bdry[[1]]$x
polygon(hpts$bdry[[1]]$x, hpts$bdry[[1]]$y, col= "#b4c7e750")
area(hpts)

```

```

legend (locator(1), c("Asilidae n = 1", "Calopterygidae n = 2", "Cerambycidae n =
3", "Cercopidae n = 1", "Chrysomelidae n = 3", "Cicadellidae n = 3",
"Coccinellidae n = 3", "Geometridae n = 1", "Hesperiidae n = 1", "Libellulidae n=
1", "Lycaenidae n = 1", "Nymphalidae n = 3", "Panorpidae n = 2", "Papilionidae n
= 3", "Pentatomidae n = 3", "Pieridae n = 3", "Corydiidae n = 1", "Scarabaeidae n
= 4", "Silphidae n = 1", "Sphingidae n = 1", "Zygaenidae n = 3"), pch=c(19, 19,
19, 19, 19, 19, 19, 19, 19, 19, 19, 19, 19, 19, 19, 19, 19, 19, 19, 19), col=
c("#786e0550", "#cccccc50", "#66660050", "#5BA9FE50", "#66330050",
"#8792F850", "#a4b9c450", "#fdcff850", "#fdc7da50", "#99660050",
"#a7e8ff50", "#dbdbdb50", "#ffb19f50", "#f8cbad50", "#b8ade950", "#ffe69950",
"#dcffc550", "#8ea68a50", "#af818150", "#c5e0b450", "#b4c7e750"), cex= 3/5,
bty= "n")

```

```

plot (plda.f$x[,1], plda.f$x[,2], pch= 19, col= hex, xlab= "LD1 - 48.6%", ylab=
"LD2 - 18.55%") # make scatterplot

```

```

text (plda.f$x[,1],plda.f$x[,2], labels= sp, col= hex, cex= 0.5, pos= 3)

# Plot Biplot for LDA Family

heads.f <- coef(lda.f)
heads.f
biplot.f <- arrows(x0 = 0, y0 = 0, x1 = heads.f[,1], y1 = heads.f[,2], length = 1/10,
  col = "red")
text(heads.f[,1:2], labels = row.names(heads.f))

# MANOVA:

Fe <- all$Fe
Zn <- all$Zn
Cu <- all$Cu
Mn <- all$Mn
Ni <- all$Ni
Ti <- all$Ti
Ca <- all$Ca
K <- all$K
Cl <- all$Cl
S <- all$S
P <- all$P

allmanovas <- manova(cbind(Fe, Zn, Cu, Mn, Ni, Ti, Ca, K, Cl, S, P) ~ species)
summary(allmanovas)

allmanovao <- manova(cbind(Fe, Zn, Cu, Mn, Ni, Ti, Ca, K, Cl, S, P) ~ Order)
summary(allmanovao)

allmanovaf <- manova(cbind(Fe, Zn, Cu, Mn, Ni, Ti, Ca, K, Cl, S, P) ~ Family)
summary(allmanovaf)

allmanovap <- manova(cbind(Fe, Zn, Cu, Mn, Ni, Ti, Ca, K, Cl, S, P) ~ pigment)

```



```
summary(allmanovap)
```

```
summary.aov(allmanovas)
```

```
summary.aov(allmanovao)
```

```
summary.aov(allmanovaf)
```

```
summary.aov(allmanovap)
```

```
# MANOVA Melanin
```

```
setwd("/Users/aidin_13_/Desktop/thesis(laptop)/XRF/SSRL_june_done/R/csv/good/forfamiliesalone/Orders-pigments/")
```

```
mel <- read.csv("Melanin.csv")
```

```
attach(mel)
```

```
names(mel)
```

```
allmanovam <- manova(cbind(Fe, Zn, Cu, Mn, Ni, Ti, Ca, K, Cl, S, P) ~ species)
```

```
summary(allmanovam)
```

```
summary.aov(allmanovam)
```

```
# MANOVA Carotenoids
```

```
setwd("/Users/aidin_13_/Desktop/thesis(laptop)/XRF/SSRL_june_done/R/csv/good/forfamiliesalone/Orders-pigments/")
```

```
car <- read.csv("Carotenoids.csv")
```

```
attach(car)
```

```
names(car)
```

```
allmanovac <- manova(cbind(Fe, Zn, Cu, Mn, Ni, Ti, Ca, K, Cl, S, P) ~ species)
```

```
summary(allmanovac)
```

```
summary.aov(allmanovac)
```

```
# MANOVA Ommochromes
```

```
setwd("/Users/aidin_13_/Desktop/thesis(laptop)/XRF/SSRL_june_done/R/csv/good/forfamiliesalone/Orders-pigments/")
```

```
omm <- read.csv("Ommochromes.csv")
```

```
attach(omm)
```

```
names(omm)
```

```
allmanovaom <- manova(cbind(Fe, Zn, Cu, Mn, Ni, Ti, Ca, K, Cl, S, P) ~ species)
summary(allmanovaom)
summary.aov(allmanovaom)
```

```
# MANOVA Pterins
```

```
setwd("/Users/aidin_13_/Desktop/thesis(laptop)/XRF/SSRL_june_done/R/csv/good/forfamiliesalone/Orders-pigments/")
pte <- read.csv("Pterin.csv")
attach(pte)
names(pte)
```

```
allmanovap <- manova(cbind(Fe, Zn, Cu, Mn, Ni, Ti, Ca, K, Cl, S, P) ~ species)
summary(allmanovap)
summary.aov(allmanovap)
```

```
# MANOVA Flavonoids
```

```
setwd("/Users/aidin_13_/Desktop/thesis(laptop)/XRF/SSRL_june_done/R/csv/good/forfamiliesalone/Orders-pigments/")
fla <- read.csv("Flavonoids.csv")
attach(fla)
names(fla)
```

```
allmanovaf1 <- manova(cbind(Fe, Zn, Cu, Mn, Ni, Ti, Ca, K, Cl, S, P) ~ species)
summary(allmanovaf1)
summary.aov(allmanovaf1)
```

```
# MANOVA for individual orders
```

```
# MANOVA Blattodea - only one species
```

```
setwd("/Users/aidin_13_/Desktop/thesis(laptop)/XRF/SSRL_june_done/R/csv/good/forfamiliesalone/Orders-pigments/")
blat <- read.csv("Blat_pig.csv")
```

```
attach(blatt)
```

```
names(blatt)
```

```
blattmanova <- manova(cbind(Fe, Zn, Cu, Mn, Ni, Ti, Ca, K, Cl, S, P) ~ species)
```

```
summary(blatmanova)
```

```
summary.aov(blatmanova)
```

```
# MANOVA Coleoptera
```

```
setwd("/Users/aidin_13_/Desktop/thesis(laptop)/XRF/SSRL_june_done/R/csv/good/forfamiliesalone/Orders-pigments/")
```

```
col <- read.csv("Col_pig.csv")
```

```
attach(col)
```

```
names(col)
```

```
colmanova <- manova(cbind(Fe, Zn, Cu, Mn, Ni, Ti, Ca, K, Cl, S, P) ~ species)
```

```
summary(colmanova)
```

```
summary.aov(colmanova)
```

```
# MANOVA Diptera
```

```
setwd("/Users/aidin_13_/Desktop/thesis(laptop)/XRF/SSRL_june_done/R/csv/good/forfamiliesalone/Orders-pigments/")
```

```
dip <- read.csv("Dip_pig.csv")
```

```
attach(dip)
```

```
names(dip)
```

```
dipmanova <- manova(cbind(Fe, Zn, Cu, Mn, Ni, Ti, Ca, K, Cl, S, P) ~ species)
```

```
summary(dipmanova)
```

```
summary.aov(dipmanova)
```

```
# MANOVA Hemiptera
```

```
setwd("/Users/aidin_13_/Desktop/thesis(laptop)/XRF/SSRL_june_done/R/csv/good/forfamiliesalone/Orders-pigments/")
```

```
hem <- read.csv("Hem_pig.csv")
```

```
attach(hem)
```

```
names(hem)
```

```
hemmanova <- manova(cbind(Fe, Zn, Cu, Mn, Ni, Ti, Ca, K, Cl, S, P) ~ species)
```

```
summary(hemmanova)
```

```
summary.aov(hemmanova)
```

```
# MANOVA Lepidoptera
```

```
setwd("/Users/aidin_13_/Desktop/thesis(laptop)/XRF/SSRL_june_done/R/csv/goo
```

```
d/forfamiliesalone/Orders-pigments/")
```

```
lep <- read.csv("Lep_pig.csv")
```

```
attach(lep)
```

```
names(lep)
```

```
lepmanova <- manova(cbind(Fe, Zn, Cu, Mn, Ni, Ti, Ca, K, Cl, S, P) ~ species)
```

```
summary(lepmanova)
```

```
summary.aov(lepmanova)
```

```
# MANOVA Mecoptera
```

```
setwd("/Users/aidin_13_/Desktop/thesis(laptop)/XRF/SSRL_june_done/R/csv/goo
```

```
d/forfamiliesalone/Orders-pigments/")
```

```
mec <- read.csv("Mec_pig.csv")
```

```
attach(mec)
```

```
names(mec)
```

```
mecmanova <- manova(cbind(Fe, Zn, Cu, Mn, Ni, Ti, Ca, K, Cl, S, P) ~ species)
```

```
summary(mecmanova)
```

```
summary.aov(mecmanova)
```

```
# MANOVA Odonata
```

```
setwd("/Users/aidin_13_/Desktop/thesis(laptop)/XRF/SSRL_june_done/R/csv/goo
```

```
d/forfamiliesalone/Orders-pigments/")
```

```
odo <- read.csv("Odo_pig.csv")
```

```
attach(odo)
```

```
names(odo)
```

```
odomanova <- manova(cbind(Fe, Zn, Cu, Mn, Ni, Ti, Ca, K, Cl, S, P) ~ species)
summary(odomanova)
summary.aov(odomanova)

# MANOVA Aeshnidae - only one species
setwd("/Users/aidin_13_/Desktop/thesis(laptop)/XRF/SSRL_june_done/R/csv/good/forfamiliesalone/Orders-pigments/")
ash <- read.csv("Aeshnidae.csv")
attach(ash)
names(ash)

ashmanova <- manova(cbind(Fe, Zn, Cu, Mn, Ni, Ti, Ca, K, Cl, S, P) ~ species)
summary(ashmanova)
summary.aov(ashmanova)

# MANOVA Calopterygidae
setwd("/Users/aidin_13_/Desktop/thesis(laptop)/XRF/SSRL_june_done/R/csv/good/forfamiliesalone/Orders-pigments/")
cal <- read.csv("Calopterygidae.csv")
attach(cal)
names(cal)

calmanova <- manova(cbind(Fe, Zn, Cu, Mn, Ni, Ti, Ca, K, Cl, S, P) ~ species)
summary(calmanova)
summary.aov(calmanova)

# MANOVA Coenagrionidae - only one species
setwd("/Users/aidin_13_/Desktop/thesis(laptop)/XRF/SSRL_june_done/R/csv/good/forfamiliesalone/Orders-pigments/")
coe <- read.csv("Coenagrionidae.csv")
attach(coe)
names(coe)
```

```
coemanova <- manova(cbind(Fe, Zn, Cu, Mn, Ni, Ti, Ca, K, Cl, S, P) ~ species)
summary(coemanova)
summary.aov(coemanova)

# MANOVA Corduliidae - only one species
setwd("/Users/aidin_13_/Desktop/thesis(laptop)/XRF/SSRL_june_done/R/csv/good/forfamiliesalone/Orders-pigments/")
cor <- read.csv("Corduliidae.csv")
attach(cor)
names(cor)

cormanova <- manova(cbind(Fe, Zn, Cu, Mn, Ni, Ti, Ca, K, Cl, S, P) ~ species)
summary(cormanova)
summary.aov(cormanova)

# MANOVA Libellulidae
setwd("/Users/aidin_13_/Desktop/thesis(laptop)/XRF/SSRL_june_done/R/csv/good/forfamiliesalone/Orders-pigments/")
lib <- read.csv("Libellulidae.csv")
attach(lib)
names(lib)

libmanova <- manova(cbind(Fe, Zn, Cu, Mn, Ni, Ti, Ca, K, Cl, S, P) ~ species)
summary(libmanova)
summary.aov(libmanova)

# MANOVA of families with statistical significant results of elemental partitioning
in the cuticle
# MANOVA Libellulidae + Corduliidae + Coenagrionidae
setwd("/Users/aidin_13_/Desktop/thesis(laptop)/XRF/SSRL_june_done/R/csv/good/forfamiliesalone/Orders-pigments/")
odf <- read.csv("Odo_fam.csv")
attach(odf)
names(odf)
```

```
odfmanova <- manova(cbind(Fe, Zn, Cu, Mn, Ni, Ti, Ca, K, Cl, S, P) ~ species)
summary(odfmanova)
summary.aov(odfmanova)

# MANOVA Nymphalidae
setwd("/Users/aidin_13_/Desktop/thesis(laptop)/XRF/SSRL_june_done/R/csv/good/forfamiliesalone/Orders-pigments/")
nym <- read.csv("Nymphalidae.csv")
attach(nym)
names(nym)

nymmanova <- manova(cbind(Fe, Zn, Cu, Mn, Ni, Ti, Ca, K, Cl, S, P) ~ species)
summary(nymmanova)
summary.aov(nymmanova)

# MANOVA Zygaenidae
setwd("/Users/aidin_13_/Desktop/thesis(laptop)/XRF/SSRL_june_done/R/csv/good/forfamiliesalone/Orders-pigments/")
zyg <- read.csv("Zygaenidae.csv")
attach(zyg)
names(zyg)

zygmanova <- manova(cbind(Fe, Zn, Cu, Mn, Ni, Ti, Ca, K, Cl, S, P) ~ species)
summary(zygmanova)
summary.aov(zygmanova)

# MANOVA of individual specimens with statistical significant results of elemental
partitioning in the cuticle from the same order

#Anthocaris cardamines vs Colotis regina
setwd("/Users/aidin_13_/Desktop/thesis(laptop)/XRF/SSRL_june_done/R/csv/good/forfamiliesalone/Orders-pigments/species/")
anth <- read.csv("anth.csv")
```

```
attach(anth)
names(anth)

anthmanova <- manova(cbind(Fe, Zn, Cu, Mn, Ni, Ti, Ca, K, Cl, S, P) ~ species)
summary(anthmanova)

# Parnassius apollonius vs Papilio glaucus vs P. nireus
setwd("/Users/aidin_13_/Desktop/thesis(laptop)/XRF/SSRL_june_done/R/csv/good/forfamiliesalone/Orders-pigments/species/")
pap <- read.csv("pap.csv")
attach(pap)
names(pap)

papmanova <- manova(cbind(Fe, Zn, Cu, Mn, Ni, Ti, Ca, K, Cl, S, P) ~ species)
summary(papmanova)

# Cercopis vulnerata vs Graphosoma lineatum vs Cicciana oblicua
setwd("/Users/aidin_13_/Desktop/thesis(laptop)/XRF/SSRL_june_done/R/csv/good/forfamiliesalone/Orders-pigments/species/")
cerc <- read.csv("cerc.csv")
attach(cerc)
names(cerc)

cercmanova <- manova(cbind(Fe, Zn, Cu, Mn, Ni, Ti, Ca, K, Cl, S, P) ~ species)
summary(cercmanova)

# Trichius sp. vs Brachyta interrogationes vs Leptinotarsa decemlineata
setwd("/Users/aidin_13_/Desktop/thesis(laptop)/XRF/SSRL_june_done/R/csv/good/forfamiliesalone/Orders-pigments/species/")
trich <- read.csv("trich.csv")
attach(trich)
names(trich)
trichmanova <- manova(cbind(Fe, Zn, Cu, Mn, Ni, Ti, Ca, K, Cl, S, P) ~ species)
```



summary(trichmanova)

