The form and function of warning signals in Lepidoptera, with a special focus on burnet moths (Zygaenidae)

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I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

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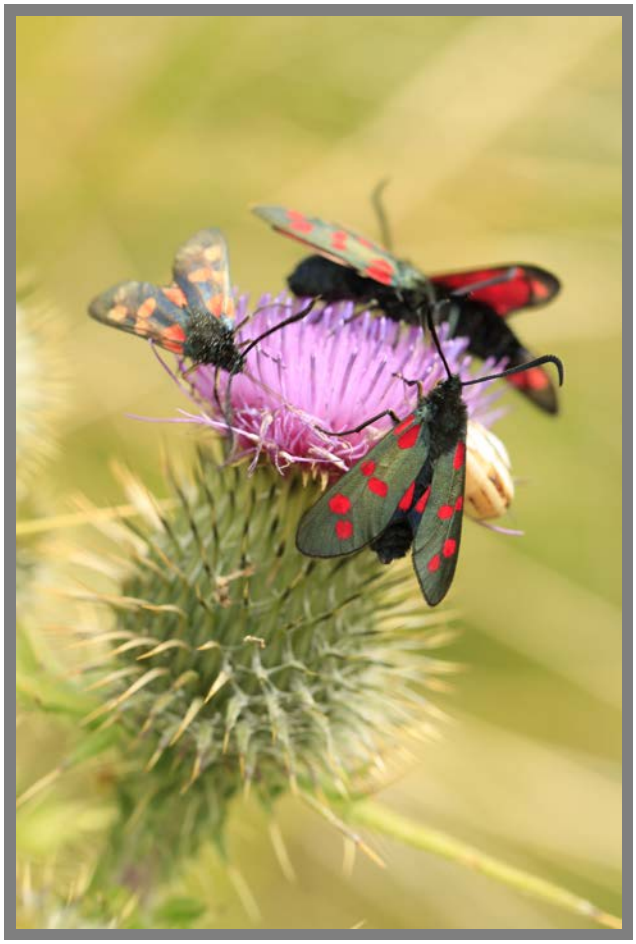

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General abstract

Many species use visual features to avoid predation by several methods, such as concealing themselves, deceiving predators and hindering capture. One of the most striking strategies is aposematism, or warning coloration, in which prey use conspicuous visual signals to advertise chemical or physical defences, and thereby deter predators from attacking. My thesis focuses on the form of these warning signals, namely which elements of visual patterns might be most effective in generating predator avoidance, as well as how these different visual features relate to defence levels and ultimately to prey survival in the wild. To address these issues, I studied the warning signals of Lepidoptera and in particular burnet moths (Zygaenidae: Zygaeninae), day-flying moths with distinctive red and black wings and the remarkable ability to both synthesise defensive compounds and sequester them from their host plants. Technological advances and a growing understanding of animal vision mean that animal signals can be studied in an increasingly precise and ecologically-relevant way. Throughout this thesis, I use sophisticated methods to quantify both the defensive chemicals and wing coloration of burnet moths, as perceived by their avian predators. I examine the key features of day-flying defended Lepidoptera, then focus on the potential for quantitative signal honesty in burnet moths. I explore the relationship between defence levels and measures of coloration, both within the six-spot burnet moth, *Zygaena filipendulae*, and across species in the Zygaenidae, then test the effects of variation in warning signals on predation risk for artificial burnet-like prey in the field. My work highlights some of the complicating factors that should be accounted for in the study of warning coloration, especially when investigating the potential for quantitative signal honesty. I hope my thesis will provide a basis for future research on the defensive strategies of day-flying moths and inspire others to pursue investigations into aposematism in the Zygaenidae.

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Author's declaration

The work presented in this thesis was carried out by the author, Emmanuelle Briolat, with the exceptions stated below:

Measurements of cyanogenic glucosides, obtained by LC-MS and featured in Chapters 4 and 5, were carried out by Mika Zagrobelny and colleagues at the University of Copenhagen.

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Specimens of Zygaenidae were primarily collected by Emmanuelle Briolat, but several entomologists and other helpers participated in field trips. In addition, some specimens were collected by entomologists during their own work, and sent to the author for analysis. Specimens are attributed to each collector in Appendices 2.1 and 5.1.

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Chapter 1

Introduction:
Conspicuous coloration as an anti-predator defence

Zygaenidae from France and the UK. Clockwise from top left*: Zygaena rhadamanthus*, *Adscita mannii*, *Z. filipendulae*, *Jordanita globulariae*, *Z. lavandulae*, *Z. transalpina*. All photographs: E. S. Briolat.

1.1 Abstract

The dazzling array of colour and patterns on display in the natural world is a never-ending source of wonder, and offers stunning visual evidence of the processes of evolution. A species' appearance fulfils a multitude of functions, both mediating its interactions with the environment, such as facilitating thermoregulation, and enabling intra- and inter-specific communication via visual signals. In particular, interactions between predators and prey play a significant role in shaping visual appearance. To avoid predation, prey species deploy a wide range of visual anti-predator strategies aimed at minimising detection or recognition as a suitable food source, reducing the likelihood of a successful attack, or otherwise deterring predators from selecting them. At one end of this spectrum, unprofitable species, possessing distasteful and toxic compounds or physical defences, advertise their aversive nature with bright and conspicuous warning, or aposematic, signals. This intuitively paradoxical strategy, attracting the attention of predators to deter attack, has been extensively studied since the pioneering work of 19th century naturalists. Yet, while the theory of aposematism is well-supported, with a wide range of examples across taxa, there are still many active areas of enquiry. Recent methodological advances, enabling new insights into the production and perception of colour, have reinvigorated the study of animal coloration, including aposematism. Questions surrounding the form of warning signals, and how specific features can influence predator behaviour, can now be addressed with increased relevance to natural predators and environmental conditions. The quantitative relationship between the strength of warning signals and the potency of the defences they advertise is an especially contentious issue, with both theoretical and empirical studies yielding conflicting conclusions. In this thesis, I use day-flying Lepidoptera, and especially the burnet moths (Zygaenidae), to explore still unresolved questions surrounding the form and function of warning signals from the perspective of their avian predators.

1.2 Visual strategies for anti-predator defence

The physical appearance of an animal is often, at least to visually-driven humans, its most obvious and fundamental characteristic, and studying visual features represents one of the best opportunities for exploring a wide range of questions in ecology and evolution (Cuthill *et al.*, 2017). Visual characteristics are shaped by a multitude of selective pressures related to how animals interact with their environment, as well as with conspecifics and heterospecifics around them. A visual stimulus can include movement (Paluh, Hantak and Saporito, 2014) and posture (e.g. in skunks and newts; Lariviere and Messier, 1996; Mochida, 2009), but most research has focused on either fixed or dynamic colour patterns. These can be produced by pigments (Chittka, 2013), by nanoscale structures scattering light, a phenomenon known as structural coloration (Vukusic *et al.*, 2001), or by light-emitting chemical reactions (bioluminescence; Wilson and Woodland Hastings, 1998). A key function of coloration in many species is to mediate thermoregulation, particularly through the dark pigment melanin (Watt, 1968), but colour patterns have generally attracted most attention as a medium for animal communication. Visual signals can be used in intra-specific communication, most famously for mate choice in sexual selection (e.g. Hill, 1990; Summers *et al.*, 1999) but also in many other capacities, such as to provide badges of status (Senar, 2006), facilitate parent-offspring communication (e.g. in nestling begging behaviour; Kilner and Davies, 1998), or enable individual recognition (e.g. in paper wasps; Tibbetts, 2002). They are similarly important for interspecific communication in a diverse range of contexts including interactions between parasites and hosts (e.g. Spottiswoode and Stevens, 2010), and predators and prey (Ruxton, Sherratt and Speed, 2004).

Predation is a key selective pressure shaping many aspects of prey phenotype, from behavioural to physical traits, including prey appearance. Prey species possess a remarkable range of visual antipredator strategies, forming layers of protection (Stevens, 2007). In the first instance, the most well-studied form of protective coloration is camouflage, a strategy aiming to prevent predators from detecting or recognising prey (Stevens and Merilaita, 2009; Skelhorn and Rowe, 2016). A cryptic appearance can be achieved in many ways, most obviously by matching the colour and pattern of the natural environment, a technique known

as background matching, but also through disruptive coloration, in which high-contrast markings break up the outline of the animal, countershading, and transparency, especially in aquatic environments (summarised in Nokelainen and Stevens, 2016). Intuitively, evading predators should select for the dull coloration and general discretion typically associated with crypsis, while bright colours are expected to be associated with sexual signalling. Yet, as an alternative to crypsis, visual signalling strategies involving extravagant visual features can be deployed to effectively deter predators.

A signal is defined as an “action or structure that increases the fitness of an individual by altering the behaviour of other organisms detecting it", and whose features have evolved to produce that effect (Maynard Smith & Harper, 1995). One such tactic is to allow detection but deceive predators by masquerading as an unprofitable item (Skelhorn *et al.*, 2010; Skelhorn, Rowland and Ruxton, 2010); well-known examples include stick insects, the leafy sea dragon (*Phyllopteryx eques*), and many Lepidoptera, from the twig-mimicking buff-tip moth (*Phalera bucephala*) to the Chinese character (*Cilix glaucata*), which resembles a bird dropping. If detection is inevitable and prey are recognised as edible, other signalling strategies can come into play. Startle, or deimatic, displays, lead to misclassification of the prey as a potential threat (Skelhorn, Holmes and Rowe, 2016), while deflective signals function to misdirect the attention of a predator to less valuable body parts (e.g. long wing extensions or false head patterns in butterflies; López-Palafox, Luiz-Martínez and Cordero, 2015; Barber *et al.*, 2015). These tactics are not mutually exclusive, and may interact to provide a flexible defence depending on circumstances. For example, the underwing moths (*Catocala* sp.) have background-matching forewings, but will reveal colourful hindwings to startle predators who persist with their attack (Sargent, 1990). Finally, defended species may use bright or otherwise conspicuous displays to warn predators of their unprofitability, a strategy known as aposematism, or warning coloration (Ruxton, Sherratt and Speed, 2004).

The theory of warning coloration was first proposed by Alfred Russell Wallace, prompted by discussions with Darwin over the function of conspicuous coloration in insect larvae (Wallace, 1867). They were intrigued by the bright colours of caterpillars, which do not reproduce at that life stage, so could not be

using their signals to attract mates. Wallace hypothesised that species which were toxic or otherwise unprofitable might benefit from advertising their defences, to avoid injury or death from mistaken attacks (Wallace, 1889). E. B. Poulton further developed this theory, supported by early evidence that conspicuous species could be distasteful and rejected by predators (Poulton, 1887). He coined the term “aposematism” to describe “an appearance that warns off enemies because it denotes something unpleasant” (Poulton, 1890). Aposematic signalling requires three key elements: a conspicuous signal, an aversive or dangerous secondary defence, and predators with the ability to learn the association between the two (Cott, 1940). The secondary defences advertised by aposematic prey can vary widely, from toxic chemicals (e.g. alkaloids and cyanogenic glucosides in tiger moths [Erebidae; Weller, Jacobson and Conner, 1999]) to a pugnacious nature (Caro, 2009). In terms of the signal itself, aposematism is not restricted to visual communication, and there is increasing interest in other warning signal modalities, such as warning odours (Rowe and Guilford, 1999; Rowe and Halpin, 2013) and acoustic aposematism, most famously in the case of defended tiger moths deterring predation by bats (Hristov and Conner, 2005; Dowdy and Conner, 2016). Nevertheless, visual warning coloration remains by far the most well-studied type of aposematic signal, and this thesis likewise focuses on aposematic colours and patterns. The association of conspicuous coloration and defences is taxonomically widespread, especially common in invertebrates, amphibians and reptiles, but now also recognised to occur in birds (in pitohuis; Dumbacher *et al.*, 1992) and mammals (e.g. the striped skunk *Mephitis mephitis*; Lariviere and Messier, 1996; Caro, 2009). While mostly studied in the terrestrial environment, warning colours have also been investigated in aquatic organisms, particularly nudibranchs (e.g. Cortesi and Cheney, 2010), despite some uncertainties over the relevant predator visual systems and lighting conditions in the marine environment (Pawlik, 2012). Aposematic signalling has also been reported in poisonous fungi (Sherratt, Wilkinson and Bain, 2005) and defended plants (Lev-Yadun, 2001; Cooney *et al.*, 2012), although these taxa have so far received comparatively little attention.

1.3 The form and function of warning, or aposematic, signals

There are several hypotheses attempting to explain why appearing conspicuous may be advantageous to defended prey. Drawing attention to physical defences would be beneficial, and could be one route by which aposematic signals might first evolve (Speed and Ruxton, 2005). In addition, cryptic strategies restrict prey to specific backgrounds or types of behaviour, so that camouflage remains effective, while a conspicuous strategy would free them from these "opportunity costs" (Ruxton, Sherratt and Speed, 2004). Yet the most important implication of conspicuous warning signals is their effect on predator behaviour. Animal signals are thought to be shaped by two fundamental considerations: the strategic message they aim to communicate, and the best means to effectively convey that message, a concept known as signal efficacy (Guilford and Dawkins, 1991). To maximise efficacy, a signal should be highly detectable, discriminable and memorable, to facilitate predator learning (Guilford and Dawkins, 1991; Ruxton, Sherratt and Speed, 2004). The bright and colourful patterns of aposematic signallers stand out from other prey and natural backgrounds, attracting the attention of predators and enhancing efficacy in a number of ways (Stevens and Ruxton, 2012). Firstly, conspicuous signals appear to provoke innate avoidance behaviour in predators. This may be linked to an initial fear of novel stimuli, or neophobia, as well as to more long-term reluctance to accept conspicuous prey even when palatable, a trait known as dietary conservatism (Marples, Roper and Harper, 1998; Thomas *et al.*, 2003, 2004). Even more importantly, the form of typical warning signals stimulates predator learning. In the most basic sense, high visibility to predators will increase the rate at which predators encounter and experience these colourful but defended prey, speeding up the development of an association between the signal and the defence. Conspicuousness may also facilitate learning via several other mechanisms, including greater memorability and easier recognition (reviewed in Speed, 2000). However, whether examining innate responses or learned aversions, the relative importance of novelty, distinctiveness and conspicuousness *per se*, defined as visibility to predators against natural backgrounds, in determining the efficacy of warning signals, is still unclear (Ruxton, Sherratt and Speed, 2004; Stevens and Ruxton, 2012). While Wallace's original idea of warning coloration was based on distinctiveness from edible prey (Wallace, 1867), conspicuousness itself does

appear to have specific advantages. More studies quantifying the conspicuousness of warning colours against natural backgrounds, and assessing the relevance of this measure to predator behaviour (e.g. Arenas, Walter and Stevens, 2015) would contribute to resolving this debate.

Focusing on the specifics of prey patterns, it can be difficult to ascertain the relevance of different signal features. Warning signals are composed of multiple visual components, including colour, lightness, overall pattern and specific pattern elements, and internal contrast between coloured patches. In general, chromatic features are considered to be more important in avoidance learning than achromatic information, at least for avian predators (Stevens and Ruxton, 2012), as demonstrated by many experiments in controlled conditions (Osorio, Jones and Vorobyev, 1999; Aronsson and Gamberale-Stille, 2008) and in the field (e.g. Finkbeiner, Briscoe and Reed, 2014). Nevertheless, there is evidence that predators do attend to achromatic patterns (e.g. Aronsson and Gamberale-Stille, 2012a,b). The role of specific shapes and features has also been investigated, especially in the context of eyespots (reviewed in Stevens, 2005). Studies of the relevance of pattern symmetry have yielded conflicting results (Forsman and Merilaita, 1999; Stevens, Castor-Perry and Price, 2008). Nevertheless, the arrangement of pattern elements is clearly important, as demonstrated by the presence of high contrast markings in both conspicuous aposematic patterns and disruptive camouflage. These two patterns have opposite effects on predator perception due to the distribution of these patches, either highlighting or concealing the outline of the body (Stevens, 2007). A given pattern may also fulfil multiple roles, from crypsis to aposematism, depending on viewing distance (Barnett, Cuthill and Scott-Samuel, 2017) or the location and posture of an animal (e.g. in the wood tiger, *Arctia plantaginis*; Honma, Mappes and Valkonen, 2015). Moreover, even for classic features of aposematic patterns, disentangling which mechanisms are at work can be complicated. Long wavelength colours are very common in warning signals, but their effectiveness may be attributed to several characteristics (Stevens and Ruxton, 2012), such as specific aversions to these colours (Roper, 1990), their high chromatic and achromatic contrasts with melanic pattern elements, their conspicuousness against foliage or their stability under a range of illuminations (Arenas, Troscianko and Stevens, 2014).

Methodological breakthroughs in the fields of animal vision and image analysis in the last 20 years present a major opportunity for researchers working on animal communication (Cuthill *et al.*, 2017), and will help to resolve many of the questions raised above, regarding the form of warning signals. Although examining signals from the perspective of the relevant receivers is not a new idea (Cott, 1940), the importance of this consideration has been increasingly emphasised (Stevens, 2007), as our understanding of animal visual systems and the differences between human and animal perception has grown (Osorio and Vorobyev, 2008). Ambient light conditions, natural backgrounds against which signals are displayed and receiver perception will all be critical to the effectiveness of visual signals (Endler, 1990), so colours should be measured in relevant natural conditions and with particular signal receivers in mind. Receiver characteristics at all stages of visual communication will be important for signal perception, from the design and composition of the eyes receiving the signal to the neural networks responsible for classifying and discriminating patterns, through processing in the retina, and there is still much to learn (Endler and Mappes, 2017). Yet it is now possible to analyse intra- and inter-specific visual signals based on our best understanding of animal perception, using visual modelling techniques (Stevens, Stoddard and Higham, 2009; Stevens, 2011). These methods are increasingly accessible, as digital photography offers a more practical alternative to expensive spectrometry as a reliable means of measuring colour (Stevens *et al.*, 2007a; Pike, 2011), and more open access tools are released to support image analysis (e.g. Troscianko and Stevens, 2015; Van Belleghem *et al.*, 2017). Throughout this thesis, I use digital photography and models of avian vision to quantify warning signals in an ecologically-relevant way.

Linking the strategic and efficacy components of signalling, conspicuousness is a uniquely appropriate property for warning signals as it provides some degree of reliability. In the case of aposematism, both predators and prey should stand to benefit from avoiding attack, yet their interests are not exactly aligned (Summers *et al*., 2015). To a predator, the net benefit of attacking defended prey will depend on individual traits, such as motivation, and environmental variables, including temperature or the availability of alternative prey (reviewed in Skelhorn and Rowe, 2016), as well as on the toxicity of the prey item.

Unprofitability itself can be highly variable within a single population (automimicry; Guilford, 1994; Svennungsen and Holen, 2007), and this variation can be enhanced by the presence of palatable (Batesian) or simply less well-defended ('quasi-Batesian'; Speed, 1993) mimics. How warning coloration can emerge as an evolutionarily stable strategy in these circumstances has been hotly debated. Early interpretations of warning coloration as a handicap signal (Zahavi, 1975, 1991) predicted honesty in aposematism, enforced by naïve or resistant predators who would attack conspicuous unpalatable prey (Grafen, 1990). Yet handicap mechanisms imply an inherent cost of producing the signal, functionally related to the strategic message conveyed by the signal. This concept of warning coloration was thus generally rejected, as there was no evidence of a physiological link between colourful signals and defences. Early models (Grafen, 1990) were further criticised for their simplistic assumptions, ignoring predator learning (Guilford and Dawkins, 1993). An alternative concept of warning colours as conventional signals was proposed, whereby signal form is geared towards maximising signal efficacy, and need not be related to the defence being advertised (Guilford and Dawkins, 1993). Nevertheless, even in this scenario, conspicuousness will generally be disadvantageous to undefended prey due to the high cost of greater predation risk through increased detectability. This enforces a level of honesty in aposematic signalling (Sherratt, 2002; Ruxton, Sherratt and Speed, 2004), such that, on average, warning colours indicate unprofitable prey.

1.4 Signal honesty in aposematism

This basic association between conspicuousness and some form of defence is inherent in the definition of aposematism, and makes warning coloration a qualitatively honest signal (Summers *et al*., 2015). More controversial is the notion of quantitative honesty in aposematism, whereby signal strength would indicate the level of an individual's defences. In a seminal model exploring the initial evolution of aposematism, Leimar, Enquist and Sillen-Tullberg (1986) suggested that conspicuousness and unprofitability should be negatively correlated in aposematic prey. They considered that predators can learn from their previous encounters with profitable or unprofitable prey, and generalise their experience along excitatory and inhibitory gradients. Under these conditions, once predators have learnt to associate warning signals and

defences, signallers should reduce their investment in defences to cut the costs of acquiring or producing defensive chemicals, leading to a breakdown of positive correlations between conspicuousness and the level of the defences advertised (Wang, 2011).

Yet many subsequent theoretical investigations suggest that the evolution of quantitative honesty in aposematic signalling is possible, given specific conditions (reviewed in Summers *et al*., 2015). If predators are more cautious when attacking conspicuous prey (the "go-slow" hypothesis; Guilford, 1994), taking more time to better evaluate their unprofitability before consuming them, quantitative honesty can be a stable strategy (Holen and Svennungsen, 2012). Even without assuming that predators treat conspicuous prey differently from the outset, some models predict reliable associations between signals and defences if unprofitable prey are more likely to survive attacks than profitable prey (Sherratt, 2002), an assumption supported by empirical evidence (e.g. Wiklund and Järvi, 1982). Similarly, Speed *et al.* (2010) predict "more-or-less" honest signalling within populations, whereby signal levels on average indicate the strength of defence, if predators can assess prey defences during attacks through taste-rejection and learn to associate their signal value with an average measure of toxicity. Using a different approach, stochastic models suggest that coevolutionary dynamics between prey with different defence levels within populations (Speed and Franks, 2014), and between defended species and their palatable mimics (Franks, Ruxton and Sherratt, 2009), may also lead to positive correlations between signal and defence levels.

Although initially dismissed, more recent work has suggested that a strict interpretation of aposematic signals as handicap signals may still be possible. In their resource-allocation model, Blount *et al*. (2009) suggested that signals and defences may compete for the same resources, leading to a positive correlation between signal strength and the potency of defence when resources are limited. They proposed that coloration and toxins might compete for energy in general, or more specifically for antioxidant function. Many pigments, including carotenoids and pteridines, have antioxidant properties, which would also be needed to detoxify the by-products of chemical defences, leading to a trade-off between producing stronger signals and accumulating more defensive

chemicals. Building on this initial model (Blount *et al*., 2009), further theoretical work suggests that resource allocation trade-offs could produce quantitative honesty without assuming that predators are innately wary of conspicuous prey or that conspicuousness confers additional fitness benefits (Lee, Speed and Stephens, 2011; Holen and Svennungsen, 2012).

Positive correlations between signal and defence levels may also arise as a consequence of other functions of coloured patches, such as thermoregulation or mate choice (Guilford, 1988; Lee, Speed and Stephens, 2011). Without considering the strategic message of aposematic signals, the economics of defence and display can predict both positive and negative correlations between these traits (Speed and Ruxton, 2007). When the fitness costs of producing signals and defences increase in parallel, positive correlations are expected, while disjunctions in fitness costs will lead to negative correlations. Production costs and available resources will be critical to the relationship between warning colours and defences, even when mechanisms of handicap signalling are invoked. For example, in the resource allocation model, signals and defences are expected to be negatively correlated when resources are abundant, as very high levels of chemical defences will provide effective protection without the need for conspicuous signals, which incur costs of detectability to naïve, highly-motivated or resistant predators (Blount *et al*., 2009). To estimate these parameters, and resolve some debates surrounding the assumptions of models of signal evolution, it is essential to study the relationship between coloration and defences in wild populations. Understanding how colour signals and defensive chemicals are produced is one important objective, as seen in studies of sequestration ability in defended newts (Mochida *et al*., 2013), colour production in stinkbugs (Fabricant *et al.*, 2013) and the relationship between alkaloid and carotenoid levels in ladybirds (Blount *et al.*, 2012; Winters *et al.*, 2014). This promising avenue of research should help determine how signals and defences are shaped by environmental conditions and if trade-offs between them are likely. A greater appreciation of how differences in signal and defence levels really affect predation risk would also be useful (Summers *et al.*, 2015), as assumptions regarding predator responses to these two traits are critical in determining the outcomes of theoretical models (Speed and Ruxton, 2007; Blount *et al.*, 2009).

Existing empirical evidence of the relationship between signals and defences in aposematic species is limited to a relatively small number of studies, primarily focusing on poison frogs (Dendrobatidae) and ladybirds (Coccinellidae). While many do find a positive correlation between quantitative measures of coloration and chemical defences, there are conflicting results both within and between populations, as well as across species (see Table 1.1). Moreover, the fairly narrow taxonomic spread of these studies and inconsistencies in the methods used to quantify both defences and visual signals mean caution is needed when attempting to draw broader conclusions from this collection of research (Summers *et al.*, 2015). In terms of measuring coloration, it is critically important to consider signals from the perspective of the relevant receivers (Stevens, 2007). Although visual modelling techniques accounting for predator perception are increasingly being adopted, coloration has been assessed by human classification or viewer-independent measures in several of these studies. The specific signal features considered also vary: while many used contrast against natural backgrounds as their measure of conspicuousness, others chose traits such as the brightness or size of specific colour patches. There is as yet little direct evidence to suggest that these features are particularly attended to by predators, and some may be involved in other functions, potentially confounding the results (Summers *et al*., 2015). For example, the yellow abdominal band in paper wasps (Vidal-Cordero *et al.* 2012) may be an intraspecific signal of dominance, and coloration in poison frogs also plays a role in mate choice (Summers *et al.*, 1999). Greater clarity in terms of which signal features are used by predators to guide their foraging decisions in the wild would be helpful, in order to test truly relevant associations between signals and defences from the predators' perspective (Summers *et al.*, 2015). Currently, there is also an imbalance between the theoretical literature, with most models addressing the issue of signal honesty within a single population, and empirical work, which tends to focus on inter-specific or inter-populational differences. Testing the importance of specific signal features in aposematic patterns, and providing more data on the relationship between colour and defence in a novel study system, including within populations, are key aims of this PhD thesis.

Table 1.1: Published empirical studies relating coloration and toxicity in aposematic animals, both across and within species and populations, with details of the metrics used, attention to predator perception and results. This table shows the conflicting conclusions of empirical work on quantitative honesty in aposematism, and highlights some of the key issues in this field of research, namely the relatively narrow taxonomic focus of these studies, primarily based on dendrobatid frogs and ladybirds, and by contrast the diversity of methods and metrics employed when measuring coloration and toxicity, making the results difficult to compare.

Study system	Measures of coloration used	Measures of defences used	Predator vision?	Correlations found?	References
INTERSPECIFIC					
Coccinellidae (ladybirds)	Conspicuousness against host plants, internal pattern contrast, luminance, saturation, area of colour	Toxicity assay in *Daphnia pulex* water fleas	Yes (birds)	Positive for conspicuousness against plants	Arenas, Walter and Stevens, 2015
Dendrobatidae (poison frogs)	Brightness contrast between frog and leaf litter (ranked by humans and computer)	Diversity, quantity (concentration) and lethality of alkaloids	No	Positive	Summers and Clough, 2001
Dendrobatidae (poison frogs)	Conspicuousness to leaf litter	Presence, quantity (concentration) and diversity of alkaloids	No	Positive – conspicuousness and chemical defences associated across phylogeny	Santos and Cannatella, 2011
Epipedobates (poison frogs)	Internal chromatic and brightness contrast of frog pattern	Toxicity assay in mice	Yes (birds)	None	Darst, Cummings and Cannatella, 2006
Opistobranchs	Chromatic contrast to natural backgrounds	Toxicity assay in brine shrimp	Yes (damselfish and triggerfish)	Positive	Cortesi & Cheney, 2010
Pachycephalidae (whistlers – songbirds)	Human impression of colour pattern	Batrachotoxin levels	No	None. The two most toxic species are most brightly-coloured, but other toxic species are not conspicuous	Dumbacher, Spande and Daly, 2000; Dumbacher *et al.*, 2008
INTRASPECIFIC, BETWEEN POPULATIONS					
Cynops pyrrhogaster (Japanese fire-bellied newt)	Area of red on ventral side	Tetradotoxin (TTX) & 6-*epi* TTX levels, sequestration ability	No	None	Mochida *et al.*, 2013
Dendrobates pumilio (strawberry poison frog)	Colour, classified by humans	Toxicity assay in mice	No	None	Daly and Myers, 1967

Study system	Measures of coloration used	Measures of defences used	Predator vision?	Correlations found?	References
Dendrobates pumilio (strawberry poison frog)	Viewer-independent overall brightness, and conspicuousness to predators against natural backgrounds	Toxicity assay in mice	Yes (birds, crabs & snakes)	Positive for brightness, and conspicuousness to birds & crabs	Maan and Cummings, 2012
Oophaga granulifera (granular poison frog)	Conspicuousness against natural backgrounds	Toxicity assay in mice	Yes (birds)	Negative	Wang, 2011
INTRASPECIFIC, WITHIN POPULATIONS (INCLUDING LABORATORY POPULATIONS)					
Coccinella septempunctata (7-spot ladybird)	Elytra brightness and colour, carotenoid levels and spot size	Coccinelline and precoccinelline levels	Yes (birds)	Positive for precoccinelline and carotenoid levels; positive for coccinelline and carotenoid levels in females, but negative in males; positive for coccinelline levels and spot size in low-diet treatment	Blount *et al*., 2012
Coccinella septempunctata (7-spot ladybird)	Egg and adult elytra saturation, brightness and hue	Coccinelline and precoccinelline levels	Yes (birds)	Positive for egg saturation, hue and precoccinelline levels, and for elytra hue and coccinelline levels; negative for elytra brightness and coccinelline levels	Winters *et al*., 2014
Dendrobates pumilio, Solarte population (strawberry poison frog)	Total reflectance, longwave chroma, luminance, chromatic contrasts and conspicuousness	Concentration and diversity of alkaloids	Yes (birds and frogs)	Negative between total reflectance and both aggregate pumilotoxin content & pumilitoxin PTX307A levels	Crothers *et al.*, 2016
Harmonia axyridis (harlequin ladybird)	Elytra redness, area of red, spot colour, and carotenoid concentration	Concentration of alkaloids	No	Positive for area of red; lighter spots associated with higher harmonine levels in females	Bezzerides *et al.*, 2007
Polistes dominula (paper wasp)	Brightness of yellow abdominal band	Size of poison gland	No	Positive	Vidal-Cordero *et al.*, 2012

1.5 Aposematism in burnet moths (Lepidoptera: Zygaenidae)

Historically, Lepidoptera have been at the heart of research on warning coloration, from the brightly-coloured caterpillars stimulating discussions between Darwin and Wallace to the tropical butterflies inspiring the concepts of

Batesian and Müllerian mimicry of defended species (Bates, 1862; Müller, 1879). To this day, work on Lepidoptera continues to contribute to our understanding of aposematism, although other species, primarily poison frogs (Dendrobatidae) and ladybirds (Coccinellidae), have also emerged as important model systems. Beyond the astounding diversity in colour and pattern that first attracted the attention of naturalists, Lepidoptera possess many qualities that make them amenable to research on visual signalling: their flat wings are ideally suited to photography and image analysis, many species can be reared in captivity, and their principal visual predators are birds, a group whose visual systems are relatively well-understood (Hart, 2001a; Ödeen and Håstad, 2003; Osorio and Vorobyev, 2008). *Heliconius* butterflies are without doubt the most important model for understanding the evolution of colour patterns, allowing crucial questions to be addressed, such as how mimicry rings evolve, how warning signals interact with sexual selection, and how diversity in warning coloration might arise and be maintained (reviewed in Jiggins, 2017). More recently, the wood tiger moth, *Arctia plantaginis*, has become established as another key species in which to investigate warning coloration. Studies of the wood tiger have yielded new insights into the selection pressures shaping the form of warning signals, examining the effect of different predator communities (Nokelainen *et al.*, 2014), seasonality (Mappes *et al.*, 2014), trade-offs with sexual selection (Nokelainen *et al.*, 2012) and functional constraints (such as temperature; Lindstedt, Lindström and Mappes, 2009) amongst others. Meanwhile, other species provide opportunities to test the roles of particular signal properties, such as iridescence in the pipevine swallowtail, *Battus philenor* (Pegram, Han and Rutowski, 2015). In this thesis, I focus primarily on a large and diverse family of Lepidoptera, which has as yet received comparatively little attention from researchers studying aposematism: the Zygaenidae, commonly known as forester and burnet moths.

The Zygaenidae form a species-rich family of mostly day-flying moths, with a worldwide distribution but especially diverse in Asia and the Palearctic region (Naumann, Tarmann and Tremewan, 1999; Niehuis, Naumann and Misof, 2006). Approximately 1000 described species fall into four recognised subfamilies: Callizygaeninae, Chalcosiinae, Procridinae and Zygaeninae (Niehuis, Naumann and Misof, 2006). In the Western Palearctic 44 species of

Procridinae, known as foresters, and 70 species of Zygaeninae (burnet moths) are found, along with a single member of the Chalcosiinae, the almond-tree leaf skeletonizer moth, *Aglaope infausta* (Naumann, Tarmann and Tremewan, 1999). Species diversity is more limited in the British Isles, with only three species of Procridinae and seven species of Zygaeninae, four of which are now restricted to Northern Scotland. One of these, the New Forest burnet, *Zygaena viciae*, is vanishingly rare, restricted to a single protected colony in Northwest Scotland after being collected to extinction from the New Forest (Young and Barbour, 2004).

The defining characteristic of the Zygaenidae is their ability to synthesise the toxic cyanogenic glucosides linamarin and lotaustralin (Figure 1.1b); these compounds have been found in every one of the 45 species tested (Davis and Nahrstedt, 1982; Zagrobelny *et al.*, 2004). Widespread defensive tools in plants, cyanogenic glucosides are known as phytoanticipins, defensive compounds constitutively expressed in plants in anticipation of herbivore attack (Pentzold *et al.*, 2014). They also occur in many arthropods, such as some polydesmoid millipedes (Diplopoda), centipedes (Chilopoda), beetles (Coleoptera), true bugs (Heteroptera) and many Lepidoptera, including *Heliconius* butterflies (Zagrobelny, Bak and Møller, 2008). Cyanogenic glucosides release cyanide when broken down by enzymes, for example in the gut of a predator (Zagrobelny, Bak and Møller, 2008), and are also bitter-tasting so may deter predation through taste-rejection (Skelhorn and Rowe, 2009). This provides an effective defence for the Zygaenidae, and early work injecting zygaenid extracts into mice, frogs, and more disturbingly, humans, demonstrated the toxicity of burnet moths even before the compounds responsible were identified (Rothschild *et al.*, 1970; Marsh and Rothschild, 1974). Experiments with birds in captivity have further confirmed that they are generally considered unprofitable by avian predators (Heikertinger, 1939; Wiklund and Järvi, 1982; Rammert, 1992), despite anecdotal records of predation in the wild (collated in Tremewan, 2006).

Since the identification of linamarin and lotaustralin in the six-spot burnet, *Zygaena filipendulae* (Davis and Nahrstedt, 1979), the chemistry of this species has been thoroughly investigated. The larvae of *Zygaena* species are

apparently unique in their ability to both synthesise cyanogenic glucosides *de novo*, from the amino acids valine and isoleucine, and sequester the same compounds from their host plants at the same time (Zagrobelny *et al.*, 2014a). *De novo* synthesis of cyanogenic glucosides is ancestral in the Zygaenidae but served as a pre-adaptation for some Zygaeninae to feed on cyanogenic plants. The evolution of sequestration ability then allowed these species to accumulate toxins more economically. Retaining the capacity for *de novo* synthesis enables fine-tuning of the ratios of linamarin and lotaustralin, compensating for the variability in toxin content in their host plants (Zagrobelny *et al.*, 2014a). *Z. filipendulae* feeds on cyanogenic Bird's foot trefoil (*Lotus corniculatus*), yet can almost completely compensate for a lack of cyanogenic glucosides in its diet, albeit at the cost of slower and reduced growth (Zagrobelny *et al.*, 2007a). Uncovering the genetic pathway for the synthesis of linamarin and lotaustralin revealed a remarkable convergence between the herbivore and its host plant, with both species following the same steps and using similar classes of enzymes, but derived independently, to produce the cyanogenic glucosides (Jensen *et al.*, 2011). These toxins play a crucial role throughout the life cycle of *Z. filipendulae* and are present at every stage (see Figure 1.1a; Jones, Rothschild and Parsons, 1962). Beyond protection from predators, cyanogenic glucosides also provide a store of nitrogen to fuel metamorphosis and serve as a nuptial gift from males to females during courtship (Zagrobelny *et al.*, 2007b).

Although primarily based on cyanogenic glucosides, the defensive arsenal of burnet moths is complex and multimodal. Effective at all life stages and against a range of predators, their defences include pyrazine warning odours (Rothschild, Moore and Brown, 1984), which enhance the aversiveness of visual warning signals (Rowe and Guilford, 1996; Lindström, Rowe and Guilford, 2001), as well as bitter-tasting fluids, and compounds that are toxic if ingested. For example, when disturbed, both larvae and adults release defensive droplets, aversive to many invertebrate and vertebrate predators (Jones, Rothschild and Parsons, 1962; Franzl and Naumann, 1985). Illustrating the multiple layers of their defences, the larval fluid's viscosity provides protection from ants, while the presence of bitter cyanogenic glucosides and the neurotoxin β-cyanoalanine should deter vertebrates. If predators continue to attack and the droplets come into contact with β-glucosidases in the *Zygaena*

haemolymph, cyanide will be released as a further step in their defence (Pentzold *et al.*, 2016). These sophisticated defences are also advertised to predators with unmistakeable, conspicuous warning signals, especially in the adult stage.

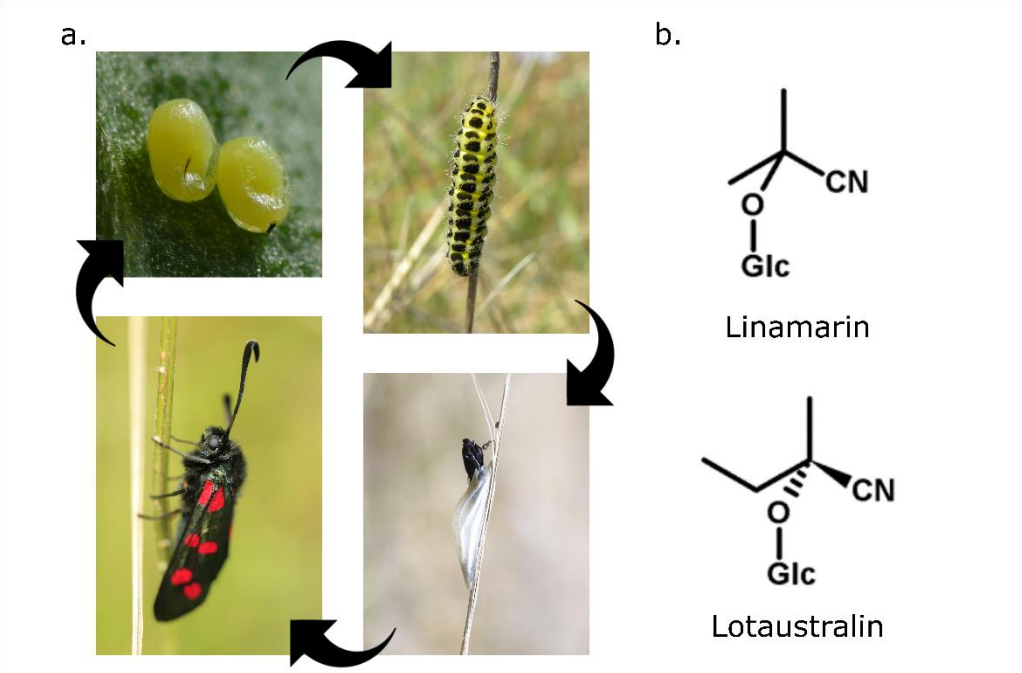


Figure 1.1: The life cycle of the six-spot burnet moth, *Zygaena filipendulae* (a, clockwise from top left: eggs, larva, cocoon and adult) and its defensive chemicals (b). Egg photograph: © Harald Süpfle, Wikimedia Commons; all other photographs E. S. Briolat. Chemical diagrams produced by Mika Zagrobelny.

While the Procridinae are generally brown or green in colour, and discreet in their behaviour, the Zygaeninae are classic examples of aposematic animals. They are characterised not only by toxicity in all life stages but also by sluggish behaviour, high local abundance and conspicuous wing patterns (Hofmann and Tremewan, 2017). The typical appearance of a burnet moth features red spots on black forewings and red hindwings, but four main phenotypes can be found: red and black, yellow and black, dichromatic (yellow, red and black), and a darker melanistic type (Figure 1.2). Moreover, these wing patterns can be extraordinarily diverse, both within and between populations of the same species. Variation in adult phenotype can take many forms, from changes in spot colour, replacement of black scales with colour and increased melanism to spot confluence and changes in abdominal pattern (Figure 1.3; Hofmann and

Tremewan, 2017). The most famous example of polymorphism in the Zygaeninae is found in *Z. ephialtes*, which possesses two pattern types (known as “peucedanoid” and “ephialtoid”), both occurring in red or yellow forms. In some locations, the prevalence of these pattern types is thought to be linked to mimicry of another distasteful moth, the nine-spotted, *Amata phegea* (Sbordoni *et al.*, 1979). Polytypisms, or differences between populations, can also be spectacular; for example, *Z. carniolica*, which typically has a dark background colour, displays white wings with red spots in Cappadocia (Turkey). Zygaenidae larvae are also highly variable both within and between species, and can be cryptic or conspicuous (Hofmann and Tremewan, 2017). Despite considerable interest in their diverse patterns from the entomological community, relatively little work has been done on the function of burnet moth coloration, and none with sophisticated modern methods accounting for predator vision. With the exception of work on mimicry in *Z. ephialtes*, and some experiments on mate choice (Zagatti and Renou, 1984; Toshova, Subchev and Toth, 2007), the Zygaenidae are a relatively untapped resource for researchers working on visual signals.

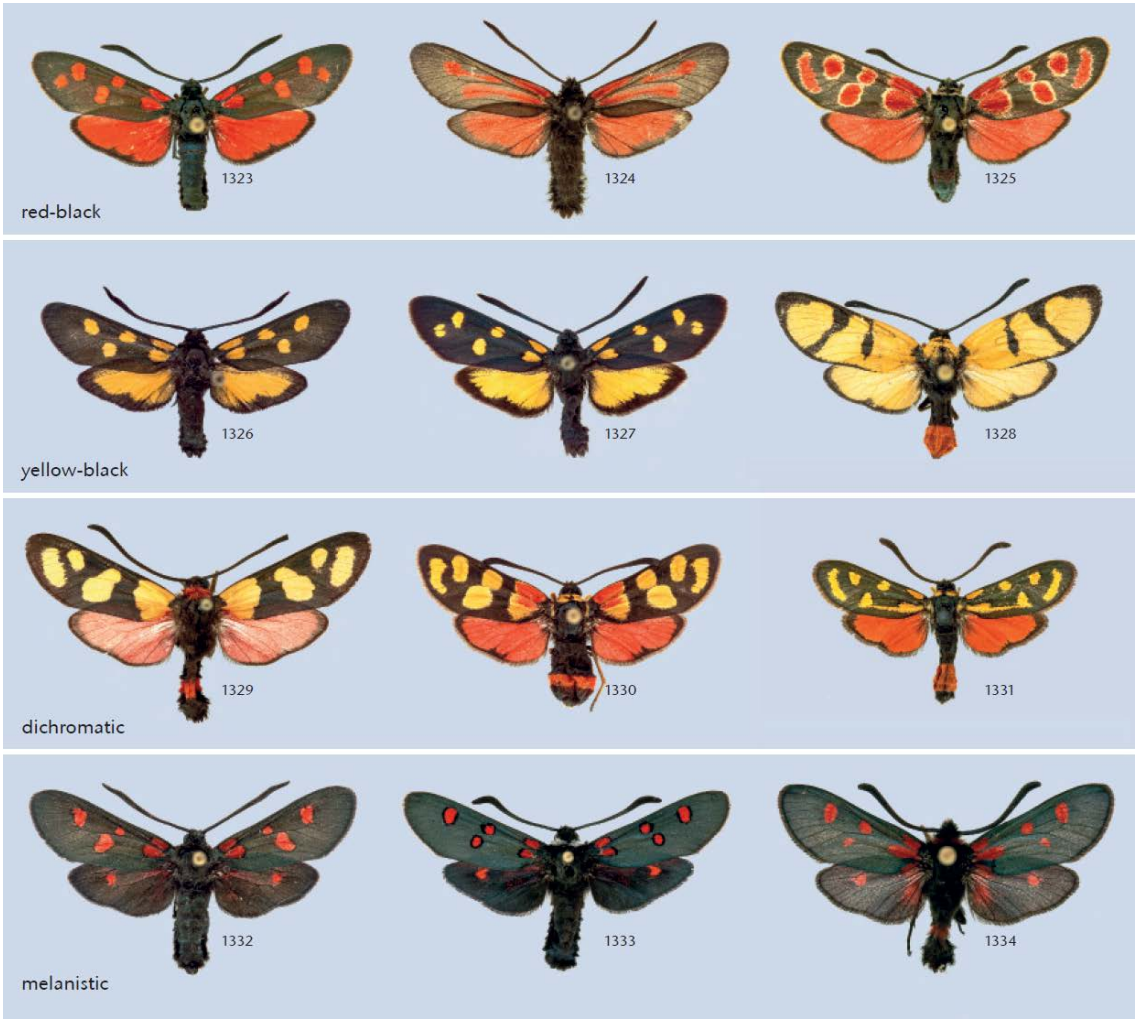


Figure 1.2: The four main phenotypes found in the Zygaeninae: red-black, yellow-black, dichromatic and melanistic, with example species. 1323, *Zygaena transalpina*; 1324, *Z. osteorodensis*; 1325, *Z. carniolica*; 1326, *Z. angelicae ternovanensis*; 1327, *Z. transalpina tilaventa*; 1328: *Z. tamara*; 1329, *Z. nocturna*; 1330, *Z. cocandica*; 1331, *Z. johannae*; 1332, *Z. lonicerae extremata*; 1333, *Z. lavandulae*; 1334, *Z. speciosa*. This figure was reproduced with permission from Hofmann and Tremewan (2017).

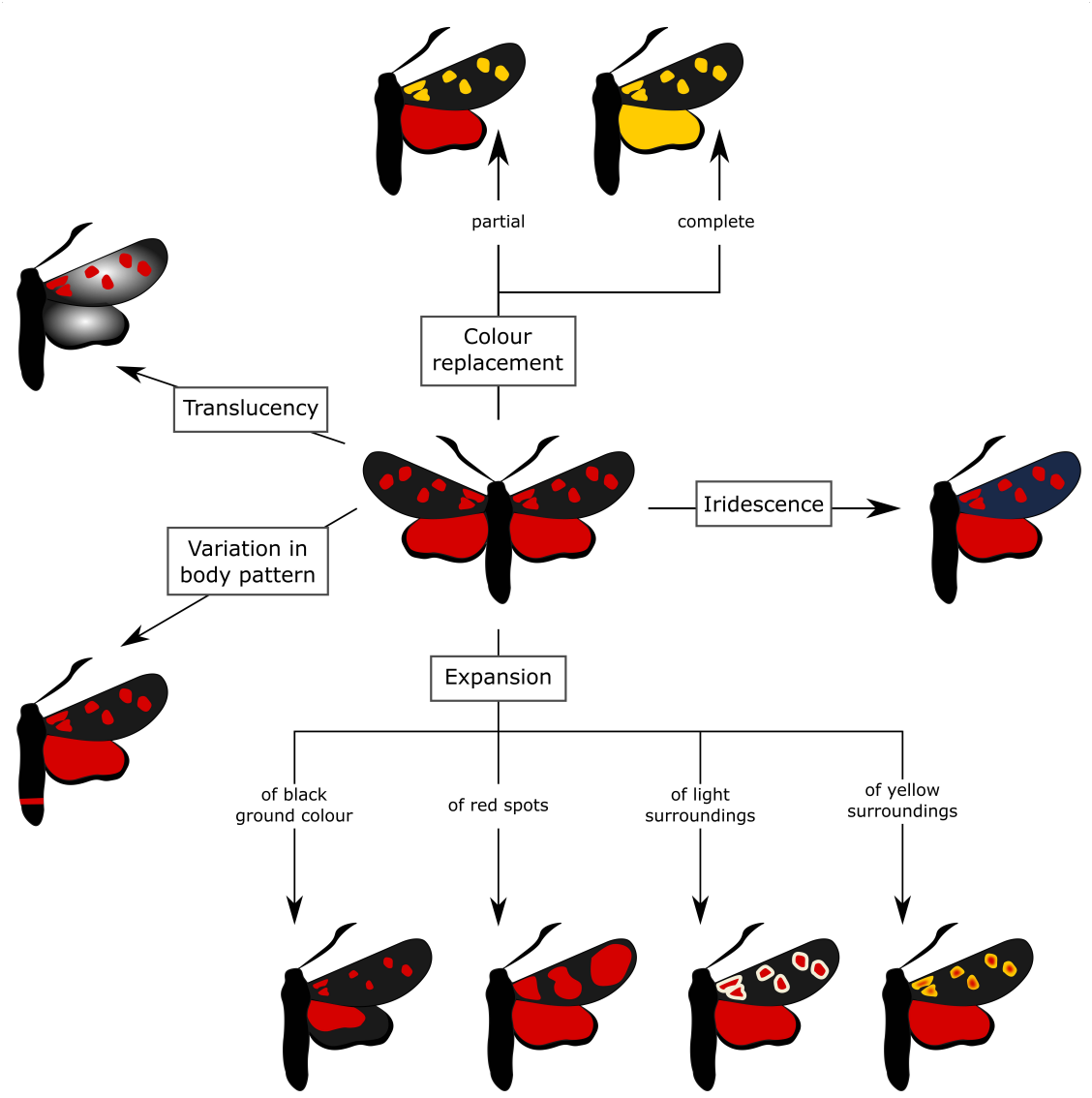


Figure 1.3: Types of variation in the phenotype of adult Zygaeninae. This figure was created by E.S. Briolat, based on Table 70 in Hofmann and Tremewan (2017).

The ability to identify and accurately quantify the chemical defences of the Zygaenidae, combined with the diversity of conspicuousness and other visual features seen in their wing patterns, makes them an attractive system in which to explore the relationship between coloration and defences. In this thesis, I explore the form of lepidopteran warning signals and how their characteristics relate to the presence and potency of defences, as well as to predation risk in the wild, using the Zygaenidae as my principal study system. I begin by presenting a detailed explanation of the photography and image analysis techniques used throughout this thesis in Chapter 2, illustrating them with some

preliminary experiments on *Z. filipendulae*. In Chapter 3, I test for broad trends in the visual features of day-flying defended Lepidoptera with a comparative analysis of museum specimens of British moths. I then focus on the six-spot burnet, *Z. filipendulae* in Chapter 4, and measure the colour and cyanogenic glucoside levels of specimens from Denmark, France and the UK to test for quantitative signal honesty in this species. In Chapter 5, I extend this study to address the question of signal honesty across species in the Zygaenidae. Finally, in Chapter 6, I test whether variation in several signal properties affects predation risk for burnet-like prey, by conducting artificial predation experiments in a natural habitat of *Z. filipendulae*. Throughout my work, I consider the perspective of relevant visual predators by analysing the warning signals of Lepidoptera as perceived by the avian visual system. In Chapter 7, I discuss the implications of my findings and the many outstanding questions that could be addressed by further research on signalling in burnet moths. Bringing these insights together, this thesis should serve to develop our understanding of the relationship between coloration and defences in aposematic animals, and highlight the great potential of burnet moths as a promising study system for researchers in the field of animal communication.

Chapter 2

Methods for measuring warning colours – an illustration with preliminary experiments on the six-spot burnet moth (*Zygaena filipendulae*, L.)

The Provence burnet, *Zygaena occitanica*, as perceived by human vision (left) and as a false-colour image (right; blue, green and red colours represent the ultraviolet-, short wavelength- and medium wavelength-sensitive channels of avian perception). Photographs: Jolyon Troscianko

2.1 Abstract

Investigating the form and function of animal signals requires techniques to quantify colour, brightness and pattern in a reliable and biologically-relevant way. In this thesis, I use digital photography as a means to measure warning colours and other wing patterns in Lepidoptera. This increasingly popular method in the study of animal coloration presents numerous advantages over more traditional spectrometry, yet several precautions must be taken to ensure that it yields accurate and repeatable measures of colour and pattern. These include considerations regarding the equipment and experimental set-up used for photography, as well as protocols for subsequently processing the images and extracting meaningful metrics of coloration from them. In addition, the accuracy and relevance of measurements obtained from digital photography will largely depend on having adequate specimens to photograph, kept in appropriate conditions. Burnet moths (Lepidoptera: Zygaenidae) are an attractive family in which to explore warning coloration and signal honesty within and between species, yet some preliminary experiments were required to verify that the six-spot burnet, *Zygaena filipendulae* (L.), would be amenable to my investigations. In particular, I established that the wing markings of this species, which, to human observers, seem relatively uniform among individuals, do appear variable to avian predators, legitimising the study of differences in coloration between individuals. Another important methodological concern, dealt with in a second experiment, was the repeatability of measurements over time under my experimental conditions. Using these two preparatory tests as examples, this chapter describes and explains the techniques I have used to quantify warning colours throughout this thesis, and presents important results underpinning the rest of my work on burnet moths.

2.2 Introduction

Coloration in the natural world has long been a subject of fascination to naturalists, scientists and artists alike. Yet, by its very nature, colour is an elusive concept and a difficult subject to study, as it depends on the perception of the observer and the lighting conditions under which it is seen (Endler, 1990, 1993). The critical importance of considering the observer's perspective is increasingly apparent as we learn more about the visual systems of other

animals with capabilities very different from those of humans. In this context, devising tools and methods to quantify the colours and patterns seen in nature is not necessarily straightforward.

The use of photography as a means of studying animal coloration has a long history, with Abbott Thayer's illustration of the principles of protective coloration and countershading one of the most famous early examples (Thayer, 1896). Yet, for accurate quantification of colour, the preferred method of researchers interested in animal coloration has long been spectrometry, measuring the spectrum of light reflected from a point sample. More recently, the explosive increase in the quality and availability of digital cameras is modernising the study of animal coloration, with digital photographs replacing spectrometry to investigate colours in a range of contexts, from aposematism and camouflage to sexual signals and maternal investment (Stevens *et al.*, 2007a; recent examples include Winters *et al.*, 2014; Arenas, Walter and Stevens, 2015; Troscianko *et al*., 2016). Digital photography has several significant advantages compared to spectrometry, previously reviewed in Stevens *et al*. (2007a) and Troscianko and Stevens (2015). These include practical considerations, as good quality cameras are more easily accessible to researchers than expensive spectrometers, as well as different capabilities for analysis. One important benefit of photography is the ability to measure whole patterns with multiple colour patches at once. Spectrometry is limited to point measures of very small areas, which are vulnerable to changes in the distance and angle of the probe with respect to the focal sample, while photographs can efficiently provide information about natural scenes and complex objects. Several methods, such as granularity analyses (Chiao *et al.*, 2009), have also been developed to enable pattern analyses from digital images.

Nevertheless, several issues must be dealt with in order to generate objective and repeatable measurements from digital photographs, yet these are not always considered or appropriately addressed, leading to incomplete or erroneous results (Stevens *et al.*, 2007a). Firstly, most cameras respond to differences in light levels across wavelengths in a non-linear way (Stevens *et al.*, 2007a): this means that the Red, Green, and Blue (RGB) pixel values recorded from a digital image may not be equally influenced by changes in light

intensity. Non-linearity occurs because digital cameras are designed to optimise the perceived quality of photographs to human observers and for the wide range of printers and monitors used to display them, which themselves possess inherent non-linearities (Westland and Ripamonti, 2004), rather than to provide accurate measurements. To complicate matters, each camera brand and model may behave in a unique way, preventing comparisons between photographs taken with different equipment. As such, methods must be implemented to linearise the camera's responses before the photographs can be used (Stevens *et al.*, 2007a; Troscianko and Stevens, 2015). Moreover, ambient light is likely to differ in intensity and colour in both natural environments (Endler, 1993), as well as in the laboratory. Lighting conditions during photography will affect measurements taken from a photograph, so these must be normalised with respect to light levels. This can be done by simultaneously measuring reflectance standards, which reflect a known percentage of light across all wavelengths, and using those to standardise the pixel values obtained from the photographs (Stevens *et al.*, 2007a). The rapid development of methods implementing these processes facilitates accurate and robust analyses based on digital photography, unlocking its full potential as a tool for research (Stevens *et al.*, 2007a; Pike, 2011; Akkaynak *et al.*, 2014; Troscianko and Stevens, 2015).

Beyond obtaining reliable and objective values, measurements of animal coloration are far more relevant to natural situations if they take into account the visual systems of the species paying attention to these stimuli (Stevens and Ruxton, 2012). When studying warning coloration, understanding how potential predators perceive the signals of aposematic species is crucial to assessing the effectiveness of the displays or the relevance of variation in colour and pattern. Fortunately, techniques exist to convert the information gained from digital photographs to the visual systems of many well-studied species (Stevens *et al.*, 2007a; Stevens, Stoddard and Higham, 2009; Stevens, 2011; Troscianko and Stevens, 2015). Throughout this thesis, I have mapped my wing photographs to avian vision, as birds are the most likely visual predators of day-flying Lepidoptera. Birds are tetrachromatic, possessing four types of single cones determining colour vision. Although they can all perceive ultraviolet wavelengths, the sensitivity of their most shortwave-sensitive cone type does vary, separating avian visual systems into two broad categories: an ultraviolet-

sensitive (UVS) group, with a peak sensitivity λ_{UVS} ranging from 355 to 380 nm in measured species, and a violet-sensitive (VS) group, in which λ_{VS} ranges from 402 to 426 nm (Hart, Partridge and Cuthill, 1999; Hart *et al.*, 2000; Ödeen and Håstad, 2003; Hart and Hunt, 2007). For adult Zygaenidae, anecdotal reports of avian predation implicate multiple species, including blackbirds and skylarks, passerine species falling into the UVS category (Ödeen and Håstad, 2003), and corvids, belonging to the VS group (Ödeen and Håstad, 2003; Håstad, Victorsson and Ödeen, 2005; Tremewan, 2006). Therefore, I chose to model warning signals primarily as perceived by the UVS visual system, but also to check my conclusions with the VS system.

The bulk of the experimental work in this thesis focuses on the Zygaenidae, a family of day-flying moths chosen as a study system because of existing literature concerning their phylogeny and chemical defences, as well as for their interesting variation in coloration between and within subfamilies (see Chapter 1). To ensure that these species were amenable to this investigation and to the digital photography methods I would be using, I conducted two preliminary experiments. These were carried out under the same conditions and using the same techniques as all my work on burnet moth coloration in the subsequent chapters of this thesis. As such, describing these preliminary tests provides an opportunity to delve deeper into the rationale behind the methods I have used throughout my thesis for measuring and analysing colour data. Firstly, while different species in the *Zygaena* genus are identifiable based on their wing pattern, there appears to be little variation to the human eye within my principle study species, the six-spot burnet, *Zygaena filipendulae* (with the exception of rare aberrant orange, yellow or black morphs; Tremewan, 2006). I therefore measured variation in colour among my samples of *Z. filipendulae*, to determine whether the species was sufficiently variable for avian predators to discriminate between individual colours. In addition, due to the timings of field collections for different species or populations in different localities, not all specimens included in my analyses of colour and toxicity could be photographed with the same delay after emergence. I thus conducted a second experiment, repeatedly photographing a subset of specimens, to verify that wing colour did not significantly change over time under the experimental conditions. The protocols

I used for photographing specimens and analysing photographs in these experiments address the methodological concerns raised above.

2.3 Methods

2.3.1 Experimental set-up

To ensure that my measurements remained as consistent as possible throughout the experimental period, all photographs of burnet moths used in this thesis were taken in the same controlled conditions, inside a darkroom. The specimens were illuminated by an EYE Color Arc® MT70 bulb (Iwasaki Electric Co. Ltd.), its UV-blocking coating removed by lightly scrubbing the bulb with a steel brush (Troscianko and Stevens, 2015). After this treatment, it emits a light spectrum close to D65 daylight irradiance, including UV wavelengths. The wings of most Zygaenidae are iridescent, so the angle of incident light reaching the wing will influence colour measurements (Meadows *et al.*, 2011). To account for this, only the colours of the right-hand wings were measured, as the direction of the wing scales will affect iridescence. The light source was also kept at a constant 50° angle relative to each wing and the specimens were photographed directly from above, at a 90° angle to the wings.

Photographs were taken with a Nikon D7000 camera, which had previously undergone a quartz conversion enabling it to be sensitive to UV wavelengths (Advanced Camera Services, Norfolk). The camera was fitted with a 105mm CoastalOptics quartz lens (Jenoptik, Jena, Germany), also sensitive to UV. In addition, a set of filters was attached to the lens, and each specimen was photographed twice: once restricting wavelengths of light to the human-visible spectrum, using a UV/infrared (IR) blocking filter (Baader UV/IR Cut Filter, transmitting between 400 and 700 nm) and once under ultraviolet wavelengths alone, using a UV pass and IR blocking filter (Baader U filter, transmitting between 300 and 400 nm). These two images per individual were then combined during image processing to cover the range of wavelengths relevant to avian visual systems. All images were taken in RAW format, with manual white balance (“cloudy” setting) and a constant ISO (typically ISO 400) and aperture (f8). Compressed image formats, such as JPEG, cannot be used, as in-camera processing and compression create irreversible artefacts, making

subsequent linearisation of pixel values unreliable (Akkaynak *et al.*, 2014). Exposure length was adjusted according to light levels and was always considerably longer for photographs taken with the UV pass and IR blocking filter than for the human-visible ones, as camera sensitivity in UV wavelengths is approximately 100 times lower than in the human-visible spectrum (Troscianko and Stevens, 2015).

Moth wings were dissected from frozen specimens, and placed flat on a background of grey ethylene-vinyl acetate (EVA), more commonly known as craft foam, for photography. Previous work in our research group investigating the properties of background materials for photography found that, of the test samples, black EVA had the lowest reflectance across all wavelengths (approximately 5% reflectance), so was most suitable to be used as a background substrate (Arenas, 2015; Arenas, Walter and Stevens, 2015). However, my pilot experiments revealed that the outline of the burnet moth wings was too difficult to distinguish from the black EVA background during image analysis, so grey EVA was substituted to facilitate wing selection. Background reflectance was especially problematic for previous work on ladybird elytra, as the light appeared to "bounce off" the translucent chitin structures making up the elytra (Arenas, 2015). This is not the case for zygaenid wings, which, in addition, do not appear transparent in most species, including *Z. filipendulae*, so the background colour should not affect measurements. For the few species with more fragile wings (see Chapter 5), additional care must be taken when interpreting the results, but keeping a consistent uniform background should enable robust comparisons, at least between individuals of these species. Each photograph also included an individual label, a scale bar and a set of reflectance standards, reflecting all wavelengths of light equally between 300 and 750 nm. These were used to standardise light levels between photographs, eliminating any residual variation in lighting arising even in these controlled conditions, and making my results comparable to measurements taken in other settings. For most of my experiments, including those presented in this chapter, I used a pair of standards reflecting 93% and 7% of light respectively, cut from Zenith Lite Diffuse Target sheets (SphereOptics, Pro-Lite Technology, Cranfield, UK). The

standards were placed in the same plane as the moth wings, an important condition for accurate measurements (Troscianko and Stevens, 2015).

2.3.2 Image processing and mapping to animal visual models

Processing digital images for analysing coloration requires a number of steps, which are now easily implemented in the open-access multispectral image analysis toolbox, run in ImageJ (Schneider, Rasband and Eliceiri, 2012) and developed in our research group (Troscianko and Stevens, 2015). Prior to analysis, the human-visible and ultraviolet photographs of each specimen must be checked to ensure they are appropriately exposed, either by inspecting the image histogram or with image processing tools in the software toolbox (Troscianko and Stevens, 2015). Over-exposure, quashing variation among high values, would especially prevent correct interpretation of measurements. The chosen images must then be linearised, using an ImageJ plugin (IJ-DCRAW; Sacha, 2013) importing images via DCRAW (Coffin, 2015), a software package which extracts pixel values from RAW camera files in a linear way (Chakrabarti, Scharstein and Zickler, 2009). The validity of this method of linearisation has previously been verified, including with the camera set-up I used for photography (Troscianko and Stevens, 2015). Finally, selecting and measuring the reflectance standards in each image as it is imported enables the software to normalise pixel values with respect to light levels. As alternating between UV/IR-blocking and UV-pass filters can cause the camera to move slightly between shots, I used automatic alignment tools to accurately merge the human-visible and ultraviolet photographs for each sample wing. The toolbox software ultimately imports the combined picture as a multispectral image, a 32-bit stack with 5 layers, corresponding to different channels, or images taken in a specific range of wavelengths: three in the human-visible spectrum (vR, vG, vB) and two in the ultraviolet (uR and uB).

The next step in the analysis process is to map the camera pixel values onto the visual system of the potential predators of day-flying Lepidoptera, namely birds in both the UVS and VS groups. To do this, I used previously published data on the spectral sensitivities of model species for the US and VS systems, the blue tit *Cyanistes caeruleus* (Hart *et al.*, 2000) and the Indian peafowl *Pavo cristatus* (Hart, 2002) respectively. Camera sensitivities in the five wavelength

channels (uB, uR, vR, vG, vB), in the presence of the lens and filters, have also been measured previously (Figure 2.1) and are highly repeatable between camera set-ups of the same type (Troscianko and Stevens [2015], using methods similar in principle to Lovell *et al.* [2005] and Garcia *et al.* [2013]). Nevertheless, to ensure maximum consistency at all stages in the process, all photographs analysed together as part of the same experiment were taken with the same camera. Converting pixel values in these five camera-vision channels to cone catch values for avian photoreceptors was achieved via a polynomial mapping technique (Troscianko and Stevens, 2015). This is essentially a multiple regression calculating the cone catch value for each photoreceptor type (ultraviolet/violet-sensitive [UVS/VS], short wavelength-sensitive [SWS], medium wavelength-sensitive [MWS] and long wavelength-sensitive [LWS]) based on the camera values for each channel; for tetrachromatic visual systems, the mapping algorithm allowed two-way interactions between channels. Cone catches are then standardised so that a grey stimulus has equal values in all cone types. The quality of this modelling technique was tested with a database of reflectance spectra from natural stimuli (Arnold *et al.*, 2010) and revealed a very high match between camera values and photoreceptor cone catches (R^2>0.996) (Stevens and Cuthill, 2006; Pike, 2011; Troscianko and Stevens, 2015).

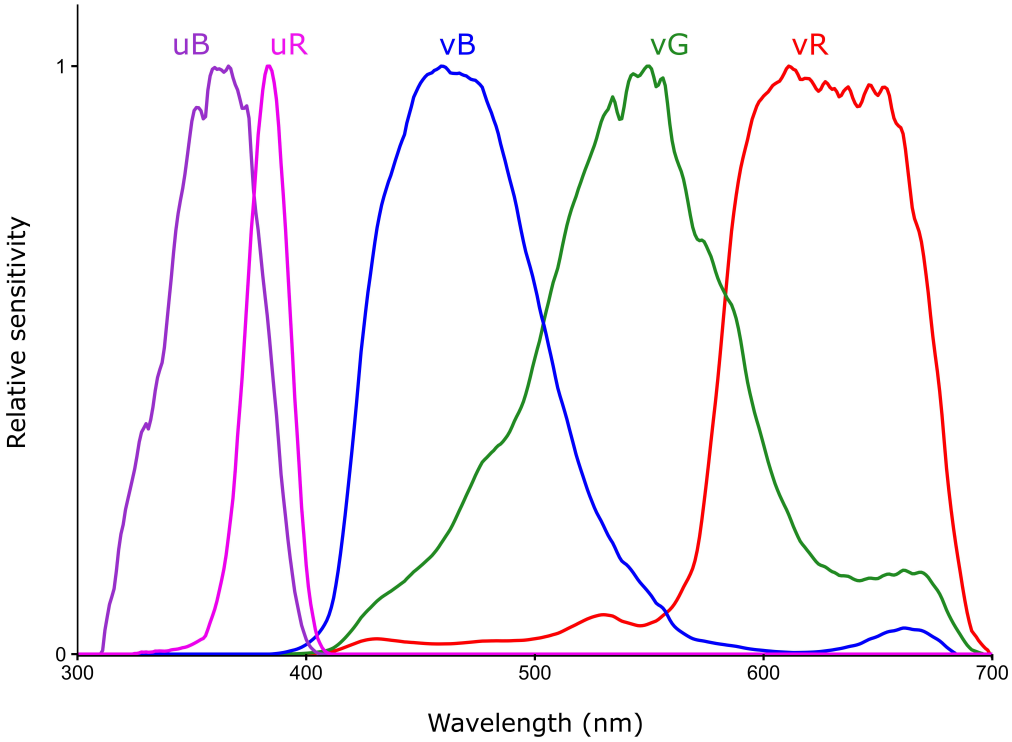


Figure 2.1: Standardised spectral sensitivities of the camera set-up, for wavelengths of light between 300 and 700 nm.

2.3.3 Quantifying colour and lightness, as perceived by relevant signal receivers

Once the images have been converted to cone catch values, useful measurements can be taken from areas of interest in the photographs. I also scaled the images using the scale bar in each photograph; although the camera is held at a fixed distance from the specimens, any slight differences in the height of the wings would otherwise affect the accuracy of measurements of wing length and area. For experiments with Zygaenidae, the images were scaled at 100 pixels/mm. On each image, I selected wing markings and background areas using the freehand tool in ImageJ (Figure 2.2), following a specific protocol for the zygaenid wings used in the experiments described here and in Chapters 4, 5 and 6. Each forewing spot was precisely outlined to allow for accurate measurements of area, and if the spot was damaged, separate measurements of undamaged sections were taken for spot colour. For the background and hindwing regions, areas as large as possible were selected, while avoiding any rubbed scales. The cone catch values for every photoreceptor type were measured, then averaged over the selected areas to

obtain a single set of values for the marking and background colours on each wing. These numbers are the raw data, from which a series of more easily interpretable metrics was subsequently calculated for each wing area.

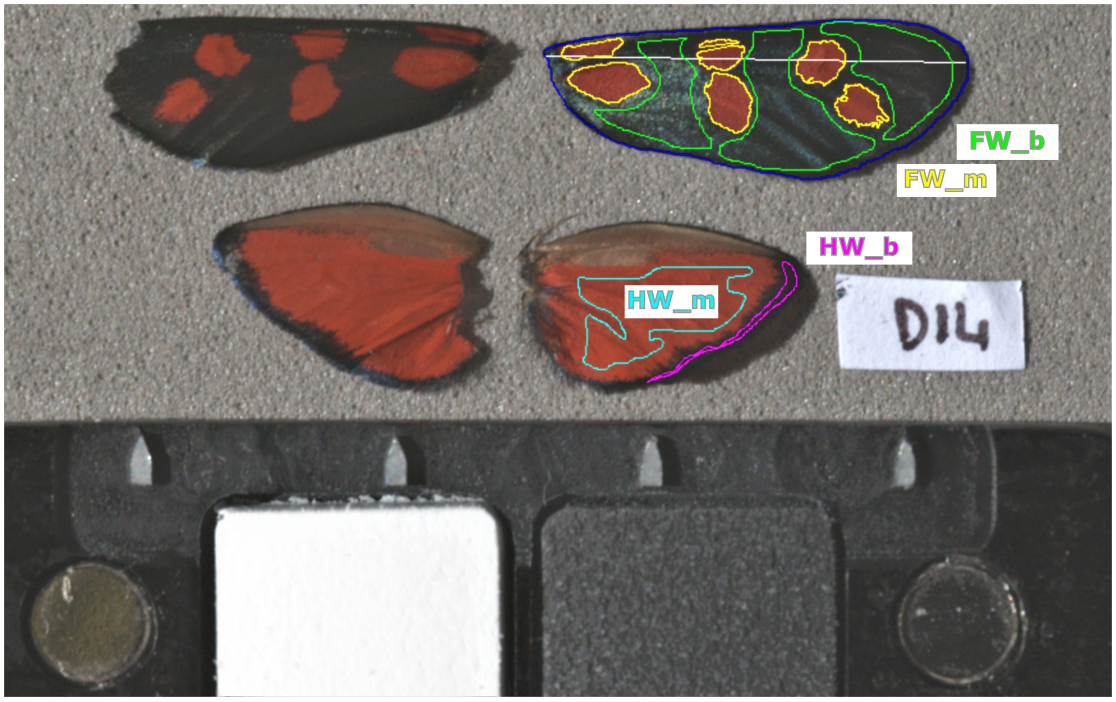


Figure 2.2: Example wing photograph, showing patch selection, scale bar (30mm) and reflectance standards. FW_b = forewing background, FW_m = forewing markings, HW_b = hindwing background, HW_m = hindwing markings.

Achromatic, or lightness, information is perceived and processed in different ways across animal taxa. While humans and primates with similar vision gain achromatic information by combining the input to their long wavelength and medium wavelength photoreceptors (Osorio and Vorobyev, 2005), and bees use signals from their long wavelength photoreceptors (Giurfa *et al.*, 1997), other species, such as flies and birds, use separate photoreceptors to code achromatic and chromatic information (Osorio and Vorobyev, 2005). In birds, experiments on chicks (*Gallus gallus*) detecting pattern differences (Osorio, Mikló and Gonda, 1999; Jones and Osorio, 2004) and on motion detection in pigeons (Campenhausen and Kirschfeld, 1998), suggest that special types of photoreceptors, known as double cones (λ_{max}=565nm) are responsible for detecting lightness cues (Jones and Osorio, 2004; Osorio and Vorobyev, 2005,

2008). I therefore used luminance values equal to the cone catch values for the double cones as an avian visual system-dependent measure of lightness.

To describe the colour of wing markings in a meaningful way, I used two additional metrics: saturation, a measure of colour intensity compared to white light (for example, saturation increases from pink to red), and hue, which provides a sense of the shade of the colour (Stevens, Stoddard and Higham, 2009; Stevens, 2011). Saturation (sometimes referred to as chroma, e.g. in Stoddard and Prum, 2008) is determined by plotting each colour of interest in a tetrahedral colour space. First suggested by Burkhardt (1989) and Goldsmith (1990), this approach has more recently been revived by Kelber, Vorobyev and Osorio (2003), Endler and Mielke (2005) and Stoddard and Prum (2008). It provides a standardised way of measuring the colours of a range of different biological objects, including those with complex reflectance spectra, such as bird plumage, as perceived by tetrachromatic visual systems (Stevens, Stoddard and Higham, 2009). Following the methods of Goldsmith (1990) and Stoddard and Stevens (2011), cone catch values for each photoreceptor type (UVS/VS, SWS, MWS, LWS) were standardised to the total cone catch value to remove overall differences in brightness, then converted to Cartesian X, Y, Z coordinates to form a tetrahedral colour space (after Endler and Mielke, 2005). The centre of the tetrahedron corresponds to equal stimulation of each photoreceptor type – a grey, black or white colour - and saturation can be measured as the Euclidean distance between this central point and the colour of interest (a value between 0 and 0.75). This method of calculating saturation has been used in several recent studies of animal coloration (e.g. Stevens, Lown and Wood, 2014a,b; Winters *et al.*, 2014; Arenas, Walter and Stevens, 2015).

Hue can also be assessed using the tetrahedral colour space, by converting Cartesian to polar coordinates to define colour vectors (Endler and Mielke, 2005; Stoddard and Prum, 2008; Stevens, Stoddard and Higham, 2009), but these can be difficult to analyse and especially to interpret (Stevens, 2011). An alternative method, used here and throughout this thesis, is to estimate hue values based on the principal of colour opponent channels. Colour opponency is crucial to neural processing of colour in animals, with colour vision depending

on the comparison of inputs to photoreceptor types with different but overlapping sensitivities. The blue-yellow and red-green opponent channels in humans and trichromatic primates are relatively well-understood, so can be used to calculate physiologically relevant measures of hue (Lovell *et al.*, 2005; Stevens, Stoddard and Higham, 2009). While opponent processing mechanisms are also important in other species, including birds and their close relatives, turtles, the exact channels are not clearly known (Ammermüller, Muller and Kolb, 1995; Osorio, Vorobyev and Jones, 1999; Twig and Perlman, 2004). In the absence of this information, ratios representing hue in the form of opponent-style colour channels can be designed based on *a priori* expectations about the principal direction of variation in colour. These colour channels are not intended to mimic actual opponent channels but rather to describe colours in an intuitive and biologically relevant manner. Komdeur *et al.* (2005) pioneered this approach in a study of plumage colour in European starlings, *Sturnus vulgaris*. Predicting that females would prefer males with more purple feathers (reflecting more strongly in the red and blue parts of the visual spectrum), they calculated a measure of hue that would explicitly test this idea:

$$Hue = \frac{LW+SW}{MW+UV} \quad (2.1)$$

Hue values can also be determined by using principal component analysis (PCA) to reveal the main axes of variation in colour in the samples of interest, a method developed to quantify egg colour among the hosts of the African cuckoo finch, *Anomalospiza imberbis* (Spottiswoode and Stevens, 2011; Stevens, 2011) and since used to investigate both camouflage and warning colours (e.g., Stevens, Lown and Wood, 2014a,b; Winters *et al.*, 2014; Arenas and Stevens, 2017). Applying the methods of Spottiswoode and Stevens (2011) to my measures of wing colour, I performed PCA on the standardised cone catch values for UV/V-, SW-, MW- and LW-sensitive photoreceptors, and used the first two components, which cumulatively explain over 99% of variance in the data, to define two hue channels, ratios of the standardised cone catch values (see specific equations for Experiment 2 below). This process was always run on a single signal type (e.g. forewing markings, hindwing markings, forewing background areas), to ensure that the hue channels were representative of variation between similar colours.

Luminance, saturation and hue do not have any specific units of measurement, so no units are included on figures representing these metrics throughout the thesis. Luminance values correspond to standardised cone catch values for the double cones (a proportion of these cone catch values to total reflectance), so are positive values, between 0 and 1. Saturation is represented by the distance between the origin and a point plotted in a tetrahedral colour space (Stoddard and Stevens, 2011), so is given by positive values between 0 and 0.75. Hue values are based on a ratio of standardised cone catch values, and are always positive.

2.3.4 Calculating visual contrasts

The colour of each part of a warning signal pattern may not necessarily be that informative *per se*, compared to the perceived difference, or contrast, between the signal and the natural background against which it is seen (Arenas, Troscianko and Stevens, 2014; Arenas, Walter and Stevens, 2015). Equally, contrast between different components of the colour pattern may also play an important role in determining the salience and effectiveness of aposematic signals (Aronsson and Gamberale-Stille, 2012b; Stevens and Ruxton, 2012; Barnett, Scott-Samuel and Cuthill, 2016). To calculate contrasts between colours measured from photographs, I used the Vorobyev-Osorio receptor noise-limited model (Vorobyev and Osorio, 1998), which estimates the discriminability of two colours for a specific visual system. In this model, the visual contrast between two stimuli is determined by unspecified colour opponent mechanisms and is primarily limited by the amount of noise in each photoreceptor channel. It assumes that, for n photoreceptor types, there are n-1 opponent channels, and that, for each one, discriminability depends on the difference in the cone catch values of the photoreceptors and on an estimate of noise in this channel. The standard Vorobyev-Osorio model ignores achromatic information, providing only a measure of how different two colours appear in a chromatic sense; for a tetrachromatic avian visual system, it makes use of the cone catch values for all the single cones (LWS, MWS, SWS and UVS/VS). Noise itself is determined by the relative abundance of each cone type in the retina, and a Weber fraction, an estimate of the smallest detectable change in stimulus intensity dependent on the initial magnitude of a stimulus, following Weber's law. In all my calculations, I chose a widely-used and conservative

estimate of the Weber fraction (ω=0.05), deemed conservative as lower values of ω would result in higher contrast values (Stevens, Lown and Wood, 2014a). Cone ratios for the blue tit (UVS=1, SWS=1.92, MWS=2.68, LWS=2.7) and peafowl (VS=1, SWS=1.9, MWS=2.2, LWS=2.1) visual systems were taken from Hart *et al.* (2000) and Hart (2001b) respectively.

The Vorobyev-Osorio model yields contrast values measured as "just-noticeable differences" (JNDs), whereby colours with JND<1 are not discriminable, those with 1<JND<3 are likely to be only perceptibly different under good lighting conditions, and those with JND>3 should be increasingly easy to tell apart even in poor conditions (Siddiqi *et al.*, 2004). A JND of 1 is typically considered the limit for differentiation between two colour stimuli (e.g. in Stobbe and Schaefer, 2008; Stevens, 2011; Cibulková, Veselý and Fuchs, 2014), and behavioural tests on domestic chickens (*Gallus gallus*) showed that stimuli with JND>1 could easily be discriminated under bright lighting conditions (Olsson, Lind and Kelber, 2015). Yet several studies adopt a more conservative threshold of JND>3 for two colours to be discriminable under most natural conditions (e.g. Nokelainen *et al*., 2012; Hegna *et al*., 2013; Stevens, Lown and Wood, 2014a; Arenas *et al*., 2015), based on the precedent of work by Siddiqi *et al*. (2004). Recent behavioural tests with domestic chickens (Olsson, Lind and Kelber, 2015) provide some support for the idea that stimuli with JND>3 are more likely to be discriminated in all conditions. In their experiments, successful discrimination of stimuli separated by small chromatic differences was reduced with increasingly dim lighting, and the level of light intensity at which discriminability was compromised depended on the magnitude of the difference between the colours. However, from a JND of around 3, increasing differences between stimuli had a reduced impact on the likelihood of correct discrimination under low light conditions.

To quantify the achromatic differences between stimuli, the principle of the Vorobyev-Osorio model can be adapted to measure differences between the double cone catch values, using the same Weber fraction (ω=0.05) to estimate noise (Siddiqi *et al.*, 2004). Throughout this thesis, I used these calculations to determine the chromatic and achromatic contrast between the wing colours of different individuals (see Experiment 1 below), but also to investigate contrasts

between patches in multi-coloured wings and their visibility against different plant types, to provide a measure of signal detectability in natural conditions.

2.3.5 Experiment 1: Individual variation in wing colour in Z. filipendulae

I first carried out this test on a pilot dataset at the very beginning of my research project, but the results presented here are based on the entire collection of six-spot burnets, *Z. filipendulae*, photographed throughout my PhD (N=115). The specimens were collected, as larvae or pupae, in 2015 and 2016, at several locations in France, the United Kingdom and Denmark, by myself and other entomologists (see Appendix 2.1 for details of locations and collectors). Similarly to methods previously used for this species (Zagrobelny *et al.*, 2007a), the insects were housed individually in plastic boxes with air holes, inside an incubator maintained at 20°C, with a 16:8hr day:night cycle, until the emergence of the adults. Larvae were fed *ad libitum* with their natural host plant (*Lotus corniculatus*, *Dorycnium pentaphyllum* or *Hippocrepis comosa*). Chapter 4 provides more information about rearing conditions and diet for these moths, most of which were used for my study of the relationship between colour and toxicity in *Z. filipendulae*. Adults were euthanised in a -80°C freezer immediately after emergence, and stored in these conditions until their wings were dissected and photographed, as described above.

To estimate the variability of wing colours in my full dataset, I calculated pairwise chromatic and luminance contrasts between the colours of all individuals, for each wing area in turn. These contrasts appeared highly skewed, so were transformed using the logit function to satisfy graphical tests of normality. I then compared them to the threshold for discrimination by predators (JND=3) using paired t-tests, for each wing area in turn. All statistical analyses for this experiment and the next were run in R 3.3.1 (R Development Core Team, 2015).

2.3.6 Experiment 2: Colour stability in zygaenid wings over time

To carry out this experiment, I collected a further 20 specimens of *Z. filipendulae*, as pupae and fresh adults, from Holywell Bay (Cornwall, UK; 50° 23’ 22.53’’ N, 5° 40’ 13.56’’ W) in June 2016. The pupae were housed in the

same way as all other moths used in my experiments, and freshly-emerged adults were euthanised in a -80°C freezer. The right forewing of each moth was dissected, then photographed using the same equipment and protocol as described above, at several time points, corresponding to delays experienced by samples in my main experiments (see Chapters 3 and 4): 1 hour, 24 hours, 72 hours, 1 week, 2 weeks, 4 weeks and 8 weeks post emergence and freezing. Six individuals were not photographed at the 2-week point, yielding a final total of 134 images. The wings were held in a -80°C freezer throughout the experimental period.

Red forewing patches were selected as described above, and the same image analysis techniques were used to determine luminance, saturation and hue values for every wing at each time point. I chose to analyse the effect of time on these colour metrics, as they form the basis for most of my analyses in subsequent chapters. For this particular experiment, only the UVS visual system was used. Hue calculations, based on measurements of the red markings for this dataset alone, yielded the following equations:

$$Hue1 = \frac{SW+LW}{UV+MW} \quad (2.2)$$

$$Hue2 = \frac{SW+MW}{UV+LW} \quad (2.3)$$

UV, SW, MW, LW = standardised cone catch values for the UV-, SW-, MW- and LW- sensitive photoreceptors respectively.

Analysis was restricted to the values of Hue1 (hereafter referred to as hue), as it accounted for over 91% of the variance in colour. To meet the assumptions of linear models, saturation and hue values were transformed using the logit function in the ‘car’ package in R (Fox and Weisberg, 2011). I then analysed these results using mixed models, with the ‘lme4’ package (Bates *et al.*, 2014), including time as a fixed effect and individual ID as a random effect in the following general formula: $Colour\ metric \sim Time + 1|ID$. To improve model diagnostics, the results reported below are based on analyses excluding one and five outlier values for saturation and hue respectively, although analyses with the full dataset yielded qualitatively similar results.

2.4 Results

2.4.1 Experiment 1: Individual variation in wing colour in Z. filipendulae

While *Z. filipendulae* typically does not appear particularly variable in colour to human observers, there were significant individual differences in coloration, which were discernible by both types of avian predators. Across my entire dataset, for all wing areas, pairwise comparisons between individuals produced luminance and chromatic contrasts with JNDs greater than 3 (Table 2.1; Figure 2.3). Chromatic contrasts were especially high: all areas except the hindwing border were overall perceptibly different between specimens, while achromatic differences were detectable only between the black areas of both wings (Table 2.1).

Table 2.1: Summary of individual differences in colour, for each wing area and visual system. UVS = ultraviolet-sensitive system, VS = violet-sensitive system; FW_m = red forewing markings, FW_b = black forewing background, HW_m = red hindwing area, HW_b = black hindwing border.

Contrast	Visual system	Wing area	Raw jnds			Logit(jnd)		T-test result: H_1 =logit(jnd)>logit(3)
			Min	*Max*	*Median*	*Mean*	*Stdev*	
Chromatic	***UVS***	FW_m	0.0743	33.520	4.125	-3.136	0.923	t=42.205, df=13109, p<0.001
		FW_b	0.143	20.680	4.113	-3.223	0.743	t=39.055, df=13109, p<0.001
		HW_m	0.104	51.840	4.114	-3.100	0.906	t=47.509, df=13109, p<0.001
		HW_b	0.0142	40.790	2.114	-3.776	0.960	t=-35.773, df=13109, p=1
	VS	FW_m	0.181	66.180	6.852	-2.676	0.950	t=90.619, df=11573, p<0.001
		FW_b	0.1007	19.240	3.253	-2.393	0.786	t=0.393, df=13109, p=0.3473
		HW_m	0.1393	86.850	7.929	-3.473	1.120	t=103.09, df=11363, p<0.001
		HW_b	0.0424	48.270	2.082	-3.782	1.057	t=-33.145, df=13109, p=1
Luminance	***UVS***	FW_m	0.00036	11.250	1.653	-4.319	1.123	t=-85.923, df=13109, p=1
		FW_b	0.00194	39.860	5.797	-2.962	1.231	t=47.793, df=13109, p<0.001
		HW_m	0.000023	8.666	1.458	-4.453	1.134	t=-98.651, df=13109, p=1
		HW_b	0.00064	47.640	4.695	-3.176	1.360	t=25.261, df=13109, p<0.001
	VS	FW_m	0.000492	11.690	1.895	-4.178	1.156	t=-69.467, df=13109, p=1
		FW_b	0.000169	39.820	5.836	-2.956	1.231	t=48.323, df=13109, p<0.001
		HW_m	0.000746	9.918	1.687	-4.305	1.171	t=-81.064, df=13109, p=1
		HW_b	0.00152	49.120	4.747	-3.167	1.368	t=25.834, df=13109, p<0.001

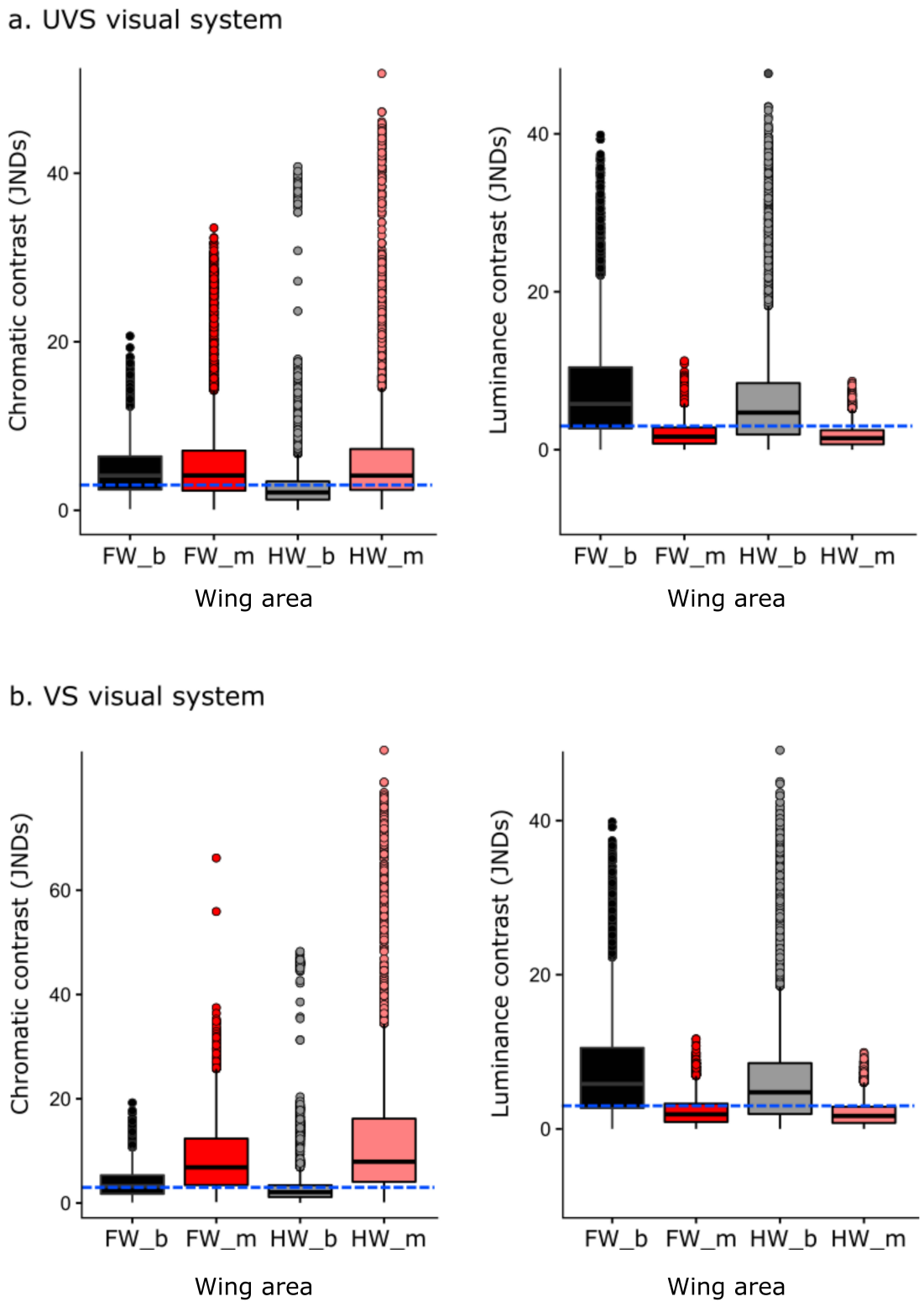


Figure 2.3: Chromatic and luminance contrasts between all individuals, for the UVS (a) and VS (b) visual systems. The dashed blue line indicates the threshold for discrimination between specimens (JND=3). UVS = ultraviolet-sensitive system, VS = violet-sensitive system; FW_m = red forewing markings, FW_b = black forewing background, HW_m = red hindwing area, HW_b = black hindwing border. Boxplots indicate the medians and interquartile ranges.

2.4.2 Experiment 2: Colour stability in zygaenid wings over time

There was no significant change in the luminance, saturation or hue of the red forewing markings over time (linear mixed-effects models, LME; $\chi^2(1)=0.0899$, $p=0.764$; $\chi^2(1)=2.290$, $p=0.130$; $\chi^2(1)=2.115$, $p=0.146$ respectively; Figure 2.4). This suggests that, despite fluctuations in my results, the colours of the wings were not consistently altered by the time spent in a -80°C freezer.

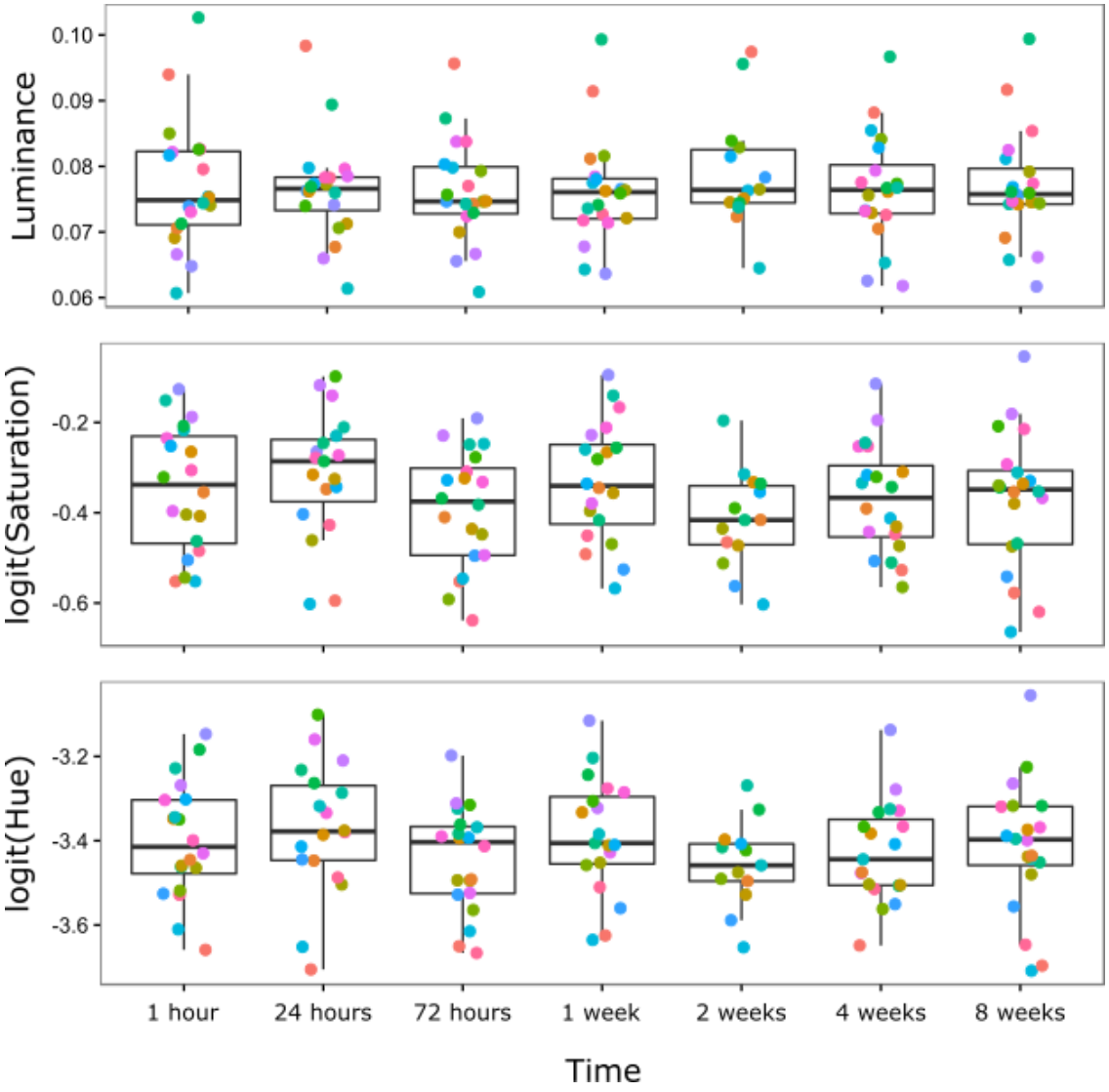


Figure 2.4: Luminance, saturation and hue values for the red markings of wings in Experiment 2. Boxplots provide a sense of the median and spread of the data across time post-termination, while each colour point represents an individual wing.

2.5 Discussion

The results of these preliminary experiments suggested that *Z. filipendulae* was amenable to my research methods and questions. Firstly, the wing colours of *Z. filipendulae* are more variable to avian visual systems than they appear to human observers. In particular, the red wing markings, which are the main focus of my research in following chapters, are variable in chromatic terms; meanwhile, there are greater lightness differences between the dark scales of individual moths. The spectral sensitivities of avian photoreceptors, including the medium wavelength-sensitive (MWS) and long wavelength-sensitive (LWS) photoreceptors have less overlap than the human MWS and LWS photoreceptors, an effect exacerbated by the presence of oil droplets filtering the light absorbed by each photoreceptor type in the avian retina. As a result, even stimuli reflecting only in the human-visible spectrum will appear different to human and avian vision (Bennett, Cuthill and Norris, 1994) and the discriminability of colours, for example in bird plumage, will be higher in birds than humans (Vorobyev *et al.*, 1998). Birds can thus perceive differences in the colours of zygaenid wings which are not apparent to human observers, much as many sexually dichromatic bird species, from an avian perspective, can be incorrectly classified as monomorphic by humans (Eaton, 2005). From a more practical perspective, the time spent in a -80°C freezer by *Z. filipendulae* samples between termination and photography, within the range experienced by the specimens in my study, did not have a significant effect on the colour of the red wing markings. As all the Zygaeninae rely on the same classes of pigments (melanin and pteridines; Tremewan, 2006), this enabled me to include several species from different localities and with varying emergence times in my analyses (see Chapter 5).

In addition, this chapter explains how the equipment, set-up and image analysis methods I used enable accurate and reliable measurements of wing coloration, from the perspective of potential avian predators. In particular, they address crucial issues that must be considered when using digital photography to study animal coloration, such as sensitivity to UV, controlled conditions for photography, linearisation and normalisation of images, as well as techniques for analysing images while accounting for animal vision. The open-access toolbox used to implement these methods and the accompanying user guide

will facilitate the correct use of digital photographs for the purpose of research on colour and pattern in nature in a wide range of contexts (Troscianko and Stevens, 2015). I used these methods throughout my thesis for measuring the coloration of Lepidopteran wings and host plant samples. They are briefly summarised again in each chapter, and any additional techniques or deviations from the protocols described above are explained in more detail.

Chapter 3

What makes an effective warning signal? A comparative study of British moths

Aposematic Lepidoptera photographed on Penhale sands, Cornwall, UK.
Clockwise from top left – Garden tiger *Arctia caja*, Scarlet tiger *Callimorpha dominula*, Cinnabar *Tyria jacobaeae*, Six-spot burnet *Zygaena filipendulae*. All photographs: E. S. Briolat

3.1 Abstract

Toxic or otherwise unprofitable animals advertise their defences with bright and conspicuous visual displays known as aposematic, or warning, signals. To effectively convey their message to predators and facilitate avoidance learning, warning signals should be highly detectable, easily recognisable and memorable. Brighter, more colourful, more conspicuous patterns, as well as larger signals, are thought to enhance these properties, increasing the overall efficacy of warning signals. I tested these predictions of efficacy theory on the form of warning signals from the perspective of avian predators, with a comparative analysis of museum specimens of British moths. The wing colours of palatable species were compared to those of both diurnal and nocturnal moths known to possess chemical defences, or considered to be aversive to avian predators. Defended moths displayed more saturated and redder colours than palatable moths in some, but not all wing areas. They were also more conspicuous, in terms of chromatic contrast, than profitable moths against general tree bark and herbaceous backgrounds, although not against their specific host plants. The forewing patterns of defended species featured larger conspicuous markings and greater contrast between colours. Overall, chromatic features appeared to be more important than achromatic information in warning signals. Based on this study, high internal chromatic contrast and a greater diversity of colours appear to be key hallmarks of aposematic Lepidoptera.

3.2 Introduction

Avoiding attack from predators is a major driving force behind the diversity of colours and patterns found in the natural world, and different species make use of a range of visual strategies. While many species stand to benefit from being inconspicuous, whether escaping their predators' notice entirely or avoiding recognition as a food source, those which possess toxic chemicals or other forms of defences often adopt a radically different tactic (Ruxton, Sherratt and Speed, 2004). The bright colours and patterns of these unprofitable species are known as warning, or aposematic signals (Poulton, 1890). While they attract the attention of predators, they have evolved to be recognised as associated with an unpleasant defence, and hence serve to warn predators away (Wallace, 1867; Ruxton, Sherratt and Speed, 2004; Stevens and Ruxton, 2012). The form

of warning signals is widely considered to be shaped by two aspects of their function: communicating a strategic 'message' concerning prey defences, and conveying this information in an appropriate and effective way, a concept known as signal efficacy (Guilford and Dawkins, 1991). According to efficacy theory, warning signals should evolve to facilitate predator learning, by maximising three key properties: detectability, discriminability, and memorability (Guilford and Dawkins, 1991).

Conspicuousness, or the high visibility of warning colours against natural backgrounds, is key to the effectiveness of aposematism, stimulating avoidance learning in a number of ways (Speed, 2000; Ruxton, Sherratt and Speed, 2004). Most basically, predators will encounter more noticeable conspicuous prey at a relatively higher frequency than cryptic items, and this will help them to more quickly learn the association between signals and defences (Gittleman, Harvey and Greenwood, 1980; Roper and Redston, 1987). Conspicuous prey may also trigger innate avoidance by predators (Smith, 1975), and would be distinguishable from alternative palatable prey (Sherratt and Beatty, 2003). Finally, being conspicuous may enhance both the initial learning speed of predators and their retention of learned associations over time (Roper and Redston, 1987), by tapping into several psychological mechanisms (Speed, 2000). Yet conspicuousness is not the only property of warning signals. Aposematic displays are often composed of multiple visual features, with several patches of different colours and luminance, which contrast against each other, and are arranged into specific patterns. They are also highly variable, both within and between species, despite the expectation, first proposed by Fritz Müller (1879) that warning signals should converge on similar forms to simplify predator learning. Unpicking the relative contribution of different properties of warning signals to deterring predators from attack, and how variation in certain aspects can arise and be maintained, are still active areas of research (Stevens and Ruxton, 2012).

Numerous laboratory experiments with captive birds have attempted to determine which visual features predators attend to and utilise when learning associations between warning signals and unprofitability (e.g. Gittleman, Harvey and Greenwood, 1980; Schuler and Hesse, 1985; Roper and Redston, 1987;

Roper, 1990; Exnerová *et al.*, 2006; Svádová *et al.*, 2009). These can be difficult to interpret, as features such as luminance, colour and conspicuousness are often tightly linked. The taste and odours of colour dyes and distasteful chemicals may also have confounded the results of early experiments (e.g. Roper and Redston, 1987). Nevertheless, this body of work has yielded several general conclusions. Overall, chromatic information is considered more important than luminance for learning and memory, at least for avian predators (Osorio, Jones and Vorobyev, 1999; Osorio, Vorobyev and Jones, 1999; Stevens and Ruxton, 2012). In particular, experiments testing the responses of birds to stink bug-like prey with manipulated patterns have suggested that colour takes precedence over pattern recognition (Exnerová *et al.*, 2006; Aronsson and Gamberale-Stille, 2008; Svádová *et al.*, 2009; Aronsson and Gamberale-Stille, 2012a). Yet achromatic contrast still plays a role, for example in distinguishing pattern textures (Osorio, Mikló and Gonda, 1999; Jones and Osorio, 2004), provoking initial avoidance of stimuli (Sandre, Stevens and Mappes, 2010), and speeding up learning (Aronsson and Gamberale-Stille, 2012b). In the wild, the effectiveness of different signal components will also depend on the perceptual and psychological characteristics of specific predators, as well as on the environmental variables, and in particular lighting conditions and the background against which the signal is displayed (Endler, 1990, 1993; Rojas, Rautiala and Mappes, 2014). Artificial predation experiments have been used to test the role of visual features such as luminance, colour and pattern in determining predation risk in more ecologically-relevant conditions (e.g.Finkbeiner, Briscoe and Reed, 2014; Arenas, Walter and Stevens, 2015; Flores *et al.*, 2015; Pegram, Han and Rutowski, 2015; Tan, Reid and Elgar, 2016), and these have tended to confirm that, while pattern can have an effect, chromatic information is generally more important (Stevens and Ruxton, 2012).

Beyond establishing the relative importance of chromatic and achromatic cues, several specific signal features have been investigated. Long wavelength colours, such as yellow and red, are prevalent among aposematic animals and are thought to be especially effective due to several characteristics (Stevens and Ruxton, 2012), such as an innate aversion of predators to these colours (Roper, 1990), their high chromatic and achromatic contrasts against natural

backgrounds, and the stability of these contrasts under a range of illuminations (Arenas, Troscianko and Stevens, 2014). In addition, experiments testing predator generalisation suggest that more saturated colours would be more strongly avoided (Gamberale-Stille and Tullberg, 1999). Larger signals, whether due to increased body size or an increase in the size of the signal specifically, have also been demonstrated to enhance avoidance learning in multiple experiments with artificial stimuli as well as natural prey items, such as the larvae of the wood tiger moth, *Arctia plantaginis* (Forsman and Merilaita, 1999; Lindström *et al.*, 1999; Lindstedt, Lindström and Mappes, 2008; Smith, Halpin and Rowe, 2014). Finally, conspicuousness against natural backgrounds is important, as several field experiments with artificial models of aposematic frogs and ladybirds found that conspicuousness was more influential than prey pattern in determining the risk of predation (e.g. Hegna *et al.*, 2011; Arenas, Walter and Stevens, 2015).

While the presence of particular visual features in many aposematic species does not provide evidence of their role in deterring predators from attacking, it does suggest which components of warning signals are likely to be important. For this chapter, I investigated the presence of key signal features in defended and undefended British moths, using a comparative analysis based on museum specimens. With only a few exceptions, defended moths in the UK fall into two families: the burnet moths (Zygaenidae) and the tiger and footmen moths (Erebidae: Arctiinae and Erebidae: Lithosiinae). The chemical defences of these families have been studied extensively, but the depth of our knowledge varies substantially, especially when considering species found in the UK. Burnet moths also possess bitter-tasting cyanogenic glucosides, which release cyanide when hydrolysed, and which they both sequester from larval host plants and synthesise *de novo* (Davis and Nahrstedt, 1982; Nahrstedt, 1993; Zagrobelny, Bak and Møller, 2008). Their specific defensive compounds, linamarin and lotaustralin, have been identified, can be accurately quantified, and the genetic pathways responsible for their synthesis and breakdown have been elucidated in the six-spot burnet, *Zygaena filipendulae* (Jensen *et al.*, 2011). However, the strength of chemical defences in the less brightly-coloured forester moths (Zygaenidae: Procridinae) is less well known. Tiger moths possess multiple chemical defences, primarily pyrrolizidine alkaloids, but also iridoid glucosides,

cardenolides and polyphenolics among others (Conner and Weller, 2004). Despite earlier interest (e.g. Bisset *et al.*, 1960; Aplin, Benn and Rothschild, 1968; Rothschild *et al.*, 1979), recent research efforts have principally focused on North American species, such as *Utetheisa ornatrix*, and moths studied for their acoustic aposematism directed towards bats (e.g. Hristov and Conner, 2005). As such, the defences of many of the less colourful members of this family found in the UK have not been precisely identified, and their effects on predators are relatively untested.

Similarly to determining unprofitability, questions of efficacy in visual signals are best addressed from the perspective of the receivers to whom the signals are directed, which in the case of warning signals are potential predators (Stevens, 2007). As birds are the principal visual predators of Lepidoptera, I analysed the colours of palatable and unpalatable moths using the two recognised types of avian visual system, the ultraviolet-sensitive and violet-sensitive systems (Hart and Hunt, 2007). Although I used museum specimens, rather than photographing animals in their environment, I did assess the conspicuousness of the specimens against potential natural backgrounds, by taking photographs of potential host plants. In this study, I explicitly tested a number of key predictions of efficacy theory, supported by previous work on warning signals and by observations of aposematic Lepidoptera from a human perspective. I expected defended species to present more saturated, redder colours and larger, more contrasting patterns, and to be more conspicuous against natural backgrounds than palatable species, which are expected to adopt crypsis as their principal anti-predator strategy. Moreover, the diurnal or nocturnal activity patterns of defended species were expected to affect their warning signals. An earlier comparative study of warning coloration in Lepidoptera suggested that warning coloration was more likely to evolve in combination with diurnal activity, as movement attracts attention, making it more difficult for diurnal species than for resting nocturnal moths to be cryptic to predators (Merilaita and Tullberg, 2005). Differences between the fore- and hindwings of these species are expected, as only the forewings of most nocturnal species will be visible at rest during the daytime. Nocturnal prey may also be expected to display lighter colours, more conspicuous in dim lighting conditions. A number of bat species have been shown to use vision to complement echolocation, in particular when

hunting for insects in cluttered environments (Svensson and Rydell, 2002; Eklöf and Jones, 2003; Rydell and Eklöf, 2003), so pale coloration could additionally act as warning signals for pteropine predators, if the moths are similarly unpalatable to them.

3.3 Methods

3.3.1 Study species

I chose UK moth species for this study on the basis of evidence of palatability or toxicity, information on their activity patterns as adults, and the existence of DNA sequences in the BOLD Systems Database for the DNA Barcode of Life (Ratnasingham and Hebert, 2007). I chose to compare three categories of moths: diurnal defended species (N=18), nocturnal defended species (N=17), and palatable species (which are also nocturnal, N=19). These groups were chosen in order to test the differences in coloration between defended and unprotected palatable species (which should respectively be avoided or preferentially-selected by predators), as well as the impact of diurnal versus nocturnal activity on the visual signals of defended species. According to aposematism theory (see section 3.2, Introduction), defended species whose predators rely on vision (such as birds) are predicted to display conspicuous and colourful markings, with saturated, longwave colours and large markings, while palatable species should employ camouflage to avoid being detected. Day-flying defended moths should be more exposed to visual predators than their nocturnal counterparts, so are expected to possess more conspicuous markings, including on their hindwings, which are usually hidden when at rest. Insufficient palatable day-flying moths with BOLD DNA sequences were found to include a palatable and diurnal category as well. Two of the defended species (*Diaphora mendica* and *Diacrisia sannio*) are sexually dimorphic and have different activity patterns, so fall into both the diurnal and nocturnal categories. In addition, one palatable species has two distinct morphs (*Biston betularia*), so a total of 55 wing patterns were included in the analysis (Figure 3.1). Classification as palatable or defended was based on an extensive literature search for records of acceptability to avian predators, presence in the diet of predator species and experimental evidence of the presence or absence of chemical defences in the literature (Appendix 3.1). I photographed 15

specimens of each chosen species or morph, from the collections of Bristol City Museum and Art Gallery, the City of Plymouth Museum and Art Gallery, in April and May 2014 and the Natural History Museum, London in October 2014 (see Appendix 3.2 for details of their provenance). Upon visual inspection, the freshest and least damaged specimens available were chosen to be photographed. I also photographed freshly-emerged specimens of four species, to validate the results based on museum specimens (see Appendix 3.3 for this analysis).

3.3.2 Photographic set-up

Photographs were taken with the same equipment and using the same techniques as described in Chapter 2, although the images were taken in museum storerooms rather than in a darkroom. The set-up was consistently illuminated with an EYE Colour Arc lamp (MT70D, Iwasaki Electric Co. Ltd.), together with a photographic umbrella to diffuse the light. Photographs were taken with a Nikon D7000 camera fitted with a Jenoptic 105 mm quartz lens, transmitting light between wavelengths of 300 and 750 nm. Images were captured in RAW format with a manually set white balance and a constant aperture (f8). Each specimen was photographed twice, using different filters: a UV/infrared (IR) blocking filter, transmitting between 300 and 700 nm, for the human-visible photographs (Baader UV/IR Cut Filter) and a UV pass and IR blocking filter (Baader U filter), transmitting between 300 and 400 nm, for the UV photographs. This yielded a set of five image layers, corresponding to different parts of the visual spectrum: long wavelength (or red, vR), medium wavelength (or green, vG), short wavelength (or blue, vB) and ultraviolet (uB and uR; see Chapter 2). Where possible, a Spectralon grey reflectance standard (Labsphere, Congleton, UK), reflecting 40% of the light equally at all wavelengths between 300 and 700 nm, was included in the photographs. For some moths tightly packed together in museum drawers, a smaller standard was used; this reflected 50% of the light at all wavelengths. Alternatively, the Spectralon standard was photographed at the start of each photography session during which the lighting conditions remained constant. Each image also included a small ruler to provide scale.

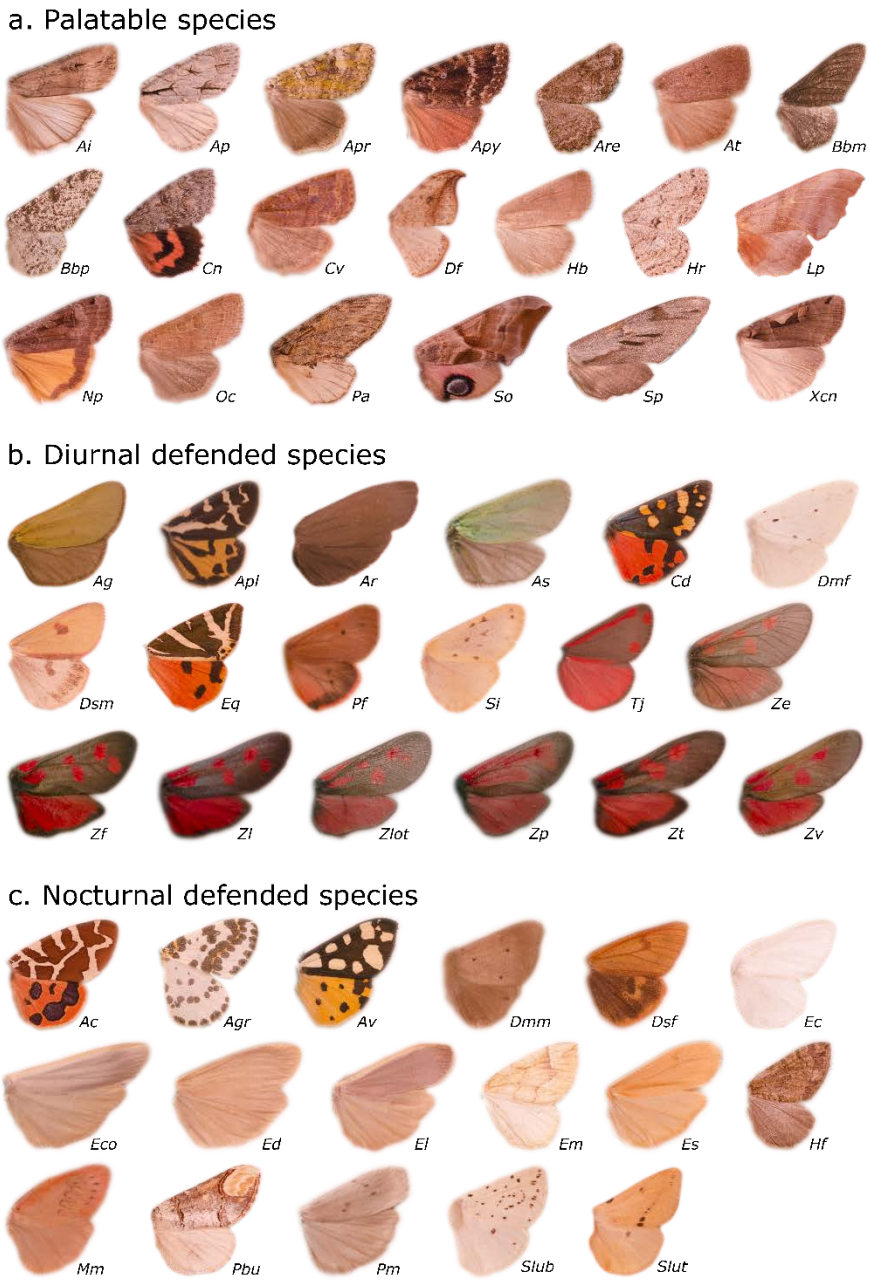


Figure 3.1: Wing patterns of the species included in the comparative analysis. Ai = *Agrotis ipsilon*; Ap = *Acronicta psi*; Apr = *Anaplectoides prasina*; Apy = *Amphipyra pyramidea*; Are = *Alcis repandata*; At = *Amphipyra tragopoginis*; Bbm = *Biston betularia* (melanic form); Bbp = *Biston betularia* (pale form); Cn = *Catocala nupta*; Cv = *Conistra vaccinii*; Df = *Drepana falcataria*; Hb = *Hoplodrina blanda*; Hr = *Hypomecis roboraria*; Lp = *Laothoe populi*; Np = *Noctua pronuba*; Oc = *Orthosia cerasi*; Pa = *Peridea anceps*; So = *Smerinthus ocellata*; Sp = *Sphinx pinastri*; Xcn = *Xestia c-nigrum*; Ag = *Adscita geryon*; Apl = *Arctia plantaginis*; As = *Adscita statices*; Cd = *Callimorpha dominula*; Dmf = *Diaphora mendica* (female); Dsm = *Diacrisia sannio* (male); Eq = *Euplagia*

quadripunctaria; Pf = *Phragmatobia fuliginosa*; Si = *Setina irrorella*; Tj = *Tyria jacobaeae*; Ze = *Zygaena exulans*; Zf = *Zygaena filipendulae*; Zl = *Zygaena lonicerae*; Zlot = *Zygaena loti*; Zp = *Zygaena purpuralis*; Zt = *Zygaena trifolii*; Zv = *Zygaena viciae*; Ac = *Arctia caja*; Agr = *Abraxas grossulariata*; Av = *Arctia villica*; Dmm = *Diaphora mendica* (male); Dsf = *Diacrisia sannio* (female); Ec = *Euproctis chrysorrhoea*; Eco = *Eilema complana*; Ed = *Eilema depressa*; El = *Eilema lurideola*; Em = *Eulithis mellinata*; Es = *Eilema sororcula*; Hf = *Hydriomena furcata*; Mm = *Miltochrista miniata*; Pbu = *Phalera bucephala*; Pm = *Pelosia muscerda*; Slub = *Spilosoma lubricipeda*; Slut = *Spilosoma lutea*

3.3.3 Image analysis

All images were analysed using the Multispectral Imaging Toolbox described in Chapter 2, and implemented in ImageJ (Troscianko and Stevens, 2015). Images were linearised and normalised (Stevens *et al.*, 2007), then mapped to the two recognised types of avian visual system (Stevens *et al.*, 2007; Pike, 2011; Troscianko and Stevens, 2015), ultraviolet-sensitive (UVS) and violet-sensitive (VS), using data on the spectral sensitivities of their respective model systems, the blue tit *Cyanistes caeruleus* (Hart *et al.*, 2000) and the Indian peafowl *Pavo cristatus* (Hart, 2002).This means that cone catch values can be measured for each photoreceptor type (ultraviolet/violet-sensitive [UV/V], short wavelength-sensitive [SW], medium wavelength-sensitive [MW], long wavelength-sensitive [LW] and the double cones) in both visual systems. I analysed one forewing and one hindwing per individual; left or right wings were chosen at random, unless one side was damaged. For each specimen, two types of coloured patches were isolated for analysis on both fore- and hindwings, corresponding to the background and main marking colours. As the colours and patterns of the species studied are highly variable, I drew up the following rules to achieve consistency in measurements across species. Background and main marking colours were chosen according to human perception alone. When the wing appeared uniform in colour, the entire wing was isolated as a single patch. In contrast, if the background colour was interspersed with markings of a different colour, five randomly distributed circular patches of the background were measured and their values averaged. The size of these patches varied with the wing size of the species and individual

moth, but was always selected to be as large as possible, without touching any other patches or markings of a different colour. To analyse the colour of the markings, up to five of the largest patches, depending on the type of pattern, were selected and their value averaged. For example, for a species with pale wings and many dark markings (e.g. *Spilosoma lubricipeda* forewings), five patches of the pale background, as large as possible without including any of the dark spots, were averaged for the background colour measurement, while the five largest markings were selected and their values averaged for the main marking colour. For species with fewer than five markings of the same type, all the markings were selected and averaged to obtain the main marking colour; for those with a single patch or border of contrasting colour, the largest possible area on this patch was selected to be measured as the main marking colour. On mottled wings, in which areas of different colour are hard to define, the background colour was obtained by averaging five circular patches distributed around the wing, excluding distinctive markings. These distinctive markings correspond to any markings that stand out against the overall mottled background, as I perceived when inspecting the wings. The most prominent (i.e. most conspicuous to my eyes) of these distinctive marking types was selected as the main marking colour. Wing veins were avoided when these appeared to be a different colour from the wing scales, for example in moths with dark wing veins on a paler wing. See Appendix 3.4 for details of the zones chosen for analysis in each species.

3.3.4 Colour metrics

Once coloured areas had been selected, I calculated a number of colour metrics and compared these patches to several other colours, using methods described in detail in Chapter 2. Figure 3.2 provides a summary of the analyses performed in this study. In brief, I computed a measure of the perceived lightness of each colour patch, or luminance, and two measures of coloration: saturation and hue. The cone catch value for the double cones was used as a measure of luminance, as these photoreceptors are thought to be responsible for achromatic vision in birds (Jones and Osorio, 2004; Osorio and Vorobyev, 2005, 2008). To calculate saturation, cone catches were plotted in a tetrahedral colour space (Endler and Mielke, 2005), and saturation was taken as the Euclidean distance between the centre of the space and each colour, following

the methods of Stoddard and Prum (2008). Hue values were computed as two ratios of cone catches, which provide a sense of the main directions of variation in colour among samples. The equations for these cone catch ratios are derived by applying principal component analysis (PCA) on the standardised cone catch values for UV/V-, SW-, MW- and LW-sensitive photoreceptors, following the methods of Spottiswoode and Stevens (2011), as described in detail in Chapter 2. PCA was run on the standardised cone catch values for each type of colour (forewing background, forewing markings, hindwing background and hindwing markings) and each visual system separately. The equations obtained are as follows:

$$Hue1_{UVS}(FW\ background) = Hue1_{VS}(FW\ background) = \frac{MW+LW}{UV+SW} \quad (3.1)$$

$$Hue1_{UVS}(FW\ markings) = Hue1_{VS}(FW\ markings) = \frac{LW}{UV+SW+MW/3} \quad (3.2)$$

$$Hue1_{UVS}(HW\ background) = Hue1_{VS}(HW\ background) = \frac{LW}{UV+SW+MW/3} \quad (3.3)$$

$$Hue1_{UVS}(HW\ markings) = \frac{MW+LW}{UV+SW} \quad (3.4)$$

$$Hue1_{VS}(HW\ markings) = \frac{LW}{UV+SW+MW/3} \quad (3.5)$$

UV, SW, MW, LW = standardised cone catch values for the UV-, SW-, MW- and LW- sensitive photoreceptors respectively. UVS = ultraviolet-sensitive (blue tit) visual system, VS = violet-sensitive (peafowl) visual system. FW = Forewings, HW = Hindwings.

Hue1 accounted for at least 79% of the variance in colour in each colour patch/visual system combination, so subsequent analyses focus solely on Hue1 values (hereafter referred to as hue). Overall, higher hue values represent a relatively higher proportion of reflectance in longer wavelengths, indicating redder colours.

3.3.5 Internal contrast and diversity in coloration

In addition, I calculated the internal chromatic and luminance contrast of the moths' forewings and hindwings, by comparing the background and marking colours of each wing. Chromatic contrast was determined with a widely-used

log version of the receptor noise-limited Vorobyev-Osorio colour discrimination model (Vorobyev and Osorio, 1998), which takes into account the sensitivity and abundance of each cone type, and noise in the photoreceptors (see Chapter 2 for further details). Relative cone abundance values for the blue tit (UV=1, SW=1.92 MW=2.68, LW=2.7, Hart *et al.*, 2000) and peafowl (V=1, SW=1.9, MW=2.2, LW=2.1 Hart, 2001b; Håstad, Victorsson and Ödeen, 2005) were used for the UVS and VS visual sytems respectively. Noise was calculated with a relatively conservative estimate of the Weber fraction, $\omega = 0.05$, for the most abundant cone type (Eaton, 2005; Håstad, Victorsson and Ödeen, 2005; Stevens, 2011; Stevens, Lown and Wood, 2014). Luminance contrast was computed as the natural logarithm of the ratio between mean double cone catch values of background and marking areas, divided by the same Weber fraction (Siddiqi *et al.*, 2004). Both chromatic and luminance contrast are measured in "just-noticeable differences" or JNDs: values between 1 and 3 indicate that colours are likely to be distinguishable only under good lighting conditions, while those below this threshold are likely to be indiscriminable, and those above 3 should be increasingly easy to tell apart (Siddiqi *et al.*, 2004).

To provide another measure of the diversity of colours found in each species and in each category of species (Palatable, Defended diurnal, Defended nocturnal), I calculated the volume occupied by these colours in a tetrahedral avian colour space (Stoddard and Prum, 2008). Volumes were calculated and plotted using the 'pavo' package in R (Maia *et al.*, 2013) and included both forewing and hindwing colours.

3.3.6 Comparisons to natural backgrounds

To assess their conspicuousness in nature, I also compared the colour of the moths' wings to those of the leaves and bark (where appropriate) of known host plants for each species, based on records from the Natural History Museum's HOSTS database (Robinson *et al.*, 2010). Appendix 3.6 provides a full list of the species used. Photographs of host plants were taken with the same camera equipment as the moths and processed using the same methods. All plants were photographed in Cornwall, UK, using plants found in and around the University of Exeter's Penryn Campus (Penryn, TR10 9FE) and the Eden Project (Bodelva, PL24 2SG). Tree trunks were photographed *in situ*, with a pair

of reflectance standards (reflecting 7 and 93% of all wavelengths, cut from Zenith Lite Diffuse Target sheets [SphereOptics, Pro-Lite Technology, Cranfield, UK]) strapped to the trunk, and a photographic umbrella to ensure uniform illumination in the photographs. Leaves were picked and photographed where possible in a dark room, illuminated by the same EYE Colour Arc lamp as used to photograph the moths, or in the field under a photographic umbrella. Five independent samples of each type of background (leaf or trunk) for each plant species were photographed, and the images were converted to avian vision. For analysis, an area of each leaf or trunk as large as possible was selected using the freehand tool in Image J, taking care to avoid any shiny patches on the leaves. The cone catch values from each of these selections were averaged, to obtain a single measure of colour per plant item. For the footmen moths (Lithosiinae), lichens were selected from photographs of tree trunks from six species (Willow, *Salix sp*.; Apple, *Malus sp*.; Beech, *Fagus sylvatica*; Alder, *Alnus glutinosa*, Scots Pine, *Pinus sylvestris*; English oak, *Quercus robur*) using the freehand tool in ImageJ. From the values obtained for each plant leaf and trunk, and each lichen, three values were subsequently calculated, and compared to the moth colours. Firstly, the cone catch values for the leaves of each moth's known hostplants (or lichens for the Lithosiinae) were averaged to obtain an average measure of likely host foliage, against which a moth may choose to rest, or a female might be seen laying her eggs. Secondly, a general average of the leaves of all herbaceous plants photographed was calculated. Thirdly, an average of all tree bark images was computed. The conspicuousness of the moths' colours was measured as chromatic and luminance contrast to these three plant colours, in JNDs, as explained above for the internal contrast between wing background and wing marking colours. For each moth species, chromatic and luminance contrast were calculated for both forewing colours, against their specific average host plant background, as well as against the average herbaceous and average tree bark backgrounds. This was designed to provide a sense of the moths' conspicuousness against their own host plants, as well as against two types of common natural background, herbaceous and woody. Only forewing colours were compared to the plant colours, as most species display only their forewings when at rest.

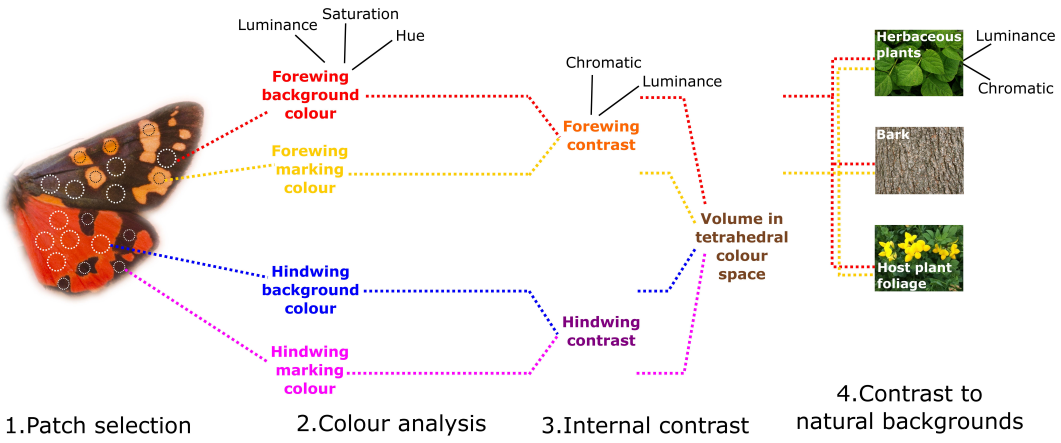


Figure 3.2: Diagram representing the colour metrics and comparisons tested in this study, using the wings of the scarlet tiger (*Callimorpha dominula*) as an example.

3.3.7 Pattern analysis

Wing pattern was analysed with a spatial frequency or "granularity" analysis, a method previously used in studies of cuttlefish body pattern (Barbosa *et al.*, 2008; Chiao *et al.*, 2009) and egg mimicry by brood parasites (Spottiswoode and Stevens, 2010; Stoddard and Stevens, 2010). For each specimen, the image layer corresponding to the avian double cones was selected for spatial frequency analysis. All images were scaled to the same size (41 pixels/mm). Using custom-made programmes in ImageJ, a Fourier transform was applied to each image, and spatial information was broken down into bands of different frequencies (24 bins from 2 to 5000 pixels, increasing on a log scale), corresponding to markings of different sizes. Plotting the energy in each of these frequency bands produces a "granularity spectrum" (Chiao *et al.*, 2009), revealing the extent to which different marking sizes contribute to the overall pattern. From these spectra, three measures of pattern were extracted for each wing: total energy, peak frequency, and proportion energy. Total energy, calculated as the total amplitude of the spectrum, provides a measure of overall contrast levels, with higher values indicative of more contrasting markings. Peak frequency is the frequency at which energy is maximal, and so corresponds to the marking size that is most prominent in the pattern. Finally, proportion energy represents the extent to which this principal marking size dominates the wing pattern.

3.3.8 Phylogenetic reconstruction

To determine the phylogenetic relationships of the species used in this study (Figure 3.3), I downloaded the only genetic data available for all species, partial DNA sequences from the cytochrome c oxidase 5P (COI-5P) gene, from the BOLD Systems Database for the DNA Barcode of Life Project (Appendix 3.7). Sequences from the spindle ermine moth (*Yponomeuta cagnagella*, Hübner 1813) were included to root the tree. To account for the great disparity in the number of sequences available for each species, each taxon's sequences were aligned using MUSCLE (Edgar, 2004) in MEGA 7.0.18 (Kumar, Stecher and Tamura, 2016) and a consensus sequence was generated, using the Ambiguity Consensus Maker tool available at http://www.hiv.lanl.gov/. IUPAC ambiguity codes (Cornish-Bowden, 1985) were used to resolve differences between sequences, although nucleotides present at each position at a frequency of less than 2% were ignored. The 53 consensus sequences were then aligned using the same protocol, and a phylogenetic tree was reconstructed using maximum likelihood methods and automatic model selection by AIC with the Smart Model Selection tool (Lefort, Longueville and Gascuel, 2017) implemented in PhyML 3.0 (Guindon *et al.*, 2010). Bootstrap values were computed for 1000 replicates.

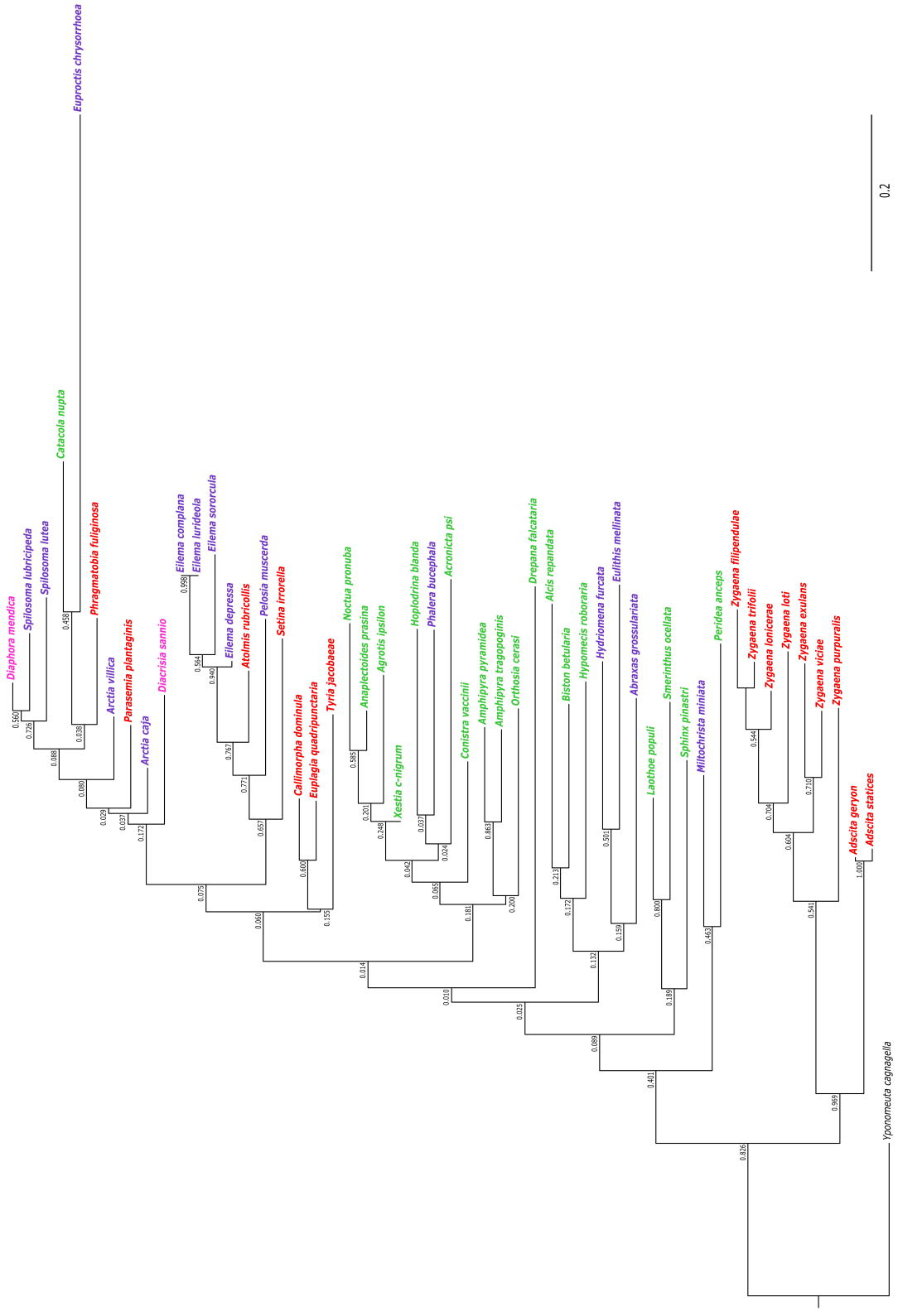


Figure 3.3: Phylogenetic reconstruction of the species used in the comparative analysis. Diurnal defended species are shown in red, nocturnal defended species in purple, palatable species in green; in the defended *Diacrisia sannio* and *Diaphora mendica* (in pink), males and females have different activity patterns. Bootstrap support values for 1000 replicates are given for each branch.

3.3.9 Statistical analyses

All statistical analyses were carried out in R 3.3.1 (R Development Core Team, 2015). Each type of colour patch (forewing background, forewing markings, hindwing background and hindwing markings) was analysed separately, and for internal contrasts, fore- and hindwings were analysed separately. The effect of category (Palatable [P]), Defended diurnal [DD] and Defended nocturnal [DN]) on each colour metric was tested with linear mixed effects models (LMEs), using the package 'lme4' (Bates *et al.*, 2014). Diagnostic plots were examined using the mcp.fnc function in the 'LMERConvenienceFunctions' (Tremblay and Ransijn, 2014), and colour metrics were transformed using the square-root or log function as appropriate, to satisfy the assumptions of linear mixed models. The pattern metrics of peak proportion and total energy were similarly analysed with linear mixed effects models, and total energy was log-transformed to meet model assumptions. Peak frequency was analysed using a generalised mixed effects model, fitting a Poisson distribution with a log link. An observation-level random effect was included to account for overdispersion (Harrison, 2014), and model assumptions were verified using the DHARMa package in R (Hartig, 2017). While the phylogenetic tree based on COI-5P sequences is poorly supported (Figure 3.3), it is clear that diurnal defended species fall into two groups: the burnets and foresters (Zygaenidae) and the tiger moths (Erebidae: Arctiinae). Similarly, most of the nocturnal defended species belong to the Erebidae family. To account for this phylogenetic non-independence without relying on a poorly-supported phylogeny, the family and species names were included as a nested random effect in all models. Significant effects were determined using likelihood ratio tests, and Tukey's post-hoc tests were carried out using the glht function in the 'multcomp' package (Hothorn, Bretz and Westfall, 2008). All tests were repeated with data corresponding to the UVS and VS visual systems, but for clarity, only the results for the UVS system are reported and plotted below. Results for the VS system are quoted below when they yielded different conclusions and can be found in full in Appendix 3.8.

3.4 Results

3.4.1 Lighter colours in nocturnal species

The main hindwing colour was lighter in both categories of nocturnal moths, whether defended or not, than for defended diurnal species (LME, $(\chi^2)_2$= 12.275, p=0.00216, Tukey's post-hoc tests: $p_{DD\text{-}P}$=0.00861, $p_{DN\text{-}P}$=0.343, $p_{DD\text{-}DN}$<0.001). Nocturnal moths also tended to have lighter hindwing marking colours and defended nocturnal species lighter forewing background colours, although these trends did not quite reach significance (Table 3.1, Figure 3.4).

Table 3.1: Results of linear mixed models testing the effect of category on luminance for the ultraviolet-sensitive visual system. Significant results are highlighted in italics.

Wing area	**(X^2)**	**df**	**p**
Forewing background	5.222	2	0.0735
Forewing markings	3.859	2	0.145
Hindwing background	12.275	2	*0.00216*
Hindwing markings	5.124	2	0.0772

3.4.2. More saturated colours in defended species

Some wing colours appeared more saturated in defended moths than in their edible counterparts (Table 3.2, Figure 3.5). Nocturnal defended moths displayed more saturated colours in their principal forewing markings than palatable moths, as did defended diurnal moths, although this latter comparison did not quite reach significance for the UVS visual system (LME, $(\chi^2)_2$= 8.160, p=0.0169, Tukey's post-hoc tests: $p_{DD\text{-}P}$=0.0556, $p_{DN\text{-}P}$=0.0158, $p_{DD\text{-}DN}$=0.947). Moreover, the main colours of the hindwings for both categories of defended moths were more saturated than the equivalent colours in palatable moths (LME, $(\chi^2)_2$= 8.910, p=0.0116, Tukey's post-hoc tests: $p_{DD\text{-}P}$<0.001, $p_{DN\text{-}P}$=0.656, $p_{DD\text{-}DN}$=0.0125).

Table 3.2: Results of linear mixed models testing the effect of category on saturation for the ultraviolet-sensitive visual system. Significant results are highlighted in italics.

Wing area	**(X^2)**	**df**	**p**
Forewing background	3.657	2	0.161
Forewing markings	8.160	2	*0.0169*
Hindwing background	8.910	2	*0.0116*
Hindwing markings	2.377	2	0.305

3.4.3 Redder colours in defended species

Forewing colours did not significantly differ in overall hue between categories, although there was a trend for lower values in markings of the palatable species. However, the main hindwing background colours had higher hue values in the diurnal defended category than in the others (LME, $(\chi^2)_2$= 9.616, p=0.00817, Tukey's post-hoc tests: $p_{DD\text{-}P}$<0.001, $p_{DN\text{-}P}$=0.839, $p_{DD\text{-}DN}$=0.00227; Table 3.3, Figure 3.6). According to the equation for hue of the hindwing background colours (equation 3.3), this suggests that diurnal defended moths had colours with a relatively greater reflectance in long wavelengths on their hindwings, indicating redder signals.

Table 3.3: Results of linear mixed models testing the effect of category on hue for the ultraviolet-sensitive visual system. Significant results are highlighted in italics.

Wing area	**(X^2)**	**df**	**p**
Forewing background	4.955	2	0.0840
Forewing markings	5.910	2	0.0521
Hindwing background	9.616	2	*0.00817*
Hindwing markings	1.056	2	0.590

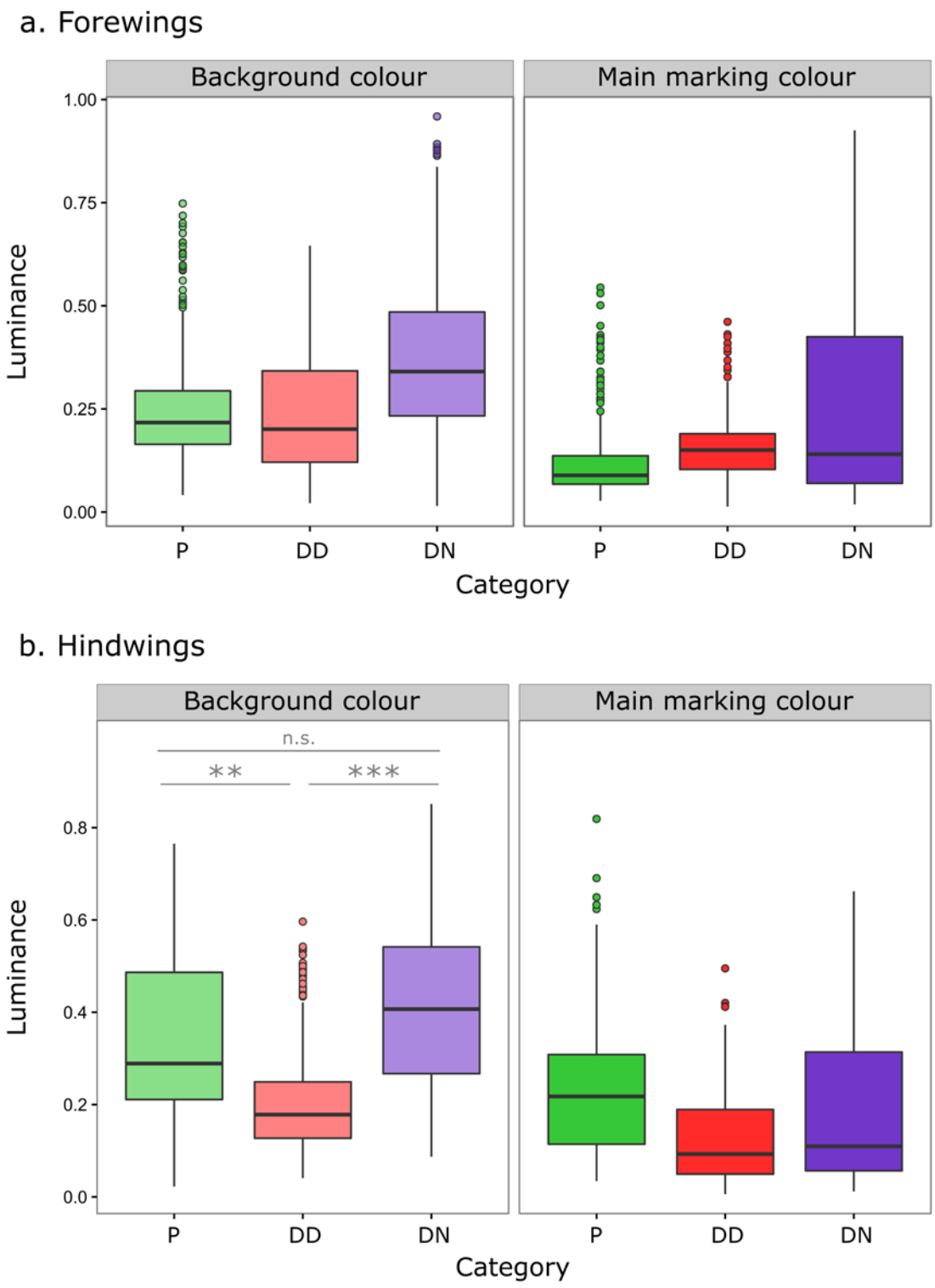


Figure 3.4: Luminance of forewing (a) and hindwing (b) colours, plotted by category. P = Palatable, DD = defended diurnal, DN = defended nocturnal. Only significant pairwise comparisons are shown. Boxplots show the median and interquartile range (IQR). In this and all subsequent plots, the whiskers extend to the maximum value, if less than 1.5 IQR away from the third quartile, and to the minimum value, within 1.5 IQR of the first quartile. Points outside this range are plotted as outliers. Significance levels: ***:p<0.001, **:p<0.01, *:p<0.05.

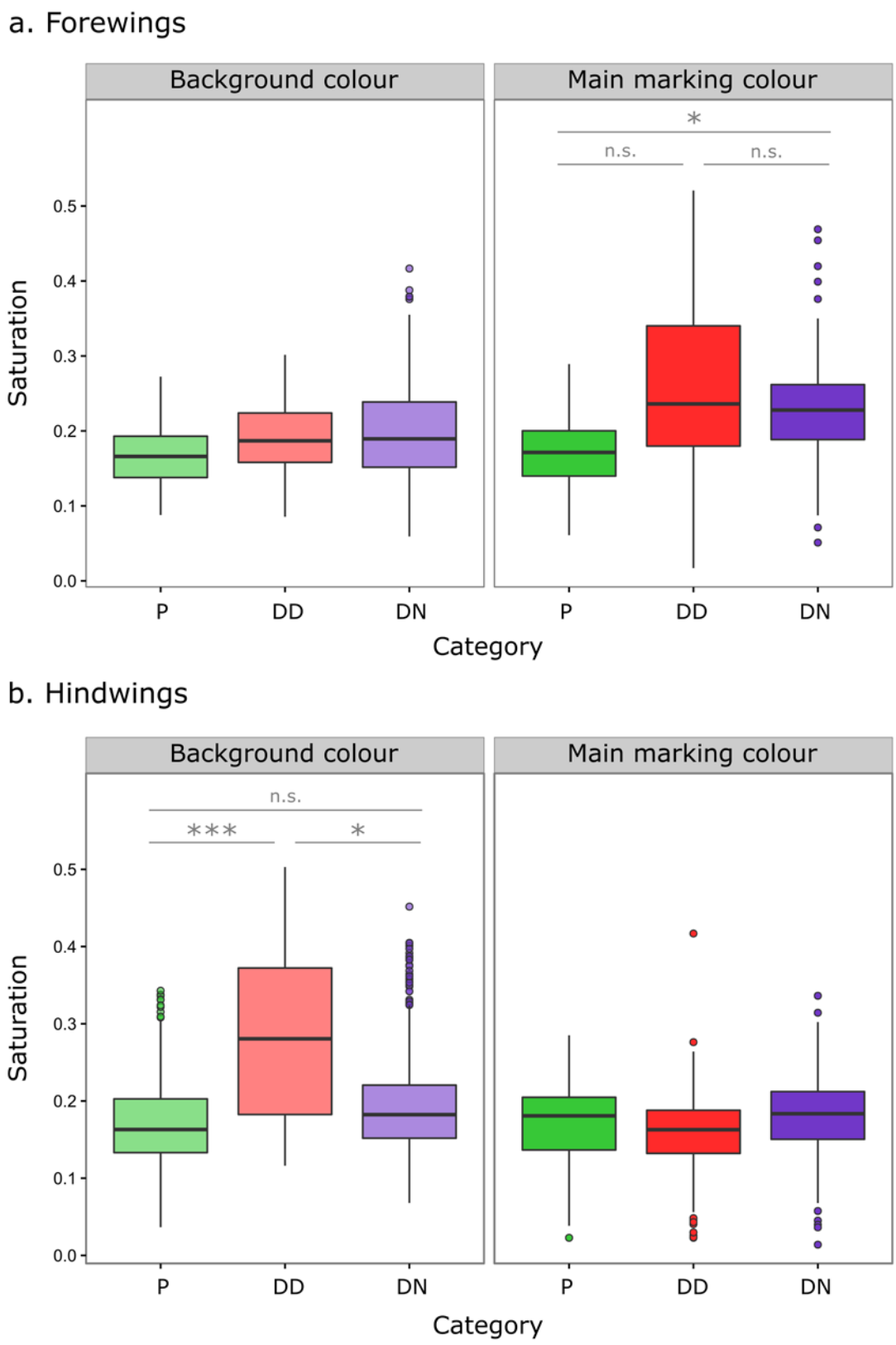


Figure 3.5: Saturation of forewing (a) and hindwing (b) colours for the ultraviolet-sensitive visual system, plotted by category. P = Palatable, DD = defended diurnal, DN = defended nocturnal. Boxplots show the median and interquartile range (IQR). Only significant pairwise comparisons are shown. Significance levels: ***:p<0.001, **:p<0.01, *:p<0.05.

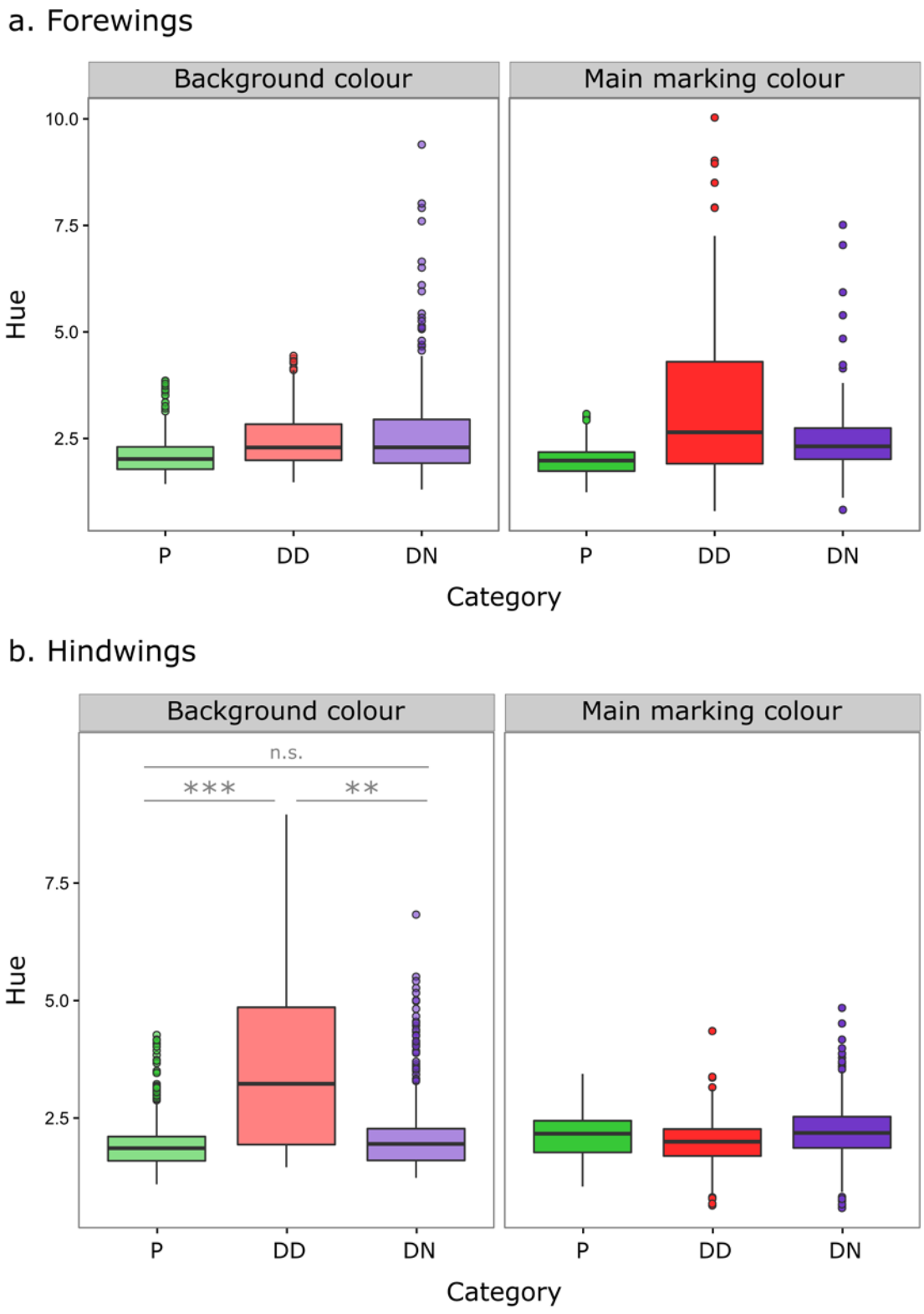


Figure 3.6: Hue values of forewing (a) and hindwing (b) colours for the ultraviolet-sensitive visual system, plotted by category. P = Palatable, DD = defended diurnal, DN = defended nocturnal. Boxplots show the median and interquartile range (IQR). Only significant pairwise comparisons are shown. Significance levels: ***:p<0.001, **:p<0.01, *:p<0.05

3.4.4 Greater colour contrast and diversity in defended species

Defended moths, both nocturnal and diurnal, displayed forewing patterns with a higher internal chromatic contrast, or contrast between their two principal colours, than palatable moths (LME, $(\chi^2)_2$= 19.674, p<0.001, Tukey's post-hoc tests: $p_{DD\text{-}P}$<0.001, $p_{DN\text{-}P}$<0.001, $p_{DD\text{-}DN}$=0.534; Figure 3.7). However, there was no difference in hindwing chromatic contrast between categories. In addition, there were no differences in luminance contrast for either the fore- or hindwings (Table 3.4).

In support of the greater chromatic differences between colour patches in defended moths, calculations of the volume occupied by the colours of moths in each category suggest that diurnal defended moths possess a greater variety of colours, both overall and on average per species (Figure 3.8). Volume in a tetrahedral colour space provides a measure of colour diversity (Stoddard and Prum, 2008), whereby larger volumes indicate a greater number of different colours found in the patterns measured. Altogether, the colours measured on moths in the diurnal defended category occupied a total volume four times larger than that occupied by the colours of palatable species (Table 3.5), suggesting that a wider range of colours is found across the defended species in this analysis than across the unprotected, edible ones. Similarly, individual species in the diurnal defended group also tended to occupy larger volumes than those in the other categories (LME, $(\chi^2)_2$= 19.674, p<0.001, Tukey's post-hoc tests: $p_{DD\text{-}P}$<0.001, $p_{DN\text{-}P}$=0.139, $p_{DD\text{-}DN}$=0.200), indicating that defended species tend to make use of a wider palette of colours on their wings.

Table 3.4: Results of linear mixed models testing the effect of category on internal chromatic and luminance contrast of the wings for the ultraviolet-sensitive visual system. Significant results are highlighted in italics.

Type of contrast	**Wing**	**(X^2)**	**df**	**p**
Chromatic	Forewings	19.674	2	*<0.001*
	Hindwings	3.698	2	0.157
Luminance	Forewings	0.553	2	0.759
	Hindwings	0.258	2	0.879

Table 3.5: Total volume occupied by the forewing and hindwing colours of all the moths in each category in the avian (ultraviolet-sensitive) tetrahedral colour space.

Category	Palatable	Toxic diurnal	Toxic nocturnal
Volume	0.00199	0.00800	0.00489

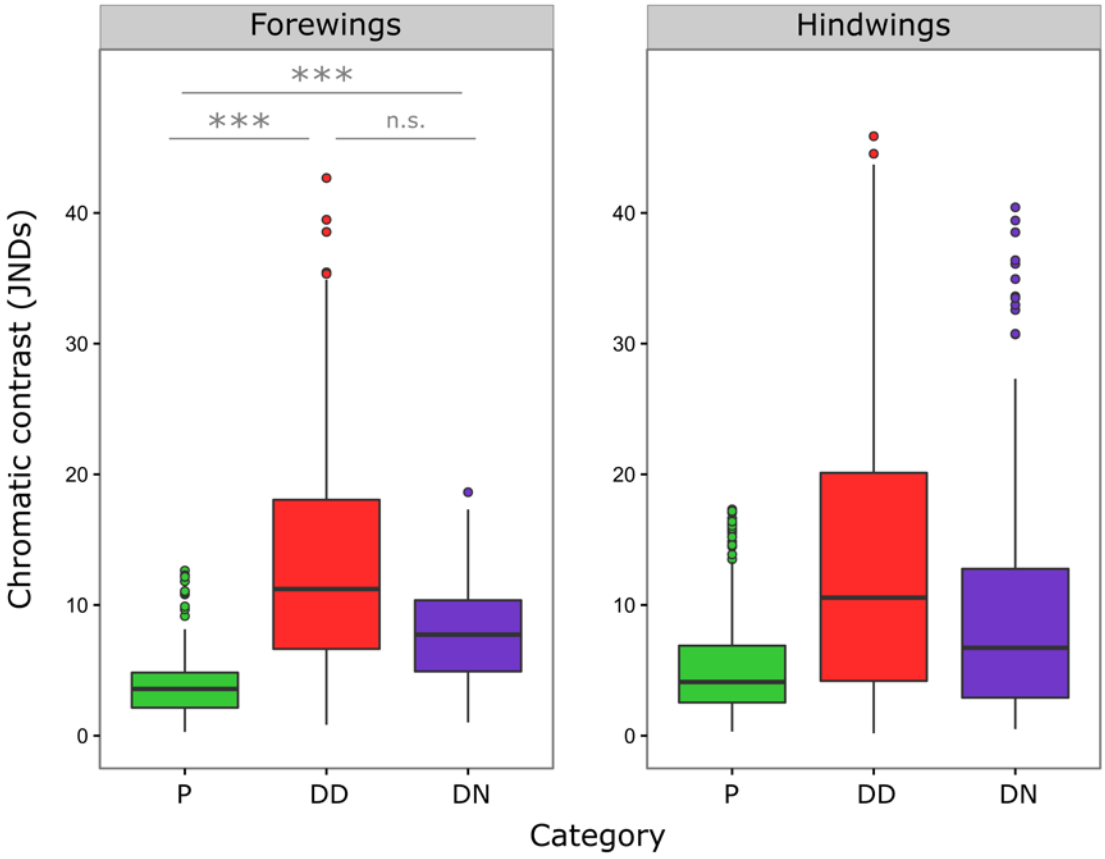


Figure 3.7: Internal chromatic contrast of forewing and hindwing colours for the ultraviolet-sensitive, plotted by category. P = Palatable, DD = defended diurnal, DN = defended nocturnal. Boxplots show the median and interquartile range. Only significant pairwise comparisons are shown. Significance levels: ***:p<0.001, **:p<0.01, *:p<0.05

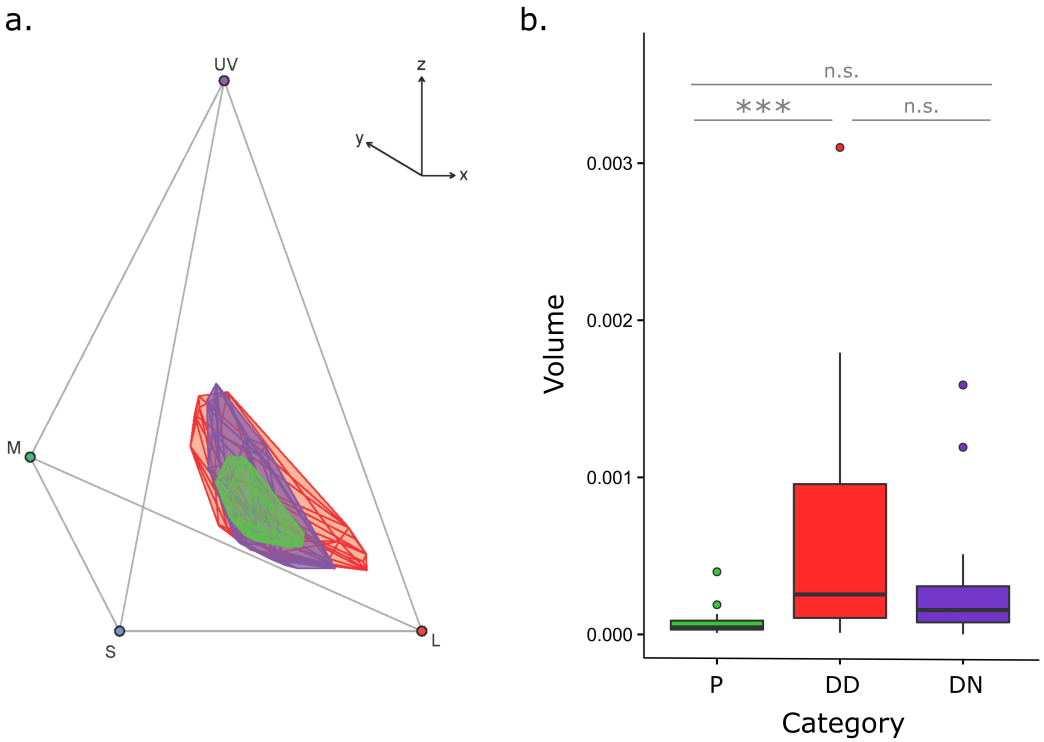


Figure 3.8: Volumes occupied by wing colours in the avian tetrahedral colour space for the ultraviolet-sensitive visual system. (a) Convex hulls depict the total volume occupied by all the wing colours measured for each category, for palatable (in green), nocturnal defended (in purple) and diurnal defended moths (in red). (b) Boxplots of the volumes occupied by all the wing colours for every species in each category, showing the median and interquartile range of each group. P = Palatable, DD = defended diurnal, DN = defended nocturnal. Significance levels: ***:p<0.001, **:p<0.01, *:p<0.05

3.4.5 Greater conspicuousness in defended species

The main background forewing colours of nocturnal defended moths tended to have higher chromatic contrast against an average herbaceous plant colour than diurnal defended moths; this difference was not significant for the UVS visual system, but it was when considering the VS system (Table 3.6 for UVS results; for the VS system: LME, $(\chi^2)_2$= 8.337, p=0.0155, Tukey's post-hoc tests: $p_{DD\text{-}P}$=0.462, $p_{DN\text{-}P}$=0.338, $p_{DD\text{-}DN}$=0.0143). However, for both visual systems, the colours of the main forewing markings of defended moths were more conspicuous against the herbaceous background than those of edible species,

in terms of colour (UVS system, LME, $(\chi^2)_2$= 7.834, p=0.0249, Tukey's post-hoc tests: $p_{DD\text{-}P}$=0.0479, $p_{DN\text{-}P}$=0.0301, $p_{DD\text{-}DN}$=0.998; Figure 3.9a).

The greater chromatic conspicuousness of defended moths is more clearly demonstrated when comparing their wing colours to an average tree bark background. The colours of both categories of defended moths presented greater chromatic contrast against bark, whether considering their forewing background or main marking colours (LME, $(\chi^2)_2$= 6.470, p=0.0394, Tukey's post-hoc tests: $p_{DD\text{-}P}$=0.0418, $p_{DN\text{-}P}$=0.0281, $p_{DD\text{-}DN}$=0.983 and $(\chi^2)_2$= 12.504, p=0.00193, Tukey's post-hoc tests: $p_{DD\text{-}P}$=0.0121, $p_{DN\text{-}P}$=0.0012, $p_{DD\text{-}DN}$=0.830 respectively; Figure 3.9b). However, there was no difference between categories in chromatic contrast against the foliage of their own specific host plants, or in luminance contrast against any of the plant background types (Table 3.6).

Table 3.6: Results of linear mixed models testing the effect of category on conspicuousness to natural backgrounds for the ultraviolet-sensitive visual system. Significant results are highlighted in italics.

a. Chromatic contrast

Plant type	**Wing area**	**(X^2)**	**df**	**p**
Average herbaceous	Forewing background	3.088	2	0.214
	Forewing markings	7.834	2	*0.0249*
Average tree bark	Forewing background	6.470	2	*0.0394*
	Forewing markings	12.504	2	*0.00193*
Average host plant foliage*	Forewing background	0.126	2	0.939
	Forewing markings	4.724	2	0.0942

* or lichen for Lithosiinae

b. Luminance contrast

Plant type	**Wing area**	**(X^2)**	**df**	**p**
Average herbaceous	Forewing background	4.381	2	0.112
	Forewing markings	4.709	2	0.0949
Average tree bark	Forewing background	1.766	2	0.414
	Forewing markings	3.602	2	0.165
Average host plant foliage*	Forewing background	0.540	2	0.764
	Forewing markings	2.706	2	0.259

* or lichen for Lithosiinae

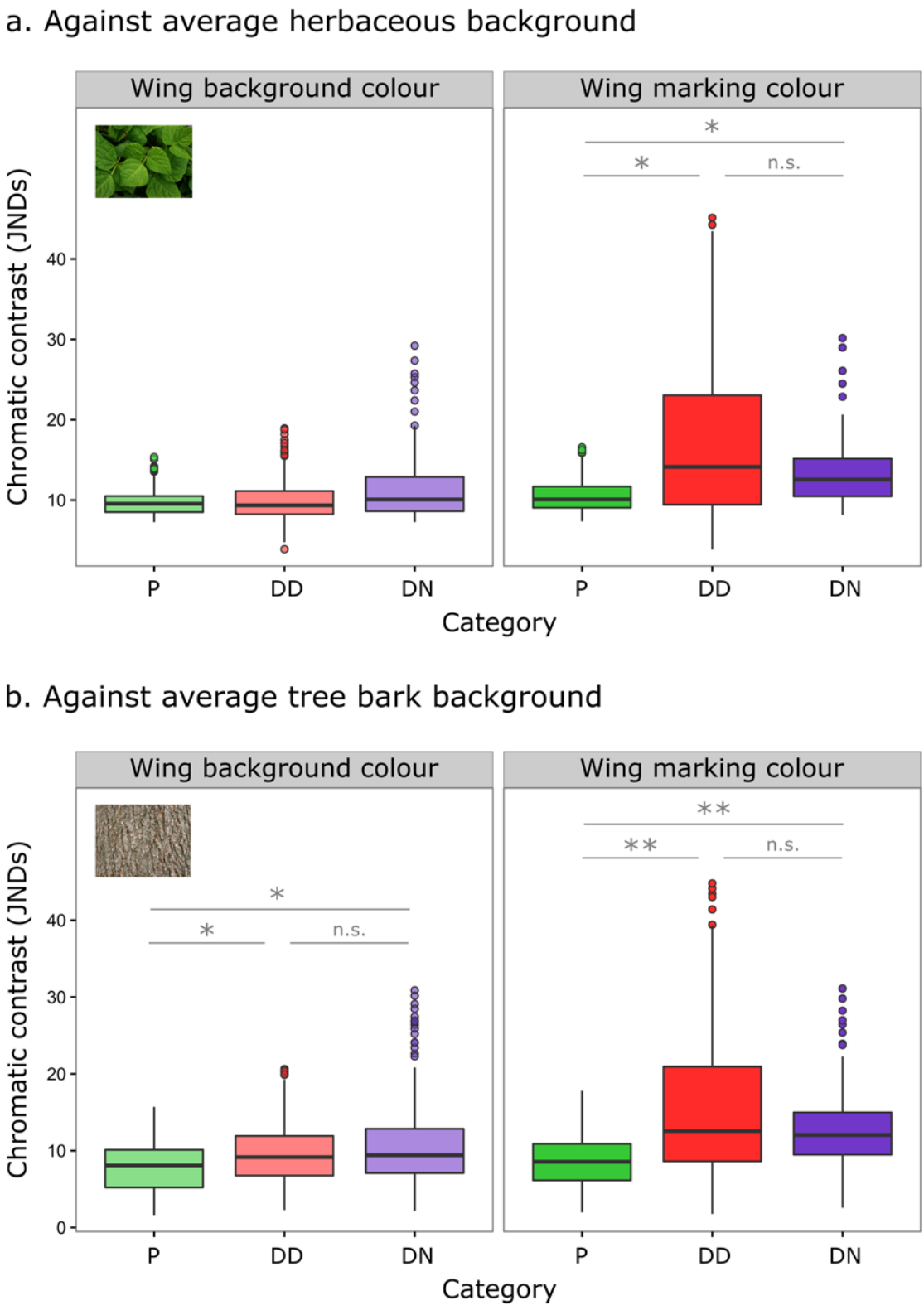


Figure 3.9: Chromatic contrast of forewing colours against natural backgrounds: herbaceous plant leaves (a) and tree bark (b), for the ultraviolet-sensitive visual system, plotted by category. P = Palatable, DD = defended diurnal, DN = defended nocturnal. Boxplots show the median and interquartile range. Only significant pairwise comparisons are shown. Significance levels: ***:p<0.001, **:p<0.01, *:p<0.05

3.4.6 Larger and more contrasting markings in defended species

Nocturnal defended and palatable moths have more contrasting patterns on their forewings than diurnal defended species, indicated by greater total energy (LME, $(\chi^2)_2$= 6.922, p=0.0314, Tukey's post-hoc tests: $p_{DD\text{-}P}$=0.216, $p_{DN\text{-}P}$=0.518, $p_{DD\text{-}DN}$=0.0207, Figure 3.10a). Peak frequency in the forewings is higher in both categories of defended moths than in palatable species, suggesting that the most prominent markings are larger in defended species (LME, $(\chi^2)_2$= 10.895, p=0.00431, Tukey's post-hoc tests: $p_{DD\text{-}P}$=0.0131, $p_{DN\text{-}P}$=0.00370, $p_{DD\text{-}DN}$=0.980, Figure 3.10b). This is not due to these categories of moths having a larger body size, as in fact the palatable species in this study are larger than both groups of defended moths (LME, log-transformed forewing length, $(\chi^2)_2$= 11.101, p=0.00389, Tukey's post-hoc tests: $p_{DD\text{-}P}$=0.0293, $p_{DN\text{-}P}$=0.00188, $p_{DD\text{-}DN}$=0.837). Similarly, the relative importance of these forewing markings is greater in the defended categories, as suggested by greater peak proportion values (LME, $(\chi^2)_2$= 12.329, p=0.00210, Tukey's post-hoc tests: $p_{DD\text{-}P}$=0.0168, $p_{DN\text{-}P}$<0.001, $p_{DD\text{-}DN}$=0.780; Figure 10c). However, there was no difference between categories in any pattern metric for the hindwings (Table 3.7).

Table 3.7: Results of mixed effects models testing the effect of category on pattern metrics. Significant results are highlighted in italics.

Wing	**Pattern metric**	**(X^2)**	**df**	**p**
Forewing	Peak frequency	10.895	2	*0.00431*
	Peak proportion	12.329	2	*0.00210*
	Total energy	6.922	2	*0.0314*
Hindwing	Peak frequency	2.283	2	0.319
	Peak proportion	0.827	2	0.662
	Total energy	4.451	2	0.108

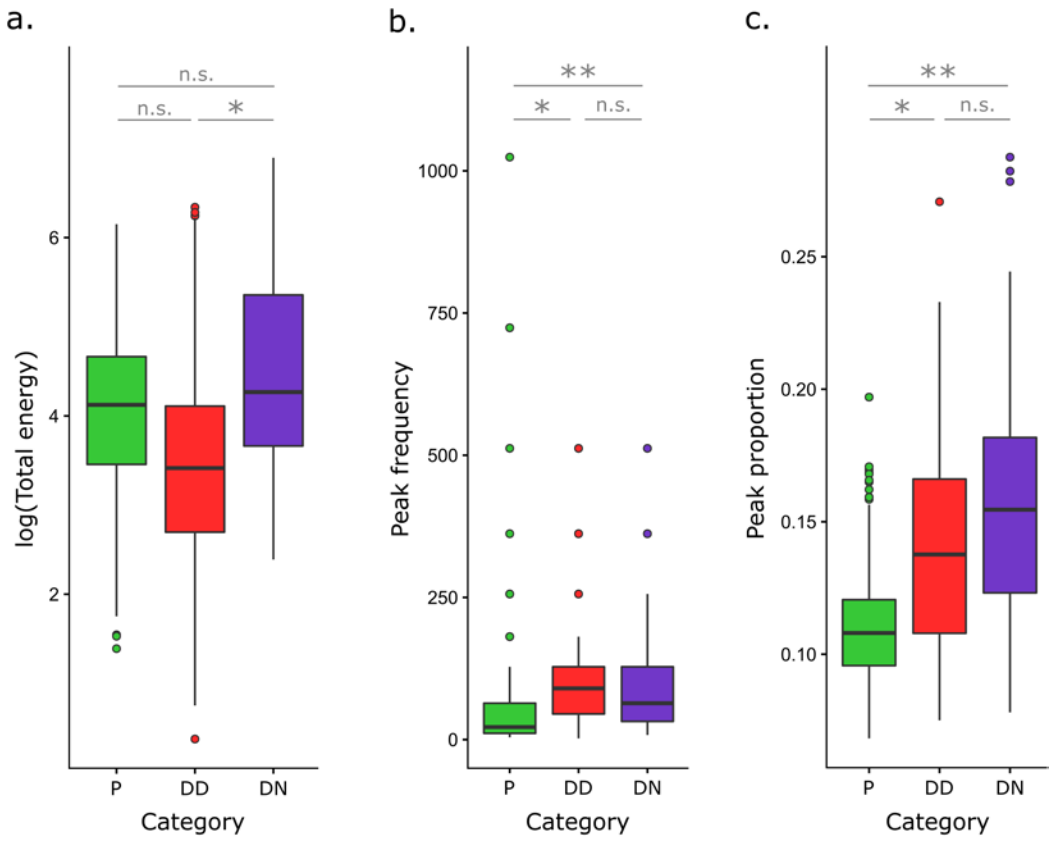


Figure 3.10: Forewing total energy (a), peak frequency (b) and peak proportion (c) plotted by category. Boxplots show the median and interquartile range. Significance levels: ***:p<0.001, **:p<0.01, *:p<0.05

3.5 Discussion

This comprehensive analysis of the colours, patterns, and conspicuousness of palatable and defended moths has highlighted a number of key characteristics of these different groups. Several, although not all, of my initial predictions were borne out. Both categories of defended moths studied here did display more saturated colours than palatable species but only for certain wing areas. Diurnal defended moths also had redder colours than palatable moths on their hindwings, along with similar trends for their forewing markings. Moreover, the forewings of both groups of defended species showed greater chromatic contrast between the wing background and wing marking areas than the edible species. Although the relevance of increased contrast beyond the threshold for discriminability (JND>3) is unclear (Eaton, 2005; Cheney *et al.*, 2014), diurnal defended species occupied a larger volume in the avian tetrahedral colour space, providing another measure of diversity in coloration (Stoddard and Prum, 2008). In terms of pattern, the most prominent forewing markings of defended

species tended to be larger and more important to the overall contrast of the wings. However, their wing patterns were not altogether more contrasting than those of palatable species in an achromatic sense, and in fact the three categories of species did not differ in many achromatic metrics. Nocturnal moths did have lighter-coloured hindwings than their diurnal counterparts, but there was no difference between defended and undefended nocturnal species. Finally, in terms of conspicuousness to natural backgrounds, only chromatic contrast between moth and plant colours varied between categories. The colours of defended moths were more conspicuous than those of palatable species against an average tree bark background, and to a lesser extent against a general herbaceous background, but there were no differences between defended and undefended species against the foliage of their own host plants.

The forewings of most moths, both nocturnal and diurnal, are more likely to be exposed to visual predators when at rest, so they should provide a more appropriate canvas for warning signals than the hindwings. In addition, palatable species may employ multiple anti-predator strategies, with crypsis in the forewings as a first line of defence, followed by secondary defences if the moths have been detected and predators approach (Edmunds, 1974). These include startle displays (for example in underwings, *Catocala* spp.; Sargent, 1990) and the use of eyespots (as seen in the eyed hawkmoth, *Smerinthus ocellata*; Stevens, 2005). As these strategies benefit from the use of bright and conspicuous colours, similar to those of warning signals, the differences between defended and undefended species should be less pronounced in the hindwings. Nevertheless, in this study, there were significant differences in colour (hue, saturation) and luminance between the hindwings of moths belonging to the three categories of prey, suggesting that hindwing colours may still be used in aposematism. In some species with brightly-coloured hindwings, such as the garden tiger, *Arctia caja*, and other tiger moths, warning coloration may be used facultatively as part of a dynamic signal, while the forewings provide disruptive camouflage (Kettlewell, 1965; Forsman and Merilaita, 1999; Skelhorn, Holmes and Rowe, 2016). However, there were no differences between defended and undefended prey in terms of internal contrast or pattern

properties in the hindwings, indicating that hindwing pattern may be less relevant.

3.5.1 The importance of chromatic information, internal contrast and marking size

In many ways, the results of this comparative study confirm existing ideas on the importance of specific signal features in warningly-coloured prey. Evidence of more pronounced differences between defended and palatable species in terms of chromatic rather than achromatic features supports previous work suggesting that colour is the most relevant feature of warning signals, including experimental evidence with natural predators in the field (Finkbeiner, Briscoe and Reed, 2014). In addition, the defended moths chosen for analysis are smaller in size than the palatable species, yet their most contrasting markings are larger. This result supports the beneficial effect of larger signals in aposematic displays, and is in line with several experiments demonstrating the greater effectiveness of larger signals, particularly in the context of Lepidopteran eyespots (Forsman and Merilaita, 1999; Stevens, Hardman and Stubbins, 2008). Testing the reactions of great tits (*Parus major*) to patterned artificial caterpillars also demonstrated that signal rather than body size was more important in determining the predators' reluctance to attack (Remmel and Tammarub, 2011).

Less widely-supported by prior experiments on warning signals is the importance of internal contrast in the wing patterns of aposematic species. Several studies have demonstrated that domestic chicks (*Gallus gallus domesticus*) attend primarily to colour rather than pattern when learning a discrimination task between palatable and distasteful prey (Aronsson and Gamberale-Stille, 2008; Aronsson and Gamberale-Stille, 2012a). In some experiments, chicks learned faster when the main object colour contrasted against the background, but internal contrast had no such effect, and chicks trained to avoid striped prey later generalised completely between striped and unstriped prey of the same colour (Aronsson and Gamberale-Stille, 2009). Nevertheless, great tits (*Parus major*) preferentially attacked edible bugs with plain brown signals rather than patterned bugs, regardless of their colour (Svádová *et al.*, 2009), suggesting that pattern can be relevant. Moreover, work

on blue tits (*Cyanistes caeruleus*) has shown that, although colour still takes precedence, internal contrast can lead to faster avoidance learning (Aronsson and Gamberale-Stille, 2012b). Domestic chicks can also use pattern for discrimination when necessary, suggesting a hierarchical use of cues, in which internal contrast would be used if colour is not sufficiently informative (Aronsson and Gamberale-Stille, 2012a). The fact that internal chromatic contrast is found to be a key characteristic of defended Lepidoptera in this study suggests that it may be more important than previously recognised. It is also worth noting that these experiments on the role of internal contrast have all used black internal markings, and thus have focused primarily on achromatic contrast, which this present study suggests may be less relevant. The importance of chromatic contrast also highlights a key difference between this present investigation and a previous study comparing the visual signals of invertebrates, which found no difference between defended and undefended species (Bohlin, 2013). In that project, internal contrasts were measured solely from grayscale images, prior to mapping to avian vision; they thus calculated only brightness differences rather than chromatic contrasts, overlooking a trait which my work suggests may be a key characteristic of aposematic patterns.

3.5.2 Conspicuousness of palatable and defended species

The conspicuousness of palatable and defended species against natural backgrounds similarly revealed a somewhat unexpected result. Defended species were expected to be more conspicuous than palatable ones against all backgrounds. Accordingly, I found that their colours had greater chromatic contrast than those of edible moths against bark backgrounds, and nocturnal defended moths were more conspicuous against an average herbaceous background. However, there was no difference between categories in their conspicuousness to the moths' specific host plant foliage. The greater conspicuousness against bark echoes the findings of a recent study on aposematic ladybird species, whose colours were overall more contrasting against an average brown background (based on photographs of bark and soil), than against an average green or specific host plant background (Arenas and Stevens, 2017). However, for ladybirds, this result depended on habitat use: specialist species were more contrasting against their specific host plants, while generalists were equally conspicuous on all types of natural backgrounds. Many

moths make use of multiple host plants, only a subset of which were photographed for this study (see Appendix 3.6), potentially contributing to the lack of differences between categories when considering host plant foliage as a natural background. In addition, the plant and moth photographs were taken in controlled conditions under D65 daylight conditions, thus not testing the conspicuousness of moth colours under a range of more ecologically-relevant lighting conditions, for example for nocturnal moths potentially exposed at dawn and dusk. More importantly, behavioural choices may further modify the conspicuousness of defended and undefended species in natural conditions. Moths, and in particular diurnal ones, can be found on a much wider variety of backgrounds than their host plant foliage, including brightly-coloured flowers. Defended moths, freed from the opportunity costs of crypsis (Speed, Brockhurst and Ruxton, 2010), are likely to be found on a wider range of backgrounds, and appear conspicuous against all of these. Conversely, palatable moths may hide out of sight under leaves or in small crevasses when at rest, so be difficult to locate even if their colours appear conspicuous against most natural backgrounds.

The absence of differences between categories in luminance contrast against any natural backgrounds offers further support for the greater importance of chromatic contrast, rather than achromatic information, to effective warning signals. Yet it may also reflect a trade-off between conspicuousness to avian predators and reduced visibility to other predators. While chromatic contrast is considered to be most important for avoidance learning in birds, achromatic contrast may be more relevant for invertebrate predators, such as mantids (Prudic, Skemp and Papaj, 2007). The effectiveness of Lepidopteran defences against invertebrates is relatively poorly-understood compared to their interactions with avian predators, although recent studies are beginning to rectify that imbalance (Pentzold *et al.*, 2016; Rojas *et al.*, 2017). If they are not protected against this class of predator, conspicuousness to invertebrates may be disadvantageous even to defended species. Many species of parasitoids are also known to make use of visual cues to locate hosts, and some, such as the ichneumonid *Pimpla turionellae* (Hymenoptera: Ichneumonidae), may use achromatic contrast to guide them (Fischer *et al.*, 2003). There is mixed evidence as to whether chemical defences such as

pyrrolizidine alkaloids can confer protection against parasitoids (Bezzerides *et al.*, 2004; Conner and Weller, 2004). As such, reducing their visibility to these enemies may also be an important consideration for all moths, especially when on host plant foliage where females will lay their eggs.

3.5.3 Limitations of this and other museum-based studies

To put the above results in context, several caveats and limitations must be considered when interpreting this study. Specimens stored in museum collections can suffer from fading, affecting brightness and colour (Starling *et al.*, 2006). This an important consideration for studies of coloration, not only for Lepidoptera but also for other types of museum collections, such as eggshells (Starling *et al.*, 2006; Cassey *et al.*, 2010). As a first precaution, I photographed the least damaged specimens available for this study. To gain a sense of whether and to what extent the fading of museum specimens might nevertheless affect the conclusions of this study, I also compared museum and fresh specimens of four species of defended moths. Overall, the results suggest that the relative differences between species are fairly consistent between fresh and collection specimens, although more caution may be needed when interpreting the results of measurements of dark hindwing markings (see Appendix 3.3). I had also hoped to precisely account for the phylogenetic relationships between species, but too little genetic information was available for a wide range of species, particularly the palatable ones, for this to be feasible. The COI sequences obtained from the DNA Barcode of Life project (Ratnasingham and Hebert, 2007) are poorly-suited to phylogenetic reconstruction (DeSalle, Egan and Siddall, 2005), although their limitations may be in future be overcome by increased taxon sampling to several hundred species per family, increasing the reliability of trees based on DNA barcodes (Wilson, 2011). In the absence of a satisfactory phylogeny for the species included in the study, I accounted for the clustering of defended species into two families (Erebidae and Arctiidae), by including family-level classification as a random effect in all models. Finally, there is some uncertainty surrounding the levels of defence in each species and in the classification of their activity patterns. In terms of activity, seasonal patterns may be relevant, as changes in the abundance of naïve predators (Mappes *et al.*, 2014) and of predator types, such as birds and bats, may alter the relative costs and benefits of investing in

aposematic signals. For example, the ability to produce ultrasonic clicks for acoustic aposematism is more widespread in North American tiger moths (Erebidae) emerging later in the season when bats are most active (Ratcliffe and Nydam, 2008). In addition, several species measured here, such as the garden tiger (*Arctia caja*) and magpie moth (*Abraxas grossulariata*) are classed as nocturnal, yet are easily disturbed and observed in the daytime (Newland, Still and Swash, 2013). Their wings display characteristics identified here as features of defended diurnal species, such as highly contrasting patterns and a diversity of colours, so their inclusion in the diurnal group would most likely only strengthen the conclusions presented here. Nevertheless, to improve the accuracy of this study, it would be useful to include a more quantitative measure of activity patterns, such as diel flight periodicity (Fullard and Napoleone, 2001; Ratcliffe and Nydam, 2008).

More difficult to overcome is the paucity of reliable and comparable data on the defences of British Lepidoptera and their relative acceptability to predators. Studies of chemical defences in Lepidoptera have understandably focused on colourful and exotic species (eg. Rothschild *et al.*, 1970; Bowers and Farley, 1990), and as a result, there is little information on the profitability of most species of dull-coloured British moths. Where palatability has been investigated, very different methods have been used to estimate the level of defences: while the defensive compounds of some species have been precisely identified and quantified (e.g. burnet moths, Zygaenidae; Davis and Nahrstedt, 1982; Zagrobelny and Møller, 2011), the assessment of other species relies on experimental evidence of rejection by avian predators (eg. Sargent, 1995), injection of extracts into mice (Marsh and Rothschild, 1974) or presence of the item in the stomach of a few individuals (eg. Campbell, 1936). There is also substantial variation in unprofitability between individuals of the same defended species (Brower *et al.*, 1968), which is not accounted for here. The potency of prey defences is important in determining predation risk, as avian predators can make educated foraging decisions, estimating the risk of consuming a prey item, potentially on a case-by-case basis using taste-rejection (Skelhorn and Rowe, 2006). They can also weigh the cost of ingesting toxins against the potential nutritional benefits and their own needs before choosing to attack (Barnett, Bateson and Rowe, 2007). As a result, weakly-defended species may

not necessarily benefit from being overly conspicuous; although not a preferred food source, they may still experience a substantial predation risk under many ecological conditions. Further research on the unprofitability of moths thought to be distasteful would thus strengthen this study, enabling a more nuanced classification of species as either strongly or weakly-defended.

Notwithstanding these caveats, this study constitutes a valuable preliminary investigation into the features likely to be most important for signal efficacy in aposematic Lepidoptera. General trends in warning signals provide useful hints to guide further work, but the role of different signal traits must be tested using appropriate predators, in the field or laboratory, to establish their relevance in natural situations. The key characteristics of defended moths picked up by this comparative analysis, and in particular the importance of internal chromatic contrast, would warrant further investigation to test their effects on predator avoidance.

Chapter 4

Testing for signal honesty in aposematic Lepidoptera – a case-study in the six-spot burnet, *Zygaena filipendulae*

Six-spot burnet moth, *Z. filipendulae*. Photograph: E. S. Briolat

4.1 Abstract

The distinctive black and red wing pattern of six-spot burnet moths (*Zygaena filipendulae*) is a classic example of aposematic coloration, warning predators of their potent cyanide-based chemical defences. While such warning signals provide a qualitatively honest signal of unprofitability to predators, the idea of quantitative honesty, whereby variation in the level of the warning signals could provide accurate estimates of individual prey toxicity, is more controversial. Combining sophisticated measures of cyanogenic glucoside content and wing colour, from the perspective of avian predators, this study investigates the relationship between coloration and toxicity in *Z. filipendulae*, to test signal honesty both within and across populations in Denmark, France and the UK. Mean cyanogenic glucoside concentration was correlated with some measures of wing coloration across populations in females, but not males. Among females, smaller and lighter forewing markings were associated with a higher concentration of cyanogenic glucosides, contrary to expectations in an honest signalling paradigm. Trends within single populations were similarly indicative of signal dishonesty, and consistent differences between the sexes were apparent. Larger females, carrying a greater total cyanogenic glucoside load, displayed larger but less conspicuous markings than smaller males, according to several colour metrics. Diverse factors may contribute to the general absence of honest signalling within and between populations, including plentiful resources, the high aversiveness of zygaenid defences, and the effect of changes in colour and toxicity over a moth's lifetime in natural conditions. Meanwhile, contrasting activity patterns and possible interactions with sexual signalling may account for the differences between males and females. These results highlight important reasons why positive correlations between toxicity and coloration might not always be expected in aposematic species.

4.2 Introduction

Warning coloration, or aposematism, is a key adaptive explanation for the bright and colourful patterns on show in the animal kingdom. Conspicuous visual signals act to warn potential predators that a prey item is toxic or otherwise unprofitable, a theory first proposed by Alfred Russell Wallace in relation to colourful caterpillars (Wallace, 1867). Despite the long history of research into

animal warning signals ever since, many issues surrounding this topic remain unresolved, perhaps most notably the question of signal honesty in aposematism. While the evolution of qualitatively honest signals to predators, reliably indicating the presence of a defence, is inherent in the definition of aposematism and has strong support from empirical work and theoretical modelling, evidence for quantitative honesty, in which the value of a signal reflects the level of the signaller's defences, remains equivocal (Summers *et al.*, 2015). Relatively few empirical studies have tested the relationship between properties of visual signals and toxicity in aposematic species, while taking into account the predator's visual perception and phylogenetic relationships where necessary (Darst, Cummings and Cannatella, 2006; Cortesi and Cheney, 2010; Wang, 2011; Blount *et al.*, 2012; Maan and Cummings, 2012; Winters *et al.*, 2014; Arenas, Walter and Stevens, 2015; Crothers *et al.*, 2016). Mirroring the contrasting predictions of the many theoretical investigations into the potential for honest signalling in aposematism (reviewed in Summers *et al.*, 2015; see Chapter 1), these studies have yielded conflicting results, with positive correlations between signals and defences emerging in some, but not all, cases. Models attempting to reconcile these observations have focused on the economics of signal and defence, proposing that correlations or disjunctions in the costs of these two strategic components of aposematism will shape the relationship between them, with honesty arising when costs increase in parallel (Speed and Ruxton, 2007). Nevertheless, a major obstacle to quantitative honesty in aposematism is the absence of a direct physiological link between signal and defence (Ruxton, Sherratt and Speed, 2004). The resource-limitation model, proposed by Blount *et al.* (2009), potentially resolves this issue by suggesting that signals and defences may be competing for shared resources, whether energy in general or specific nutrients, such as carotenoids or other antioxidants. Going some way towards addressing these ideas, recent studies have begun to measure the physiological underpinnings of colour signals and toxicity, such as hormone and carotenoid levels (Blount *et al.*, 2012; Crothers *et al.*, 2016) or sequestration ability (Mochida *et al.*, 2013). However, more empirical work is needed to truly understand when and why honest signals may or may not be observed in nature.

In the case of signal honesty, relating theoretical models to empirical data is made more difficult by a mismatch in focus: while many modelling studies relate to variation within populations, relatively few studies have investigated this in the wild (Summers *et al.*, 2015; Crothers *et al.*, 2016). In this study, I aim to start redressing the balance by focusing on variation within a single species, the six-spot burnet moth, *Zygaena filipendulae* L. (Lepidoptera: Zygaenidae). *Zygaena* species are classic examples of aposematic Lepidoptera, combining striking red and black wing patterns with potent chemical defences based on the cyanogenic glucosides linamarin and lotaustralin (Davis and Nahrstedt, 1979, 1982), which release hydrogen cyanide (HCN) when brought into contact with enzymes in larval haemolymph or in the gut of predators. Present throughout the Western Palearctic (Naumann, Tarmann and Tremewan, 1999), *Z. filipendulae* is also locally abundant in Cornwall (UK), enabling the collection of specimens from very distant populations in distinct habitat types, as well as of large samples from some local populations. Moreover, the cyanide-based defences of *Z. filipendulae* have been extensively studied since they were first identified (Davis and Nahrstedt, 1979), down to the genetic pathway controlling their synthesis (Zagrobelny *et al.*, 2009; Jensen *et al.*, 2011). For this investigation, measures of cyanogenic glucoside levels were obtained with an LC-MS technique refined for identifying linamarin and lotaustralin in zygaenid moth samples, and previously employed in numerous studies of their chemical defences (Zagrobelny *et al.*, 2004, 2007a,b, 2014, 2015; Fürstenberg-Hägg *et al.*, 2014a; Pentzold *et al.*, 2015, 2016).

Considering the importance of the relative costs of signals and defences in determining the evolution of quantitative signal honesty, understanding these costs in *Z. filipendulae* is crucial to predicting the relationship between colour and toxicity in this species. Uniquely among insects, the larvae of *Zygaena* species can acquire the same defensive compounds by both sequestering them from their host plants and synthesising them *de novo* (Fürstenberg-Hägg *et al.*, 2014b). The ability to synthesise linamarin and lotaustralin and tolerate these toxic compounds evolved first in the Zygaenidae; this then allowed *Zygaena* species, pre-adapted to handle cyanogenic glucosides, to adopt cyanogenic plants of the Fabaceae family as their hosts, thereby accessing the same chemical defences more cheaply (Niehuis *et al.*, 2007; Fürstenberg-Hägg *et al.*,

2014b). While larvae fed on acyanogenic host plants can largely compensate for the lack of these compounds in their diet, they develop more slowly, reach a lower mass at pupation and incur a higher mortality than individuals fed on cyanogenic plants, confirming that *de novo* synthesis is indeed energetically costly (Zagrobelny *et al.*, 2007a). *De novo* synthesis is thought to have been maintained to enable the larvae to keep a tight control over the ratio of linamarin to lotaustralin, relevant to other behaviours such as mate choice, regardless of the diversity in the cyanogenic content of their host plants (Fürstenberg-Hägg *et al.*, 2014a). The costs of producing the cyanogenic glucosides may be linked to nitrogen limitation, as investment in chemical defences competes with other products for nitrogen. Highlighting this trade-off, cyanogenic glucoside content decreases significantly during pupation, suggesting that these compounds are broken down to fuel metamorphosis, and especially the synthesis of chitin, the main constituent of the cocoon and pupal case (Zagrobelny and Møller, 2011; Fürstenberg-Hägg *et al.*, 2014a).

In addition, like the reds, oranges and yellows of pierid butterflies (Watt, 1964) and wood tiger moths, *Arctia plantaginis* (Lindstedt, 2016), the red colours of the Zygaeninae are generated by pterin, or pteridine, pigments, (Tremewan, 2006). Pterins are rich in nitrogen, so there may be a direct energetic trade-off between producing signals and additional defences *de novo* (Morehouse and Rutowski, 2010). Moreover, pterins are known to have antioxidant functions, playing an important role in protecting immune cells (McGraw, 2005). This suggests a potential trade-off between antioxidant function, safeguarding against stored toxins, and pigmentation, as has been proposed for carotenoids (Blount *et al.*, 2009, 2012). Although this study primarily focuses on the red wing markings, the dark background colour of the forewings may also be costly to produce. The melanin required to produce black scales is involved not only in pigmentation, but also in immune defences against parasitoids and thermoregulation in Lepidoptera (Lindstedt, Lindström and Mappes, 2009; Hegna *et al.*, 2013; Nokelainen, Lindstedt and Mappes, 2013), while iridescence is thought to be an expensive, condition-dependent form of coloration (Doucet and Meadows, 2009).

Signals and defences are thus both potentially costly for *Z. filipendulae* to produce, suggesting that honest signalling could arise in this system, depending on how these costs relate to each other. In this study, I examine the relationship between cyanogenic glucoside content and several measures of coloration to determine whether this species does display quantitatively honest signals, across 12 distinct localities, and in more detail within 3 populations with larger sample sizes. Diversity in warning signals between and within populations may be determined by different mechanisms and selective pressures (Summers *et al.*, 2015), so it is important to consider both levels of variation. Within populations, predator learning may be important to the maintenance of signal honesty, while differences in the predator community, habitat or other characteristics could alter the relative costs of signals and defences between populations (see Speed and Ruxton, 2007). I also carried out an experiment manipulating the availability of cyanogenic glucosides in the larval diet, to test whether limiting resources would affect the moths' relative investments in toxins and pigments. Along with precise quantification of toxins by LC-MS, visual system-dependent measures of coloration, based on models of avian vision, were used to assess variation in colour as perceived by potential predators. As such, these results should contribute new insights into honest signalling within and between populations of a single aposematic species.

4.3 Methods

4.3.1 Specimen collection and rearing

Although my work focuses on adult coloration and toxicity, all individuals included in this study were collected at the larval or pupal stages, to ensure that only virgin moths were used. This is critical to obtaining meaningful results, as the levels of cyanogenic glucosides fluctuate greatly during reproduction: males transfer a significant nuptial gift of linamarin and lotaustralin to females during mating, accounting for approximately 30% of their body mass and cyanogenic glucoside content (Zagrobelny *et al.*, 2007b, 2013), while females deposit cyanogenic glucosides in their eggs (Zagrobelny *et al.*, 2007a). Larvae and pupae of *Z. filipendulae* were collected from April to June 2015, at a range of sites in the United Kingdom, France and Denmark (Figure 4.1; Appendix 2.1, restricted to specimens from 2015). The insects were reared in the laboratory

until emergence of the adults. They were individually housed in plastic boxes with air holes, inside an incubator at 20°C with a 16:8h day:night cycle, similarly to previous work on this species (Zagrobelny *et al.*, 2007a). Larvae were reared with the same host plant species as they were found on in the field. For larvae from France, three different host plants were used (common bird's foot trefoil, *Lotus corniculatus* L., prostrate canary clover, *Dorycnium pentaphyllum* Scop. and horseshoe vetch *Hippocrepis comosa* L.); where possible, cuttings from plants on local field sites were used, as well as *D. pentaphyllum* plants from a commercial nursery (Les Senteurs du Quercy, Mas de Fraysse, 46230 Escamps, France). Larvae found on *L. corniculatus* were fed cuttings from plants grown in greenhouses from commercially-sourced plugs (Wildflower Shop, Elm House, Green Street, Suffolk, IP21 5AZ, UK). All larvae were fed *ad libitum*, with food replaced daily for freshness. A total of 107 adults emerged with undamaged wings and were used in subsequent photography and toxicity analyses (N_{TOTAL}=107 $N_{DENMARK}$=25, N_{FRANCE}=18, $N_{UNITED\ KINGDOM}$=64).

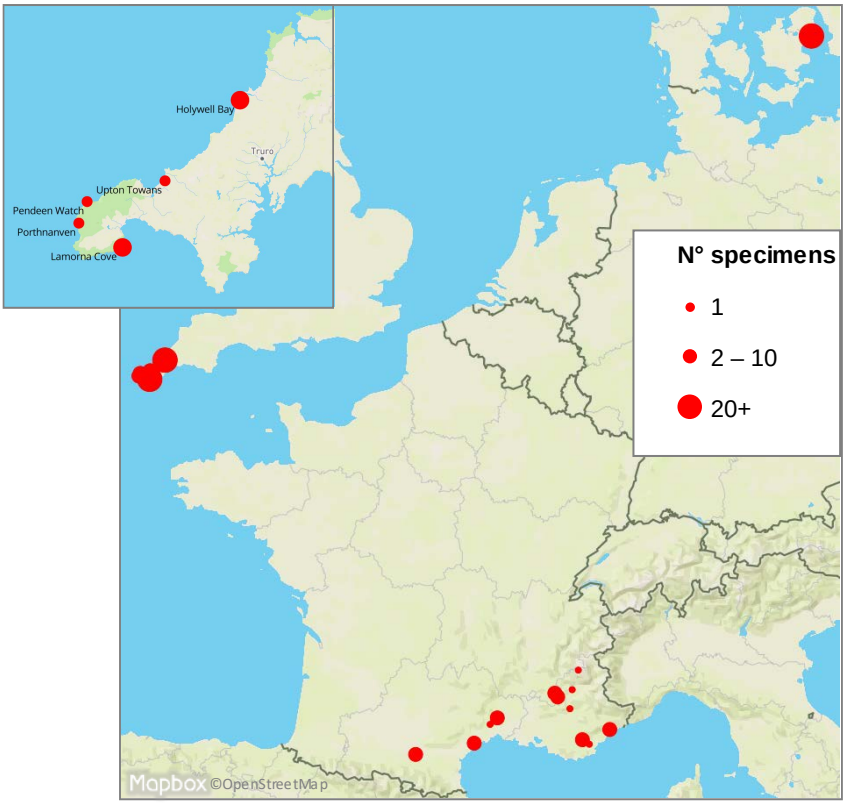


Figure 4.1: Map of field sites on which *Z. filipendulae* larvae and pupae were collected, with inset details of Cornish sites (GPS coordinates in Appendix 2.1). Specimen numbers refer to adults photographed from each site.

4.3.2 Wing photography and image analysis

As soon as the adults emerged and their wings were fully expanded, they were euthanised by placing them in a -80°C freezer. I determined the sex and mass of each individual, before dissecting and photographing their wings with a calibrated, UV-sensitive digital camera (Nikon D7000 fitted with a 105mm CoastalOptics quartz lens). Photographs were taken in controlled conditions in a dark room, illuminated by an EYE Color Arc® MT70 bulb (Iwasaki Electric Co. Ltd.), emitting a spectrum of light similar to D65 daylight conditions. Each image included a scale bar, label, and a set of reflectance standards, reflecting 7% and 93% of all wavelengths of light respectively (Zenith Lite Diffuse Target sheets, SphereOptics, Pro-Lite Technology, Cranfield, UK), so as to further control for any variation in lighting conditions. As the wings of *Z. filipendulae* are iridescent, and thus the angle of incident light on the scales affects the colour of the wings, the light source was fixed in a constant position, at a 50° angle relative to the wings, in all photographs, and only the right-hand wings were used for colour measurements. Each specimen was photographed twice, using different filters (a UV/infrared blocking filter [Baader UV/IR Cut Filter], transmitting between 300 and 700nm, and a UV pass and IR blocking filter [Baader U filter], transmitting between 300 and 400nm). Combining these photographs yields a set of five image layers, or channels, corresponding to different parts of the visual spectrum: vR, vG, vB, uR and uB (see Chapter 2 for further details).

I performed all subsequent image analysis with a dedicated image calibration and analysis toolbox in ImageJ (Troscianko and Stevens, 2015). To account for the camera's non-linear response to different wavelengths of light, and changes in ambient light conditions (Stevens *et al.*, 2007a), images were linearised and normalised as per the methods described in the software guide (see Chapter 2 for more details). The wing colours were then analysed from the perspective of potential predators, which in this case are most likely to be birds, with reports of attacks on burnet moths attributed to a range of species, including blackbirds (*Turdus merula*), skylarks, (*Alauda arvensis*), cuckoos (*Cuculus canorus*), house sparrows (*Passer domesticus*), starlings (*Sturnus vulgaris*) and meadow pipits (*Anthus pratensis*) (Tremewan, 2006). In order to do this, I mapped the moth wing images to the two known categories of avian visual system, which

differ in the sensitivity of their most shortwave-sensitive cone type, the violet-sensitive (VS) and ultraviolet-sensitive (UVS) groups (Hart *et al*. 1999). I used data from their respective model species, the blue tit *Cyanistes caeruleus* (Hart *et al.*, 2000) and the peafowl *Pavo cristatus* (Hart, 2002). With the same software package (Troscianko and Stevens, 2015), linearised and normalised images were transformed to avian vision via a polynomial mapping technique with a D65 irradiance spectrum (Westland and Ripamonti, 2004; Stevens *et al.*, 2007a; Pike, 2011; Troscianko and Stevens, 2015), yielding five image layers, with predicted cone catch values for each photoreceptor type: ultraviolet (UV or V), short wavelength (SW), medium wavelength (MW) and long wavelength (LW) sensitive photoreceptors, as well as the double cones. I selected the wing markings and background areas on each photograph using the freehand tool in ImageJ, as described in Chapter 2. While the position of the camera relative to each specimen was the same for all photographs, all images were also scaled to 100 pixels/mm to eliminate any small differences, which would affect size measurements. Each forewing spot was precisely outlined to allow for accurate measurements of its area, and if the spot was damaged separate measurements of undamaged sections were taken for spot colour. To measure the dark scales of the forewings and the red scales of the hindwings, zones as large as possible were selected, while avoiding damaged areas and creases in the fragile wings. Cone catch values for every photoreceptor type were measured from each selected patch, then averaged to obtain a single measure of colour per wing marking type. My analysis focuses primarily on the moths' red markings, as red coloration is a widespread and particularly effective aposematic signal (Stevens and Ruxton, 2012; Arenas, Troscianko and Stevens, 2014). However, I also measured the dark background colours to calculate chromatic and luminance contrasts between the markings and background areas of each wing.

From the cone catch values, I calculated three metrics for the red markings of the fore- and hindwings: luminance, saturation and hue. The rationale behind, and methods used to obtain these values are described in detail in Chapter 2. In brief, luminance provides a visual system-dependent measure of brightness, while saturation and hue respectively describe the intensity and perceived shade of a colour. Luminance is equal to the cone catch value for the double

cones, thought to mediate the perception of brightness contrasts in birds (Jones and Osorio, 2004; Osorio and Vorobyev, 2005). To derive a measure of saturation, colours were plotted in a tetrahedral colour space, with the coordinates corresponding to the proportion of total cone catch values to each channel: ultraviolet (UV-), short wavelength (SW-), medium wavelength (MW-) and long wavelength (LW-) sensitive. Saturation then corresponds to the Euclidean distance between the colour of interest and the centre of the colour space (Endler and Mielke, 2005; Stoddard and Prum, 2008). Finally, as seen in previous studies of animal coloration (Spottiswoode and Stevens, 2011; Stevens, Lown and Wood, 2014a,b), estimates of hue were based on the concept of colour opponency. Similarly to human vision, opponent mechanisms are known to be important for processing colour signals in birds (Osorio, Vorobyev and Jones, 1999), although the exact opponent channels have not as yet been elucidated. Nevertheless, principal component analysis (PCA) can be used to estimate the principal axes of variation in colour between samples. Following Spottiswoode and Stevens (2011), I performed PCA on a covariance matrix of the standardised values of all colour patches for the four photoreceptor channels (UV, SW, MW, LW) for each visual system. The first two principal components thus obtained were used to calculate ratios of cone catch values, forming logical colour channels (Hue1 and Hue2) – these do not represent actual opponent channels but provide meaningful measures of hue, broadly inspired by opponent mechanisms. The specific equations for hue used here are as follows:

$$Hue1_{UVS} = \frac{LW}{(UV+SW+MW)/3} \quad (4.1)$$

$$Hue2_{UVS} = \frac{(SW+MW+LW)/3}{UV} \quad (4.2)$$

$$Hue1_{VS} = \frac{LW}{(UV+SW+MW)/3} \quad (4.3)$$

$$Hue2_{VS} = \frac{MW+LW}{UV+SW} \quad (4.4)$$

UV, SW, LW, MW = standardised cone catch values for the UV-, SW-, MW- and LW- sensitive photoreceptors respectively. UVS, VS = ultraviolet-sensitive (blue tit) visual system, violet-sensitive (peafowl) system.

The hue channels were identical for fore- and hindwing markings. Based on these equations, high values of Hue1 correspond to colours with relatively greater reflectance in the long wavelength (LW) colour channel than in the short, medium and ultraviolet wavelength channels (SW, MW, UV), so represent redder colours. Only Hue1 values were used in subsequent analyses, as the principal components from which Hue1 ratios are derived (henceforth referred to as hue) account for 81-95% of the variance in colour in the fore- and hindwing markings. Further information on the methods for computing hue values can be found in Chapter 2.

In addition, I calculated two measures of visual contrast to provide a sense of the perceived differences between red and black areas on the moths' forewings and hindwings. The salience of these internal contrasts constitutes an important feature of warning signals, affecting predator learning (Aronsson and Gamberale-Stille, 2012b; Barnett, Scott-Samuel and Cuthill, 2016). Chromatic contrast was calculated with a widely-used log version of the receptor noise-limited Vorobyev-Osorio colour discrimination model (Vorobyev and Osorio, 1998), which takes into account the sensitivity and abundance of each cone type (relative cone abundance is UV=1, SW=1.92 MW=2.68, LW=2.7 for the UVS system (Hart *et al.*, 2000) and V=1, SW=1.9, MW=2.2, LW=2.1 for the VS system (Hart, 2002; Håstad, Victorsson and Ödeen, 2005), as well as the noise in the photoreceptors. Noise was calculated with a widely-used and relatively conservative estimate of the Weber fraction, $\omega = 0.05$, for the most abundant cone type (Eaton, 2005; Håstad, Victorsson and Ödeen, 2005; Stevens, 2011; Stevens, Lown and Wood, 2014a). Luminance contrast was computed as the natural logarithm of the ratio between mean double cone catch values of background and marking areas, divided by the same Weber fraction (Siddiqi *et al.*, 2004). Contrast values are measured in "just-noticeable differences" or JNDs: values between 1 and 3 indicate that colours are likely to be distinguishable under good lighting conditions, while those below this threshold are likely indiscriminable. Colours with JNDs above 3 should be increasingly easy to tell apart (Siddiqi *et al.*, 2004). More details concerning these calculations can be found in Chapter 2.

4.3.3 Contrast to natural backgrounds

Conspicuousness of prey to the natural backgrounds on which they are found is a key component of aposematic signalling (Stevens and Ruxton, 2012; Arenas, Walter and Stevens, 2015), which should be measured more often in empirical studies of honest signalling (Arenas, 2015). To address this issue, I calculated chromatic and luminance contrasts between the moth's wing markings and three likely natural backgrounds: the leaves and flowers of their principal host plant (*Lotus corniculatus,* Fabaceae) and a popular nectaring flower, field scabious (*Knautia arvensis*, Dipsacaceae) (Naumann, Tarmann and Tremewan, 1999; Zagrobelny *et al.*, 2015). I photographed five independent samples of each plant, collected in Cornwall (UK), with the same equipment and under the same conditions as the moth wings. *L. corniculatus* flowers were dissected so that the upper and lower petals (known as the banner and wings respectively) could be photographed as flat as possible. Plant areas for analysis were once again selected using the freehand tool in Image J: each of the three leaflets of every *L. corniculatus* leaf, each petal from the *L. corniculatus* flowers, and three outer petals and the central area of *K. arvensis* flowers. I then averaged these colour measurements to obtain a single value per plant background type, and calculated contrasts between these values and those of the moth forewing markings using chromatic and luminance JNDs, as described above.

4.3.4 Quantifying cyanogenic glucosides

The cyanogenic glucoside content of each specimen was determined by Dr. Mika Zagrobelny, in the Department of Plant and Environmental Sciences at the University of Copenhagen. Measurements were obtained with a specific liquid chromatography – mass spectrometry (LC-MS) protocol optimized for detecting cyanogenic glucosides, such as linamarin and lotaustralin in extracts from plants and insects. Prior to LC-MS analysis, the frozen samples were each ground up in 1ml ice-cold 55% MeOH, containing 0.1% formic acid and 0.044mM amygdalin, a cyanogenic glycoside not present in the Zygaenidae, as an internal standard. All samples were subsequently passed through an Anopore 0.45µm filter (Whatman) and analytical LC-MS was carried out using an Agilent 1100 Series LC (Agilent Technologies, Germany), interfaced with a Bruker HCT-Ultra ion trap mass spectrometer (Bruker Daltonics, Bremen,

Germany). Chromatographic separation was performed with a Zorbax SB-C18 column (Agilent; 1.8µM, 2.1x50 mm) at a flow rate of 0.2 ml/min, increased to 0.3 ml/min from 11.2 to 13.5 min. Oven temperature was maintained at 35°C and the mass spectrometer was run in positive electrospray mode. The mobile phases were A (H_2O with 0.1% (v/v) HCOOH, 50 µM NaCl) and B (MeCN with 0.1% (v/v) HCOOH), with a gradient program as follows: 0 to 0.5 min, isocratic 2% B; 0.5 to 7.5 min, linear gradient 2 to 40% B; 7.5 to 8.5 min, linear gradient 40% to 90% B; 8.5 to 11.5 isocratic 90% B; 11.6 to 17 min, isocratic 2% B. Mass spectral data were analysed with the native data analysis software, to detect sodium adducts of linamarin (retention time [RT] 2.6 min, $[M+Na]^+$ at *m/z* 270), lotaustralin (RT 5.5 min, $[M+Na]^+$ at *m/z* 284), and amygdalin (RT 6.6 min, $[M+Na]^+$ at *m/z* 480), then compare them to authentic standards (Møller, Olsen and Motawia, 2016). The total amount of each compound was estimated according to its Extracted Ion Chromatogram (EIC) peak areas and quantified based on calibration curves of linamarin, lotaustralin, and amygdalin standards.

4.3.5 Dietary manipulations

To test the effect of increasing the costs of acquiring chemical defences, I collected additional larvae (at L5-L6 stage) from three locations in Cornwall (Holywell Bay, Porthnanven and Upton Towans) in May and June 2015, to participate in a dietary experiment. These larvae were housed as above, but were fed *ad libitum* with plants grown from cuttings of an acyanogenic *L. corniculatus* plant originally collected at the Botanical Garden of the University of Copenhagen. This plant contains no cyanogenic glucosides and has previously been used in dietary manipulations with *Z. filipendulae* larvae (Zagrobelny *et al.*, 2007a). Due to differences in larval stage when collected and high mortality when feeding on the acyanogenic host, only 25 larvae survived to adulthood, with 10 fed on acyanogenic plants for at least 10 days before pupation. Colour and toxicity measurements were taken from the adult moths as described above. For the purposes of this study, I compared these 10 individuals to moths from the same populations, which had been fed on cyanogenic *L. corniculatus* (henceforth wild-type, WT; N=30). For the image analysis, I selected the colour patches while blind to dietary treatment.

4.3.6 Statistical analyses

I analysed all results using R 3.3.1 (R Development Core Team, 2015). Forewing and hindwing data were treated separately, and all analyses were repeated with data from both the UVS (blue tit) and VS (peafowl) visual systems. For all linear models in both within and between population analyses, assumptions were checked with diagnostic plots, and minimal adequate models were obtained via stepwise model simplification. If any outliers were identified (Cook's distance >1 in diagnostic plots), the models were run with and without these data points to test their influence; results are only reported without outliers if their removal significantly affected the model output. Tukey's post-hoc tests were implemented to determine significant pairwise comparisons, using the glht function in the 'multcomp' package in R (Hothorn, Bretz and Westfall, 2008).

Following a similar approach to a previous study of signal honesty among poison frog populations (Maan and Cummings, 2012), I examined correlations between mean cyanogenic glucoside levels and mean colour values between populations, for all colour metrics. Linear models testing the relationship between colour and toxin levels were run for each sex separately, as varying numbers of males and females were sampled in each population, potentially affecting the outcome of models based on a single average per population.

To explore the question of honesty in aposematic signalling within populations, I investigated three populations in more detail (Holywell Bay, UK, Lamorna Cove, UK, and Taastrup, Denmark, where N>20). I used multiple linear regressions to test the relationship between the concentration of cyanogenic glucosides in each sample and wing coloration, in each population separately. The presence of two outlier points significantly affected results in Lamorna Cove, so analyses for this species were performed with a reduced dataset (N=23). Each model included all relevant colour metrics for either the forewing or hindwing markings, with one exception. Saturation and hue values were calculated from the same cone catch values, so as expected, were highly correlated (Pearson's correlation > 0.99). Linear regression models thus included either saturation or hue, to avoid the problem of high collinearity in the analysis; models including only one of these measures of colour had variance inflation factors (VIFs) below the recommended threshold of 10 (Dormann *et al.*, 2013) and yielded the same

conclusions (see Appendix 4.1). Stepwise model simplification was carried out to identify the minimal model in each case. I then investigated sex differences in coloration in these three populations, with linear models allowing population and sex to interact. I also analysed contrasts between forewing markings and natural backgrounds with linear mixed effects models (LME), including sex, population and plant type as fixed effects and individual ID as a random effect, using the package 'lme4' (Bates *et al.*, 2014). Model diagnostics were checked using the mcp.fnc function in the 'LMERConvenienceFunctions' package (Tremblay and Ransijn, 2014). To fit model assumptions, luminance contrast was transformed with the logit function in the 'car' package (Fox and Weisberg, 2011). Finally, I also used mixed effects models, including population as a random effect, to test the effect of dietary treatment on cyanogenic glucoside concentration, body mass and coloration. Concentration of toxins was log-transformed to fit the assumptions of linear mixed effects models.

4.4 Results

For clarity, the following result descriptions, tables and graphs are all based on the ultraviolet-sensitive (UVS, or blue tit) visual system only. Results for the violet-sensitive (VS, or peafowl) visual system were qualitatively similar; details can be found in Appendix 4.4.

4.4.1 Variation between populations

There was no significant relationship between colour metrics and toxin concentration across populations for males (Table 4.1). In addition, measures of hindwing coloration were not significantly associated with cyanogenic glucoside levels in either sex. However, for females, there were some relationships between defences and forewing coloration: the mean concentration of defensive compounds per population was significantly positively correlated with marking luminance but negatively correlated with relative spot area, and there were other trends towards a negative relationship between colour metrics and toxin levels (Figure 4.2, Table 4.1).

Table 4.1: Correlations between colour metrics and cyanogenic glucoside concentrations across populations. Significant results are highlighted in italics. A relatively high R^2 value for the positive correlation between toxicity and luminance in females suggests this may be the most relevant result, while low R^2 values for relationships with p-values near the significance threshold ($p<0.05$) indicate that these are unlikely to be biologically important. FW=forewing, HW=hindwing.

Colour metric	**Males**	**Females**
FW luminance	No correlation, $F_{1,7}=0.451$, $p=0.523$, $R^2=-0.0737$	*Positive correlation, $F_{1,9}=16.466$, $p=0.00285$, $R^2=0.607$*
FW saturation	No correlation, $F_{1,7}=0.238$, $p=0.641$, $R^2=-0.105$	Trend towards negative correlation, $F_{1,9}=4.349$, $p=0.0667$, $R^2=0.251$
FW hue	No correlation, $F_{1,7}=0.114$, $p=0.746$, $R^2=-0.125$	Trend towards negative correlation, $F_{1,9}=4.061$, $p=0.0747$, $R^2=0.234$
FW chromatic contrast	No correlation, $F_{1,7}=0.812$, $p=0.397$, $R^2=-0.0240$	Trend towards negative correlation, $F_{1,9}=5.032$, $p=0.0516$, $R^2=0.287$
FW luminance contrast	No correlation, $F_{1,7}=0.414$, $p=0.541$, $R^2=-0.0791$	No correlation, $F_{1,8}=1.064$, $p=0.329$, $R^2=0.00639$
Proportion of red in FWs	No correlation, $F_{1,7}=0.0034$, $p=0.955$, $R^2=-0.142$	*Negative correlation, $F_{1,9}=5.252$, $p=0.0476$, $R^2=0.298$*
HW luminance	No correlation, $F_{1,7}=0.291$, $p=0.606$, $R^2=-0.0972$	No correlation, $F_{1,9}=1.782$, $p=0.215$, $R^2=0.0725$
HW saturation	No correlation, $F_{1,7}=0.183$, $p=0.682$, $R^2=-0.114$	No correlation, $F_{1,9}=0.0005$, $p=0.983$, $R^2=-0.111$
HW hue	No correlation, $F_{1,7}=0.0908$, $p=0.772$, $R^2=-0.128$	No correlation, $F_{1,9}=0.0047$, $p=0.947$, $R^2=-0.111$

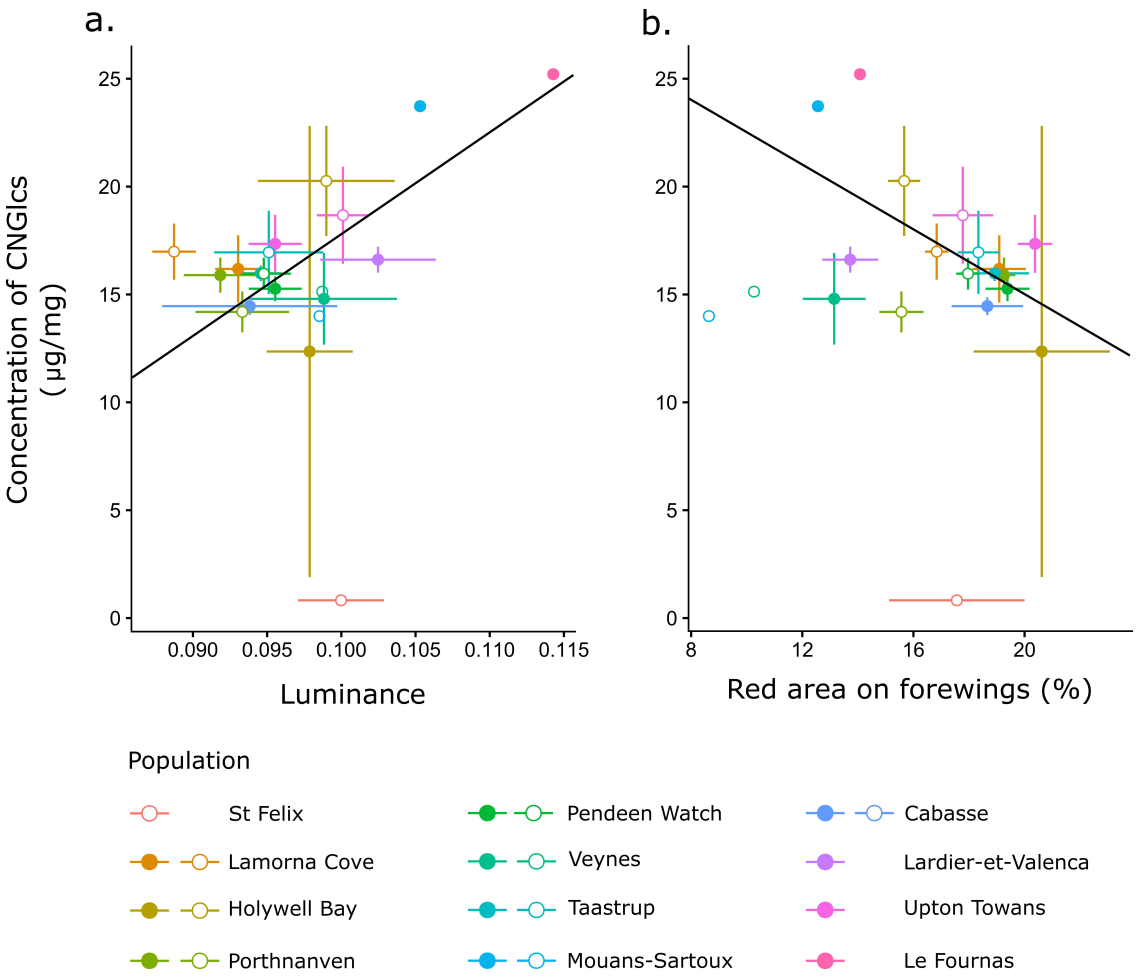


Figure 4.2: Mean cyanogenic glucoside concentration and marking luminance (a) and size (b) across populations, for males (open circles) and females (filled circles). Error bars correspond to standard errors for both colour metrics and toxin concentration. CNGlcs = cyanogenic glucosides. Lines represent the linear relationship between colour metrics and cyanogenic glucoside concentration for females.

4.4.2 Variation within populations

4.4.2.i Cyanogenic glucoside concentration and coloration

In both the Holywell Bay and Taastrup populations, forewing luminance was positively correlated with cyanogenic glucoside concentration (linear models, luminance, $F_{1,20}$=4.358, p=0.0499, and $F_{1,23}$=6.768, p=0.0160 respectively; Figure 4.3a, Appendix 4.1). Moreover, in the Holywell Bay population, chromatic contrast between the forewing background and marking colours was negatively correlated with cyanogenic glucoside concentration (linear model, chromatic contrast, $F_{1,20}$=5.645, p=0.0276; Figure 4.3b). In contrast, cyanogenic glucoside

levels were not correlated with any colour metrics in Lamorna Cove (Appendix 4.1).

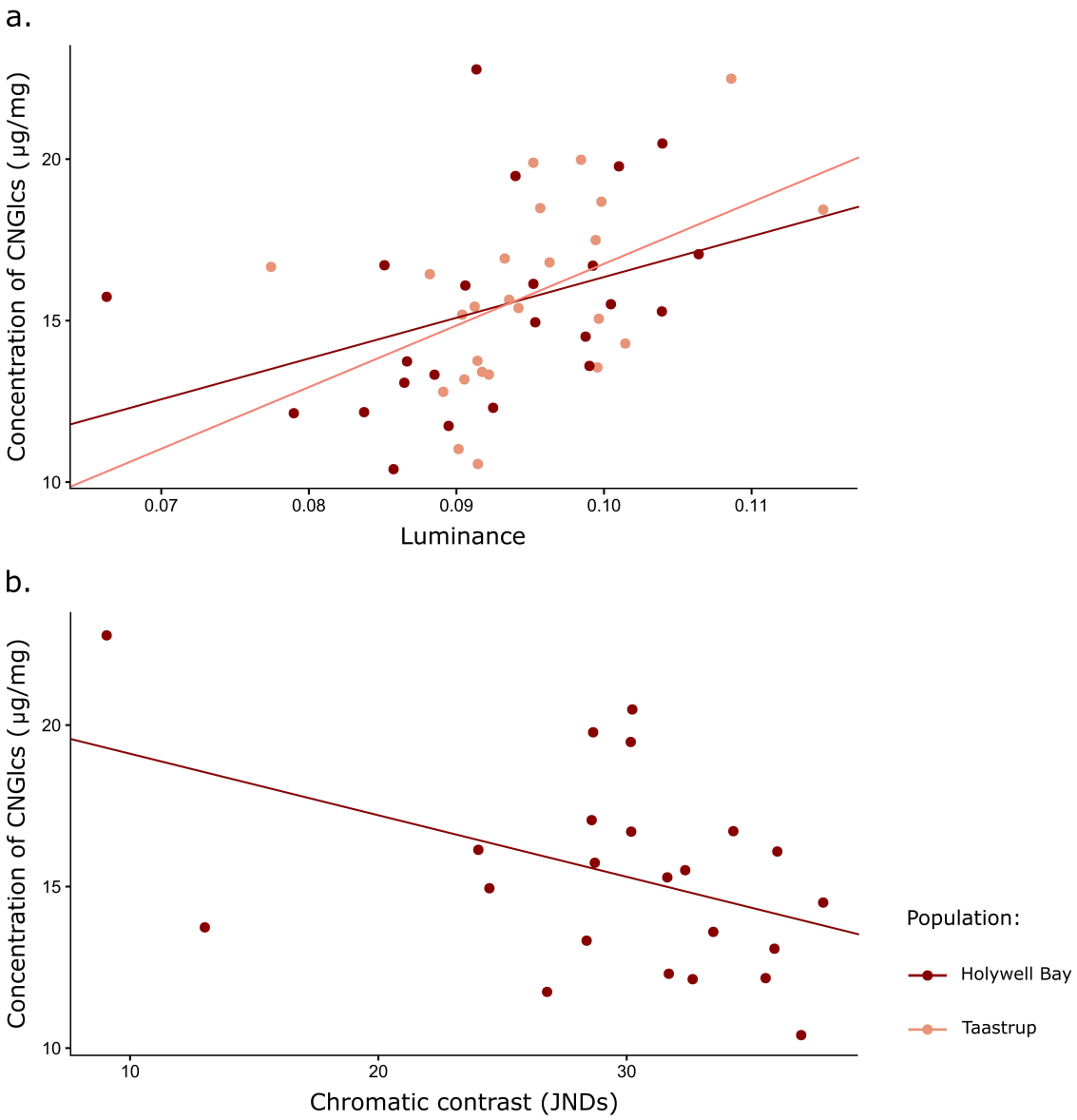


Figure 4.3: Relationship between forewing luminance (a), chromatic contrast (b) and the concentration of cyanogenic glucosides, in the Holywell Bay and Taastrup populations. CNGlcs = cyanogenic glucosides.

4.4.2.ii Differences between sexes

Toxin levels did not significantly differ between sexes (linear model, sex, $F_{1,69}$=0.0002, p=0.990). However, the total amount of cyanogenic glucosides was significantly different: larger females possessed consistently greater amounts of these compounds than males, in all three populations (linear model,

sex, $F_{1,69}$=107.31, p<0.001). Moreover, males and females differed in all colour metrics, with the exception of forewing luminance. Saturation, hue and chromatic contrast of the red forewing markings were higher in males than females, while female markings were larger relative to total wing area (Table 4.2a, Figure 4.4a). Forewing luminance contrast was higher in males than females in Lamorna Cove, but not in the other populations (Table 4.2a, Figure 4.4a). In the hindwings, luminance was higher in females, but saturation and hue values were again greater in males (Table 4.2b, Figure 4.4b). Populations also differed overall in some metrics: chromatic contrast was higher in Lamorna Cove than in the Taastrup population (Tukey's HSD: $p_{Lamorna\text{-}Holywell}$=0.116, $p_{Taastrup\text{-}Holywell}$=0.897, $p_{Lamorna\text{-}Taastrup}$=0.0334) and hindwing luminance was lower in the Lamorna Cove population than in the others (Tukey's HSD: $p_{Lamorna\text{-}Holywell}$=0.00939, $p_{Taastrup\text{-}Holywell}$=0.999, $p_{Lamorna\text{-}Taastrup}$=0.00739).

Table 4.2: Results of linear models examining sex and population differences in colour metrics. Significant results are highlighted in italics.

a. In the forewings

Factor	**F**	**df**	**p**	**F**	**df**	**p**	**F**	**df**	**p**
	Luminance			Saturation			Hue		
Sex:Population	0.843	2,67	0.435	1.166	2,67	0.318	1.285	2,67	0.283
Population	2.388	2,69	0.0993	1.945	2,69	0.151	1.347	2,69	0.267
Sex	0.0824	1,71	0.775	4.073	1,71	*0.0472*	4.696	1,71	*0.0336*
	Proportion red			Chromatic contrast			Luminance contrast		
Sex:Population	1.509	2,67	0.229	0.475	2,67	0.624	4.565	2,67	*0.0138*
Population	2.283	2,69	0.110	3.786	2,69	*0.0276*	-	-	-
Sex	17.766	1,71	*<0.001*	12.902	1,69	*<0.001*	-	-	-

b. In the hindwings

Factor	**F**	**df**	**p**	**F**	**df**	**p**	**F**	**df**	**p**
	Luminance			Saturation			Hue		
Sex:Population	0.246	2,67	0.770	1.208	2,67	0.305	1.183	2,67	0.313
Population	6.614	2,69	*<0.01*	1.011	2,69	0.369	0.690	2,69	0.505
Sex	16.199	1,69	*<0.001*	36.192	1,71	*<0.001*	12.19	1,71	*0.001*

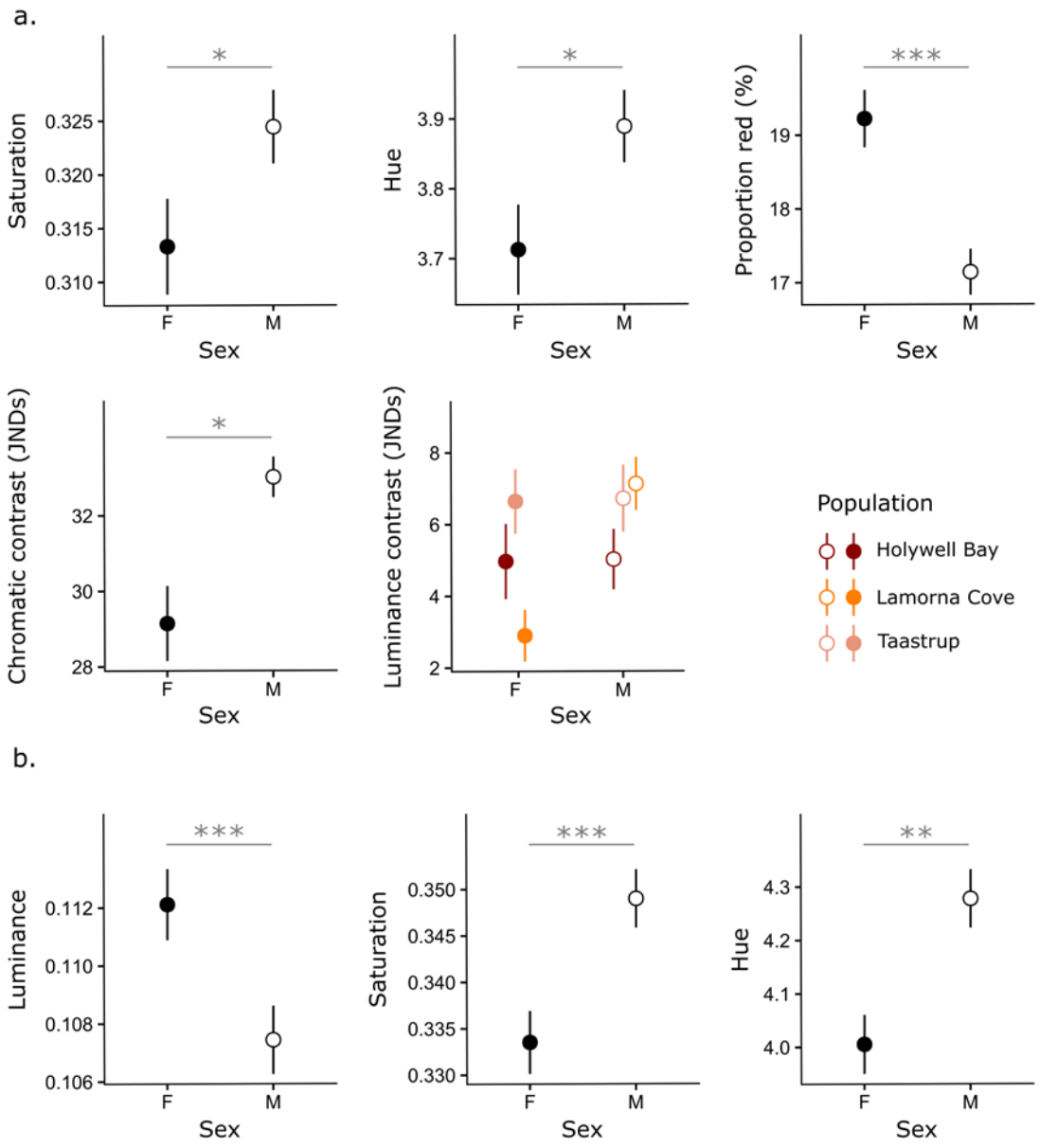


Figure 4.4: Mean and standard error for colour metrics in the forewings (a) and hindwings (b) of specimens from Holywell Bay, Lamorna Cove and Taastrup. Filled circles represent females, open circles males. Luminance contrast is plotted by population, as the relationship between sex and this metric varies between localities. Significance levels: ***:p<0.001, **:p<0.01, *:p<0.05.

4.4.2.iii Contrast to natural backgrounds

Chromatic contrast to plant tissues on which *Z. filipendulae* are likely to be observed was higher in males than females (LME, sex, $(\chi^2)_2$=4.752, p=0.0293), and lowest overall in the Taastrup population (LME, population, $(\chi^2)_2$=20.23, p<0.001, Tukey's HSD: $p_{Lamorna\text{-}Holywell}$=0.163, $p_{Taastrup\text{-}Holywell}$=0.0253, $p_{Taastrup\text{-}Lamorna}$<0.0001; Figure 4.5a). However, luminance contrast did not vary

according to population or sex (LME, sex:population, $(\chi^2)_2$=1.667, p=0.435; sex: $(\chi^2)_1$=0.48, p=0.488; population, $(\chi^2)_2$=4.429, p=0.109). The forewing markings were consistently least conspicuous against the leaves of their host plant, *Lotus corniculatus*, and most conspicuous against its flowers (LME, plant type, $(\chi^2)_2$=768.12, p<0.001 and $(\chi^2)_2$=705.96, p<0.001 for chromatic and luminance contrast respectively; Tukey's HSD, p<0.001 for all pairwise comparisons; Figure 4.5). It is important to note that contrast values were consistently greater than the threshold for discrimination (JND=3), and were especially high in chromatic terms, making all forewing markings conspicuous, regardless of population, sex and plant type differences.

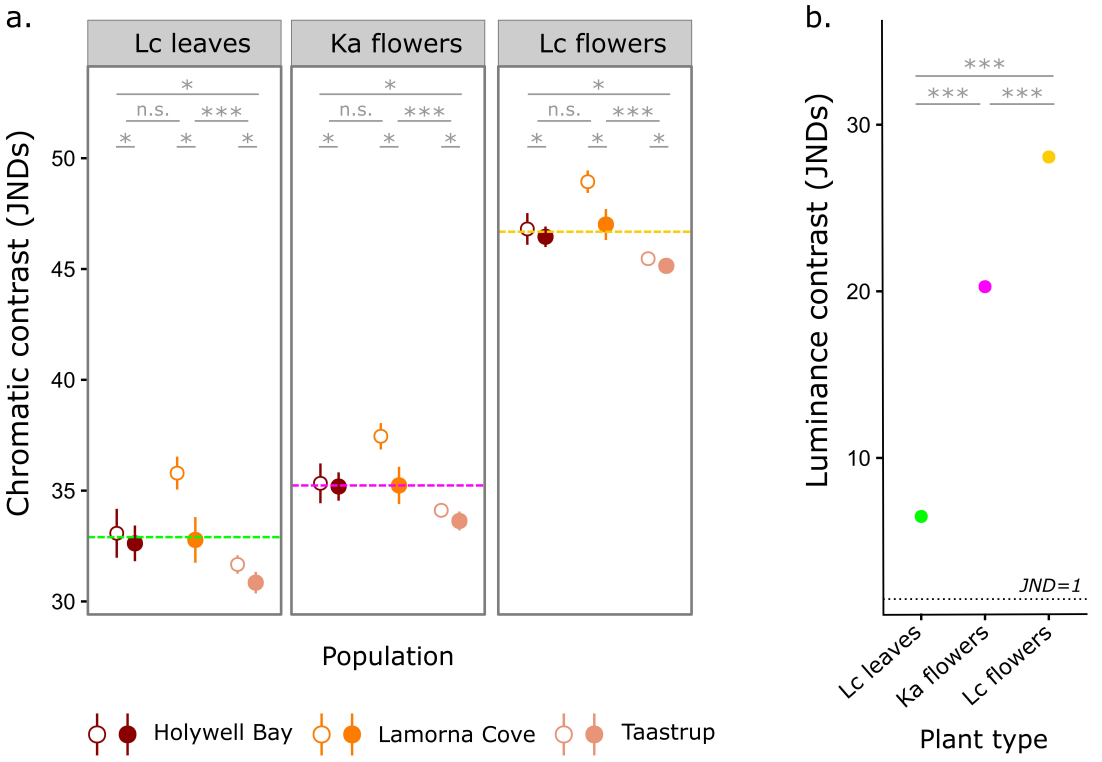


Figure 4.5: Mean and standard errors for chromatic (a) and luminance (b) contrast between forewing markings and natural backgrounds. In (a), filled circles represent females, open circles males, and dashed lines represent the mean chromatic contrast for each plant type. In (b), mean values were calculated across males and females, as there were no significant differences between sexes. The dotted line represents the threshold for easy discrimination, JND=3. Lc=*Lotus corniculatus*, Ka=*Knautia arvensis*. Significance levels: ***:p<0.001, **:p<0.01, *:p<0.05.

4.4.3 Consequences of dietary manipulations

Individuals fed acyanogenic host plants compensated for their poor diet with *de novo* synthesis of cyanogenic glucosides: in fact, the concentration of glucosides in adult tissues was higher in moths fed the acyanogenic diet (LME, diet, $(\chi^2)_1$ =4.241, p=0.00395; Figure 4.6a). Nevertheless, moths in the acyanogenic treatment had a smaller body mass at emergence (LME, diet, $(\chi^2)_1$ =8.100, p=0.00443), and as a consequence, females fed acyanogenic plants possessed a smaller total amount of cyanogenic glucosides than females in the WT treatment (LME, sex:diet, $(\chi^2)_1$ = 6.589, p=0.0103; Figure 4.6b).

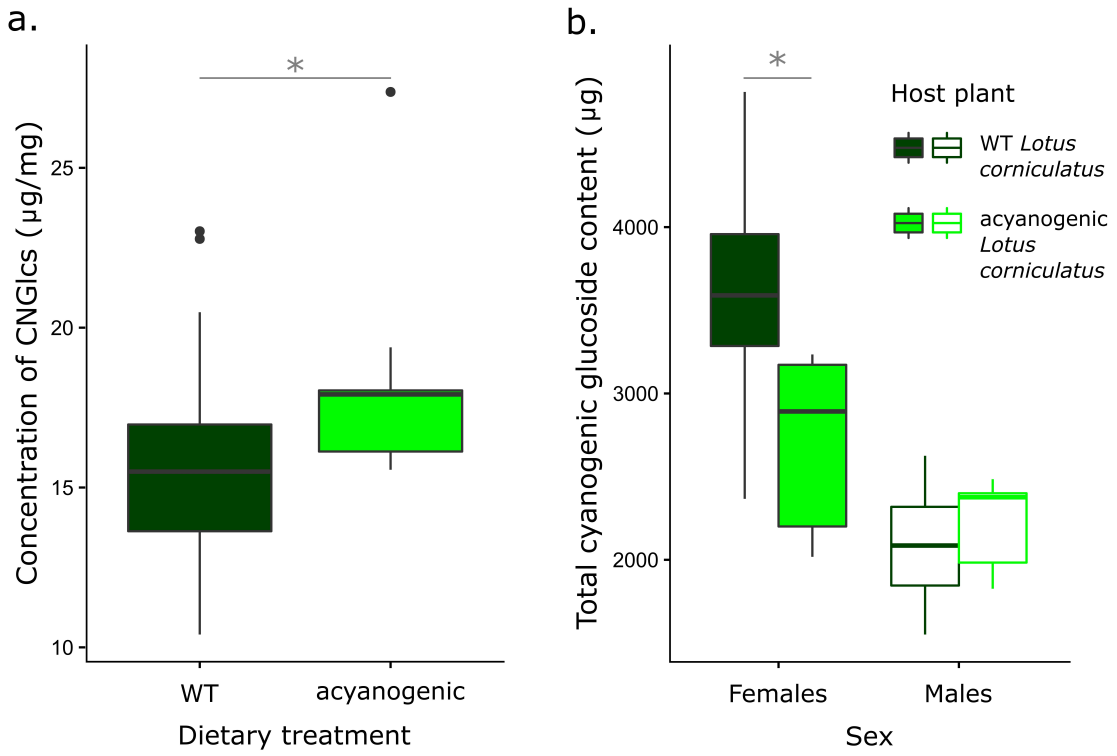


Figure 4.6: Concentration of cyanogenic glucosides across both sexes (a) and total cyanogenic content in males and females (b), plotted by dietary treatment. Boxplots show median and interquartile range. CNGlcs=cyanogenic glucosides. Significance levels: *:p<0.05.

There was no overall difference in colour metrics between moths fed different diets (see Appendix 4.2), yet the dietary treatment did affect the relationship between some measures of colour and cyanogenic glucoside levels (see Appendix 4.3). As seen in the Holywell Bay and Taastrup populations, forewing luminance and the concentration of cyanogenic glucosides were positively

correlated in the wild-type treatment, but this relationship was reversed in the acyanogenic treatment, especially for males (LME, luminance:diet:sex $(\chi^2)_1$=4.715, p=0.0299; Figure 4.7). Moreover, the relative size of the red markings on the forewings and cyanogenic glucoside levels were positively correlated in the wild-type treatment, and negatively in moths fed acyanogenic plants (LME, percent red:diet $(\chi^2)_1$=5.099, p=0.0239; Figure 4.8).

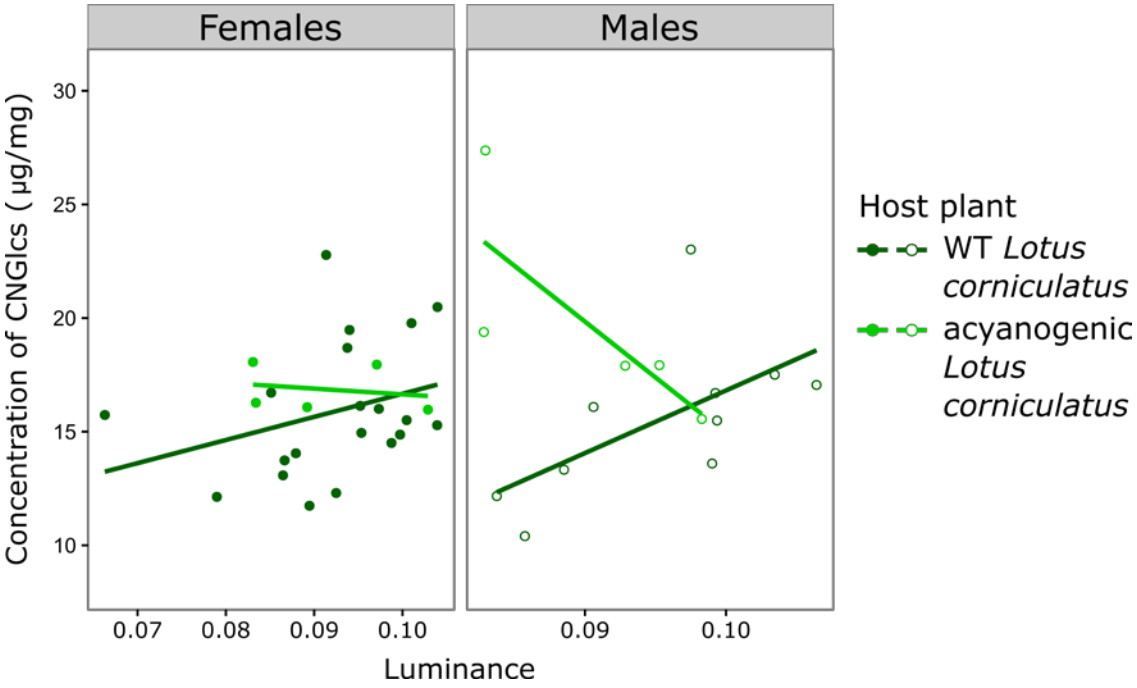


Figure 4.7: Relationship between forewing marking luminance and the concentration of cyanogenic glucosides, plotted by sex and dietary treatment. Filled circles = females, Open circles = males. CNGlcs=cyanogenic glucosides. Lines correspond to the results of linear mixed-effects models.

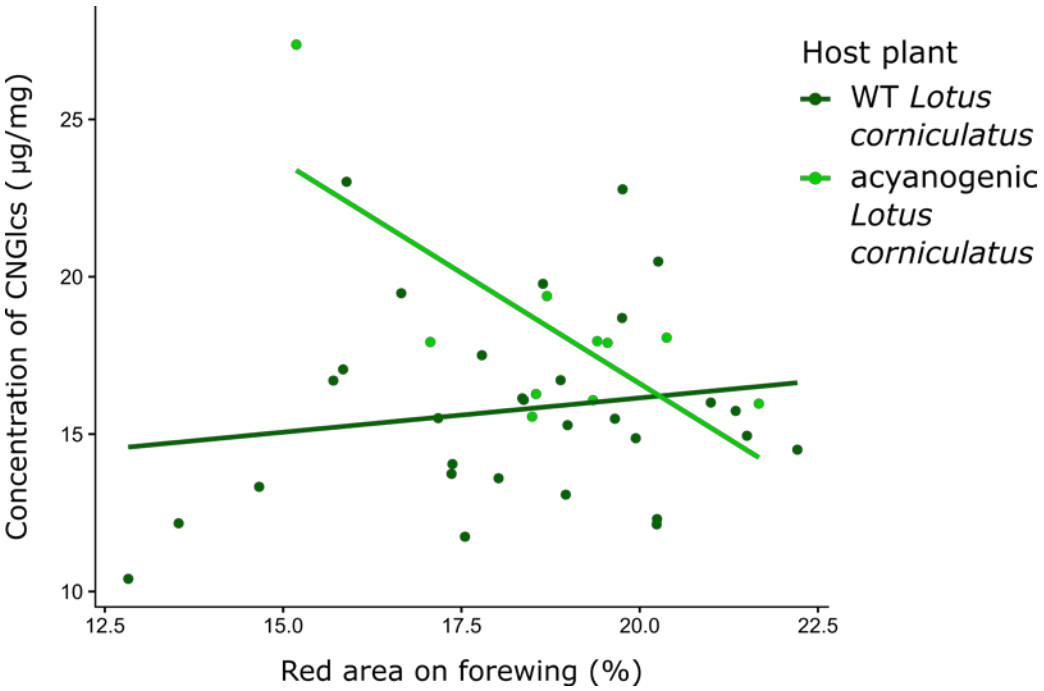


Figure 4.8: Relationship between relative forewing marking size and the concentration of cyanogenic glucosides, plotted by dietary treatment. CNGlcs=cyanogenic glucosides. Lines correspond to the results of linear mixed-effects models.

4.5 Discussion

The principal aim of this study was to test for quantitative honesty in the warning signals of *Z. filipendulae*. It is important to note that all individuals were highly toxic and conspicuous, so any differences between individuals, sexes, and populations could merely act to provide more detailed information about the level of defence. Across populations, correlations between measures of colour and toxin levels were only found in female specimens, for whom higher concentrations of cyanogenic glucosides were associated with lighter and smaller markings. Within populations, sex appeared to be the primary determinant of coloration: female markings were larger, but also lighter, less saturated, less red and less contrasting than those of males. Few colour metrics were correlated with cyanogenic glucoside concentration within populations: I found positive correlations between toxin levels and luminance in two localities, and a negative correlation between toxicity and chromatic contrast in only one population. Trends were similar for both types of avian visual system, so conclusions are applicable to a variety of potential avian predators of *Z.*

filipendulae. Collectively, these results primarily indicate either a lack of association or a dishonest relationship between measures of colour and toxin concentration, at both the individual and population levels.

Disentangling the specific roles of chromatic and achromatic information in influencing predator behaviour, along with specific colours, internal contrasts and conspicuousness to natural backgrounds, is an important area for future research in the field of visual communication (Stevens and Ruxton, 2012). In this study, most colour metrics indicated signal dishonesty or a lack of any quantitative relationships with defence levels, but larger females did display larger markings than males, which could be an informative cue for predators, and positive correlations between lightness and cyanogenic glucoside levels could be interpreted as an honest signal. In one population, marking luminance and internal chromatic contrast related to cyanogenic glucosides in opposite ways, so the potential for signal honesty would depend on which measure of coloration predators attend to. Both chromatic and achromatic information is thought to influence predator behaviour; luminance cues are considered to be more important for initial avoidance, while chromatic cues are more critical for avoidance learning, at least in birds (Osorio, Jones and Vorobyev, 1999), though further research on this topic is needed (Stevens and Ruxton, 2012). In the absence of more information on predator behaviour, it is possible that they could use lightness as a cue for the level of unprofitability. However, this could also be considered dishonest, as darker colours, requiring more pigments and hence more costly to make, would intuitively be expected to indicate more highly-defended individuals.

4.5.1 Resource allocation trade-offs and dishonest signalling

Several empirical studies in other taxa have previously found either no relationship or negative correlations between aposematic signals and the strength of the defences they are advertising, including both within and between populations of single species of poison frogs. Populations of orange and green *Oophaga granulifera* (Dendrobatidae), less conspicuous to avian predators against natural backgrounds than red populations, were found to possess greater levels of toxic alkaloids than red frogs (Wang, 2011). In this case, migration of populations into areas where more potent alkaloids were available

in the poison frog diet is thought to have driven a subsequent reduction in visual conspicuousness, reflecting a strategic trade-off between signals and defences in aposematism (Darst, Cummings and Cannatella, 2006; Speed and Ruxton, 2007). The aversiveness of highly toxic prey will in itself stimulate predator learning, reducing the incentive for displaying obvious signals, which also carry costs, such as visibility to naïve predators. Such a pattern also fits in with the predictions of resource-limitation models of signal honesty in aposematism; if resources are plentiful, so toxins can be acquired cheaply, prey should invest primarily in these rather than visual signals, while signals should be honest when resources are limited (Blount *et al.*, 2009). Alternatively, differences in predator and prey communities between populations could be responsible for a lack of signal honesty across populations (Endler and Mappes, 2004). For example, prey exposed to predators relying less on vision when hunting will be selected to reduce investment in visual signals (Wang, 2011). A wide range of other factors, from the availability and profitability of alternative prey to differences in predator experience or tolerance for toxins will also affect the relative costs and benefits of conspicuous signalling for defended prey (Mappes, Marples and Endler, 2005; Skelhorn, Halpin and Rowe, 2016).

Strategic and metabolic trade-offs may also explain negative relationships between coloration and toxicity within populations. In the highly toxic and conspicuous Solarte population of the poison frog *Oophaga pumilio*, several measures of coloration were found to be negatively correlated with pumiliotoxin levels (Crothers *et al.*, 2016). This particular population is highly toxic and overall very conspicuous compared to other *O. pumilio* populations; as these prey items are extremely aversive, the powerful selective pressure for predators to avoid them will encourage strong levels of generalisation, in turn allowing for the frogs to invest less in their warning signals (Crothers *et al.*, 2016). This situation draws parallels with *Z. filipendulae*, which are also very conspicuous and especially toxic among Lepidoptera (Rothschild *et al.*, 1970; Sbordoni *et al.*, 1979). For such unprofitable prey, there may be little to gain by communicating additional information to predators by the means of quantitatively honest signals, since anything resembling the toxic prey will be strongly avoided. Finally, effective generalisation due to perceptual limitations of the predators cannot be ruled out (Crothers *et al.*, 2016). Although individual moth colours are

perceptibly different to avian predators (Chapter 2), this does not necessarily mean that they are capable of distinguishing them and making foraging decisions based on these differences in the field. As all the specimens measured here appeared conspicuous against natural backgrounds, natural variation in colour may not necessarily be relevant. This question is addressed in Chapter 6.

The dietary experiment suggests that resource allocation trade-offs may be relevant to aposematic signalling in *Z. filipendulae*, although it is difficult to make definitive conclusions. Toxin concentration was not adversely affected by the acyanogenic diet, and was in fact higher in these moths than in those fed a host plant containing cyanogenic glucosides. Nevertheless, the adult moths in the acyanogenic treatment were smaller, leading to a reduction in total cyanogenic glucoside content in females, compared to the wild-type treatment. Coloration did not differ between diets, suggesting that moths in the acyanogenic treatment prioritised the maintenance of toxin concentration and wing colour, at the expense of growth. The relationship between most colour metrics and toxicity was generally not affected by diet, but there were two interesting exceptions. Forewing marking luminance and toxin levels were positively correlated in the WT treatment, but negatively so in the acyanogenic treatment, especially for males. This means that, when moths had to synthesise their own defences *de novo*, those with higher toxin levels invested more in pigments (which would make their markings darker) than moths with fewer toxins. This result hints at a more honest relationship between coloration and toxin levels in the acyanogenic treatment, when resources are limited, as predicted by the resource-limitation model (Blount *et al.*, 2009, 2012). However, this was not supported by parallel trends in other colour metrics; on the contrary, the relative size of red forewing spots was negatively correlated with cyanogenic glucoside levels in the acyanogenic treatment, suggesting dishonesty in signalling. High mortality rates, and in particular a very high level of larval parasitism in one sample population, unfortunately led to a very small sample size in my dietary experiment, limiting the interpretation of its results. It would be interesting to repeat this experiment with more samples to properly explore the resource allocation trade-offs in this species. Testing the toxicity of eggs laid by females in these dietary treatments, as well as the toxicity and

coloration of their offspring at adulthood, would also be a valuable test of any knock-on maternal effects of the dietary restriction.

4.5.2 Sexual dimorphism and sexual signalling

More clearly than cyanogenic glucoside levels, sex emerged as a key factor underlying variation in appearance in *Z. filipendulae*, in all studied populations. Differences in activity patterns between sexes may expose them to unequal predation pressures, as the more active males, flying to seek out females (Naumann, Tarmann and Tremewan, 1999), may be more likely to encounter predators and hence gain greater benefits from investing in conspicuous warning signals. However, *Z. filipendulae* often occur in large numbers and calling females are also highly conspicuous, exposed on flowers such as *K. arvensis* (Zagrobelny *et al.*, 2013; pers obvs.). Perhaps more relevant is the size dimorphism between males and females: for males, redder and more saturated markings might compensate for their smaller marking size, improving their salience to predators. By contrast, females may benefit from prioritising investment in toxins to ensure protection, as predators balance the risk of consuming toxic prey with the nutritional benefit gained from consuming larger, more nutritious prey (Smith, Halpin and Rowe, 2016).

Evidence of sexual dichromatism also raises the possibility that colour could be involved in sexual selection and mate choice. While pheromones are recognised as the principal means for intra-specific communication in the Zygaenidae, several observational and experimental studies suggest that visual cues might also be relevant in certain species, including *Z. filipendulae* (reviewed in Subchev 2014; Sarto i Monteys *et al*. 2016). Both *Z. filipendulae* and *Z. trifolii*, potentially along with other European species (Hofmann and Kia-Hofmann, 2010), are thought to employ two alternative mating strategies, with males relying on pheromone plumes to locate calling females in the afternoon, but using optical cues to find mates in the morning when females are not producing pheromones (Naumann, Tarmann and Tremewan, 1999; Subchev, 2014). Multiple cues might also be used in different phases of mate localisation: Zagatti and Renou (1984) observed that males of *Z. filipendulae* rely on pheromone plumes to locate mates, then use visual cues to orient themselves at close range (approximately 50cm away from the females), a strategy also seen in *Z. niphona* and *Z. fausta* (Koshio, 2003; Friedrich and Friedrich-Polo,

2005; Sarto i Monteys *et al.*, 2016). Studies seeking to manipulate visual cues during courtship in the Zygaenidae have found some limited evidence for their use in male mate choice (Zagatti and Renou, 1984; Toshova, Subchev and Toth, 2007). Monitoring the approach and copulatory attempts of wild *Z. filipendulae* males to an array of artificial female stimuli, Zagatti and Renou (1984) reported that, although males did not discriminate between mounted female specimens of closely-related species, the presence of red coloration generally encouraged copulation, and fresher specimens were preferred, leading them to conclude that males favour more saturated colours. Although the males were likely to perceive the crude differences between the artificial baits used in that study, little is known about visual perception in the Zygaenidae, so more systematic discrimination tests would be needed to establish whether colour difference on the scale measured here could be relevant to mate choice. Moreover, I found no evidence of positive correlations between colour and cyanogenic glucoside levels in either sex in natural populations, so colour would not provide quantitative information about the defences of potential mates. However, male preference for more saturated colours, as proposed by Zagatti and Renou (1984), may suggest an important reason why quantitative honesty between colour and the levels of defensive compounds at emergence may not be favoured in this species.

4.5.3 Variable and multimodal signalling

Over the lifetime of an adult burnet moth (and many Lepidoptera in general), wing scales are progressively brushed off, such that older individuals are visibly faded (pers. obvs.). In the orange sulphur butterfly (*Colias eurytheme*), wing colours fade with age (Kemp, 2006), and the decline in UV reflectance in particular may help females select younger males (Papke, Kemp and Rutowski, 2007), an advantageous strategy as male age is negatively correlated with the protein content of nuptial gifts in this and other butterfly species (Rutowski and Gilchrist, 1986; Rutowski, Gilchrist and Terkanian, 1987). In *Z. filipendulae*, females receive nuptial gifts of cyanogenic glucosides (Zagrobelny *et al.*, 2007a) and are known to reject smaller and less well-defended suitors (Zagrobelny *et al.*, 2007b, 2013), a bias which can be overcome if the males are injected with extra cyanogenic glucosides or painted with linamarin (Zagrobelny *et al.*, 2015). How females gauge male quality is still unclear, but could be

similar to female assessment of pyrrolizidine alkaloid nuptial gifts in the arctiid *Utetheisa ornatrix*, relying on compounds exposed on the males' abdominal brushes, or corremata (Iyengar, Rossini and Eisner, 2001; Zagrobelny *et al.*, 2007b, 2013). However, chemical cues are not always reliable: in fact, males emit higher levels of HCN if previously mated due to the presence of residual compounds on their corremata, despite having fewer cyanogenic glucosides to offer (Zagrobelny *et al.*, 2007b; Zagrobelny *et al.*, 2015). Since the cyanogenic glucoside reserves of older males are more likely to have been depleted by successive matings, wing colour could assist female choice as a useful proxy for male age. Likewise, female *Z. filipendulae* can mate multiple times (Naumann, Tarmann and Tremewan, 1999), but males will benefit from mating with younger females, with a greater number of eggs available for fertilisation. As a result, both sexes should prefer younger mates, and brighter, more saturated wing colours could act as reliable indicators of quality. From a predator's perspective, wing colour could similarly be used as a crude signal of toxin content in the wild. Taking toxin and colour measurements from a range of individuals at a given date in any given population, effectively a snapshot of prey items available to predators, would help test this hypothesis.

Rather than focusing on visual signals alone, observations in *Zygaena filipendulae* demonstrate the importance of considering these as elements of a more complex multimodal and multicomponent signalling system (see Rowe and Halpin, 2013). In this study, I found that the red markings of *Z. filipendulae* do not function as straightforward quantitatively honest signals of the levels of defensive compounds, neither within nor between populations. However, these visual cues are likely to be used in combination with pheromone emission, deposits on corremata and, for predators, with the bitter taste of the cyanogenic glucosides, to evaluate the profitability of individuals. Further research into the volatiles emitted by zygaenids, including degradation products of cyanogenic glucosides (HCN and ketones; Zagrobelny *et al.*, 2015) and pyrazines (Rothschild, 1961; Rothschild, Moore and Brown, 1984; Moore, Brown and Rothschild, 1990; Tremewan, 2006), odours often associated with warningly-coloured insects (Guilford *et al.*, 1987), will help develop a more comprehensive picture of their defensive strategy. Testing the response of natural predators to these volatiles, as well as taste-rejection due to the bitter cyanogenic glucosides, and how these cues interact with each other and visual signals, are

the next logical steps towards establishing the relevance of these strategic components to survival in the wild. In this and other study systems, integrating the effects of multiple cues, especially visual and chemical, is a major route towards a deeper understanding of aposematic signalling strategies.

Chapter 5

Colour and toxicity in burnet moths (Zygaenidae) – cautionary tales in the study of signal honesty across species

All wing photographs: E. S. Briolat

5.1 Abstract

How levels of signals and defences should compare across closely-related aposematic species is a largely unresolved question, addressed by some theoretical models and a limited number of empirical studies. This study tests for evidence of a quantitatively honest relationship between measures of wing marking coloration, as perceived by avian predators, and levels of toxic cyanogenic glucosides across 14 species of burnet and forester moths (Lepidoptera: Zygaenidae), collected in Denmark, France, and the UK. Broad differences in coloration and toxicity between field seasons, and variation between sexes in each species, suggested that sex-specific trends and ecological conditions should be accounted for in studies of signal honesty. The relationship between coloration and levels of defensive chemicals across species varied, depending on the colour metric considered and the year of sampling, but were generally similar across sexes, although the significance of these relationships did vary. Overall, there was no evidence of signal honesty across species in the Zygaenidae, contrary to expectations based on recent studies in ladybirds and nudibranchs. Altogether, these results indicate that the relationship between colour and toxicity in aposematic species may be more intricate and dynamic than has been suggested by previous empirical work.

5.2 Introduction

Traditional theories of warning coloration, based primarily on the concept of Müllerian mimicry (Müller, 1879), suggest that warning signals should converge on similar forms, as signal monomorphy would facilitate predator learning. Yet, polymorphic and polytypic variation is widespread in aposematic species (Arenas and Stevens, 2017), and understanding how this variation is maintained is an important and active area of research (Rojas, Devillechabrolle and Endler, 2014; Stevens, 2015; Summers *et al.*, 2015). Similarly unresolved is the relationship between these variable signals and the potency of the defences they advertise, prompting theoretical and empirical investigations at the level of individuals, populations, and species. The key question concerns how investment in the two strategic components of aposematism, signals and secondary defences, should be portioned out once an aposematic strategy has evolved (Cortesi and Cheney, 2010). According to initial models of warning

coloration as a handicap signal (Grafen, 1990), an honest relationship might be expected if stronger signals, such as more conspicuous markings, are too costly for poorly-defended individuals to maintain. This hypothesis was criticised due to the lack of a clear physiological link between signal expression and defence production in aposematic species (Guilford and Dawkins, 1993), although the resource competition model, whereby defences and signals compete for a shared resource, such as antioxidants, may offer a solution (Blount *et al.*, 2009, 2012). In contrast, some modelling approaches instead find that associations between conspicuous coloration and defences will break down (e.g. Leimar, Enquist and Sillen-Tullberg, 1986) or that defended species should prioritise investment in defences, which do not carry the detection costs associated with warning signals (e.g. Speed and Ruxton, 2007). This would lead to negative correlations between signals and defences. However, further theoretical studies, incorporating a greater awareness of the importance of predator behaviour (Endler and Mappes, 2004), suggest that signal honesty could occur even without the need for a strictly-Zahavian handicap mechanism (e.g. Guilford, 1994; Speed *et al.*, 2010; Speed and Franks, 2014). Considering the relative costs of signal and defence production may also explain why honest and dishonest relationships between signals and defences occur: positive correlations between these two strategic elements should arise when their respective costs increase in parallel, while negative correlations are expected when they do not (Speed and Ruxton, 2007).

Across species, several evolutionary mechanisms have been proposed to underpin the relationship between visual signals and secondary defences (Summers *et al.*, 2015). Interactions with other species mimicking the defended prey (Franks, Ruxton and Sherratt, 2009), cautious or “go-slow” behaviour on the part of predators (Guilford, 1994), exaptation through other functions of visual signals (Lee, Speed and Stephens, 2011), and resource allocation trade-offs (Blount *et al.*, 2009), are all thought to have the capacity to lead to honest signalling between populations or species (Holen and Svennungsen, 2012; Summers *et al.*, 2015). However, empirical tests across species are restricted to very few taxa, focusing primarily on poison frogs (Dendrobatidae) and ladybirds (Coccinellidae). While most of these studies revealed a positive correlation between the strength of the signals and defences, or signal honesty (Summers

and Clough, 2001; Cortesi and Cheney, 2010; Santos and Cannatella, 2011; Arenas, Walter and Stevens, 2015), others did not (Darst, Cummings and Cannatella, 2006). There is also substantial variation in the methods used to assess coloration, increasingly but not always accounting for predator vision, and chemical defences, either quantifying known toxins or measuring the effects of extracts in bioassays with mice, brine shrimp or *Daphnia* water fleas. These inconsistencies are likely to contribute to the contrasting trends found in the same or closely-related species, such as poison frogs (Daly and Myers, 1967; Summers and Clough, 2001; Darst, Cummings and Cannatella, 2006; Santos and Cannatella, 2011; Maan and Cummings, 2012), and make it difficult to compare between studies. Further work in other study systems is needed to put conflicting results in context and gain a better sense of general trends in the relationship between coloration and defence in the wild (Stevens, 2015; Summers *et al.*, 2015).

Burnet moths (Zygaenidae) provide a valuable opportunity to test the relationship between signals and defences across closely-related species. These diurnal aposematic moths are chemically-defended, with at least 45 species known to contain the cyanogenic glucosides linamarin and lotaustralin (Davis and Nahrstedt, 1982; Zagrobelny *et al.*, 2004), rendering them highly distasteful to predators. In the Western Palearctic, the Zygaenidae are represented by three subfamilies: the Zygaeninae, Procridinae and Chalcosiinae. Variation in wing coloration is subtle within the Zygaeninae, and more dramatic between subfamilies: while the Zygaenidae display classic warning signals, with red markings on a dark background, temperate species of Procridinae, or forester moths, are characterised by iridescent green or dull brown coloration (Drouet, 2016), which is generally considered cryptic (Efetov and Tarmann, 1999). The single representative of the Chalcosiinae in Western Europe, *Aglaope infausta* (L.), has brown forewings with discrete red markings and red hindwings. In addition, molecular data and recent phylogenies of the Zygaenidae and the genus *Zygaena* are available (Niehuis, Naumann and Misof, 2006a, 2006b, 2006c; Niehuis *et al.*, 2007), enabling evolutionary relationships to be accounted for when comparing between species.

My study provides meaningful measures of wing coloration in 14 zygaenid species, from the perspective of their most likely predators (Tremewan, 2006), using digital photography and models of avian vision (Endler and Mielke, 2005; Stevens *et al.*, 2007a). Individuals were collected over two field seasons, in 2015 and 2016, from a range of locations in Denmark, France and the UK. For their chemical defences, a liquid chromatography – mass spectrometry (LC-MS) protocol refined for the detection of cyanogenic glucosides in the Zygaenidae and their host plants was used to accurately measure the levels of linamarin and lotaustralin in each sample (see Chapter 4). Put together, these results allow a number of questions to be addressed, from variation in signals and defences over time to the relationship between coloration and toxicity both within and between species. Moreover, although many species of Zygaenidae are known to be aposematic, comprehensive studies of their chemical defences have focused on *Z. filipendulae* (e.g. Zagrobelny and Møller, 2011) and, to a lesser extent, *Z. trifolii* (Franzl, Nahrstedt and Naumann, 1986; Holzkamp and Nahrstedt, 1994). The data shown here represent the first detailed exploration of the chemical defences and coloration of multiple species in this family, and add to a relatively small number of studies investigating the relationship between signals and defences across species. The combined use of sophisticated objective measures of defence strength, quantification of coloration as perceived by relevant predators and phylogenetic controls make this a particularly valuable contribution to the field of honest signalling in aposematism.

5.3 Methods

5.3.1 Specimen collection and rearing

Individuals of 14 species were collected in spring and summer 2015 and 2016, from a range of locations in Denmark, France, and the UK (Table 5.1, Figure 5.1; see Appendix 5.1 for full details of collection numbers and localities). Specimens were collected at the larval or pupal stage then reared to maturity in individual boxes with air-holes, ensuring that only virgin individuals were used for analysis. After collection, larvae and pupae were kept in an incubator at 20°C, with a 16:8h day:night cycle, following protocols from previous work on *Z. filipendulae* (Zagrobelny *et al.*, 2007a). Each species was provided with sprigs

of the same host plant as they were found on in the field, *ad libitum* and checked daily for freshness (Table 5.1). After emergence, and once their wings were fully expanded, the imagines were euthanised by placing them in a -80°C freezer. Their wings were dissected for photography, then the entire sample was placed in 1ml 80% methanol in preparation for LC-MS analysis of cyanogenic glucoside content. Due to the difficulty of finding larvae or pupae of certain species, and high mortality, five species are limited to very small sample sizes (N=1 or N=2, see Table 5.1).

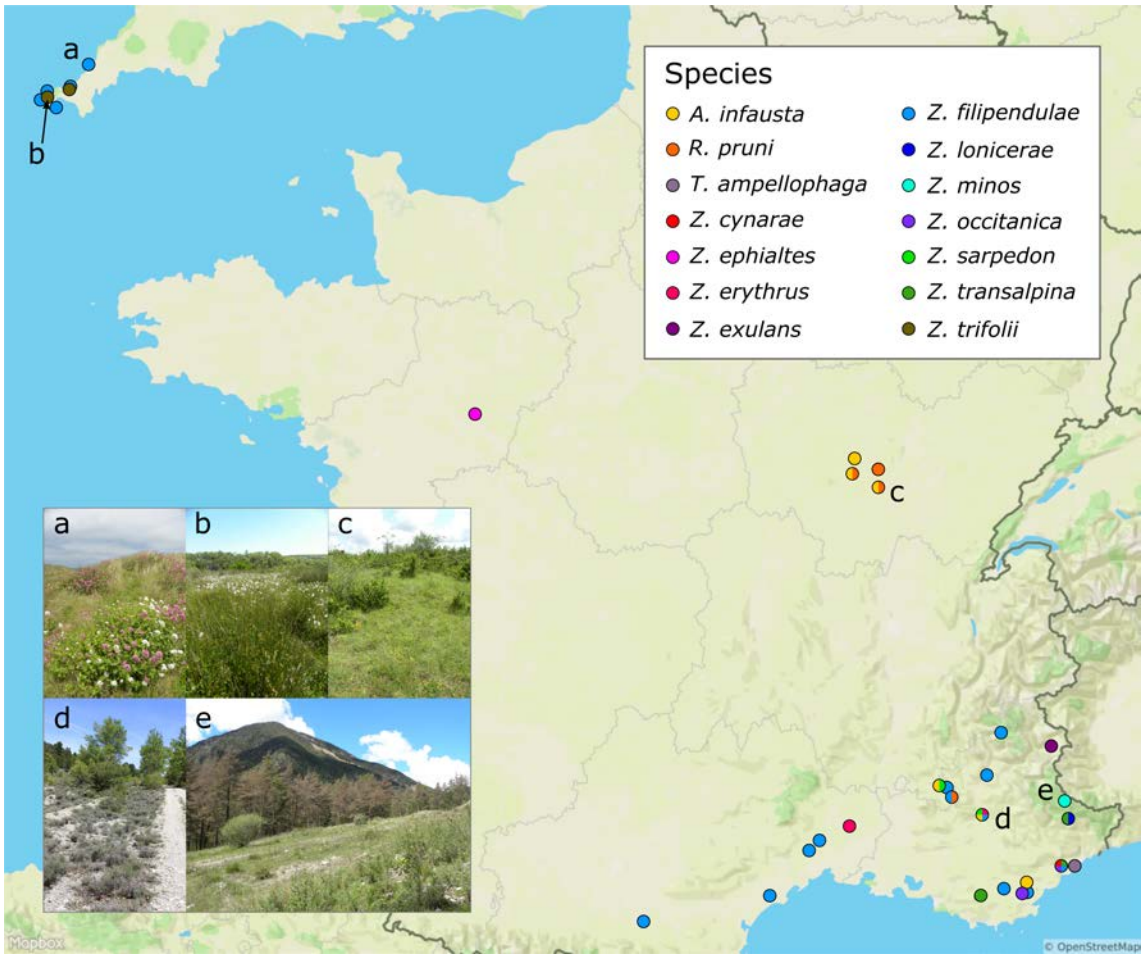


Figure 5.1: Map of collection localities colour-coded by species, and illustrating five example habitats: (a) Holywell Bay, UK, (b) Bostraze Bog, UK, (c) Antigny, France, (d) Le Fournas, France, (e) Le Cialancier, France. The Danish locality where *Z. filipendulae* was collected is not represented. The colour of each circle represents the species collected in that locality (see key); circles with multiple colours indicate that several different species were found there.

Table 5.1: Number (N), species and host plants of photographed specimens.

Species	Country of collection	Host plants at collection sites	N	
			2015	2016
Aglaope infausta	France	*Cotoneaster* sp., *Crateagus* sp., *Prunus* sp. (Rosaceae)	21	17
Rhagades pruni	France	*Prunus spinosa* (Rosaceae)	8	8
Theresimima ampellophaga	France	*Vitis* sp. (Vitaceae)	0	1
Zygaena cynarae	France	*Peucedanum cervaria* (Apiaceae)	1	0
Zygaena ephialtes	France	*Securigera varia* (Fabaceae)	21	0
Zygaena erythrus	France	*Eryngium campestre* (Apiaceae)	0	11
Zygaena exulans	France	Polyphagous – host plant unknown	0	5
Zygaena filipendulae	Denmark, France, UK	*Lotus corniculatus, Dorycnium pentaphyllum, Hippocrepis comosa* (Fabaceae)	107	8
Zygaena lonicerae	France	*Trifolium* sp. (Fabaceae)	0	1
Zygaena minos	France	*Pimpinella saxifraga* (Apiaceae)	1	1
Zygaena occitanica	France	*Dorycnium pentaphyllum* (Fabaceae)	0	2
Zygaena sarpedon	France	*Eryngium campestre* (Apiaceae)	6	2
Zygaena transalpina	France	*Hippocrepis comosa*, *Securigera varia* (Fabaceae)	3	13
Zygaena trifolii	UK	*Lotus pedunculatus* (Fabaceae)	9	14

5.3.2 Photography and image analysis

As described previously (see Chapter 4), the right-hand wings were photographed in controlled conditions inside a dark room, with a calibrated, UV-sensitive digital camera (Nikon D7000 fitted with a 105mm CoastalOptics quartz lens). Light was provided by an EYE Color Arc Lamp MT70 bulb (Iwasaki Electric Co. Ltd.), equivalent to D65 daylight conditions, and kept in a constant position at a 50° angle relative to the wings. To control for any remaining variation in illumination, a set of reflectance standards, reflecting 7% and 93% of all wavelengths of light respectively (Zenith Lite Diffuse Target sheets, SphereOptics, Pro-Lite Technology, Cranfield, UK), were included in each photograph. Each specimen was photographed twice, with a UV/infrared blocking filter (Baader UV/IR Cut Filter), and a UV pass and IR blocking filter (Baader U filter).

Image analysis was performed using the multispectral image analysis toolbox in ImageJ (Troscianko and Stevens, 2015), according to the same protocols as

the analysis of *Z. filipendulae* wing photographs (see Chapters 2 and 4). Photographs taken with both filters were aligned and merged to produce a multispectral image with five layers, corresponding to reflectance in different parts of the visual spectrum: vR, vG, vB, uR and uB (see Chapter 2 for details). Images were linearised and normalised (Stevens *et al.*, 2007a), scaled to 100 pixels/mm, then mapped to avian vision to describe coloration from the perspective of the moths' most likely visual predators (Tremewan, 2006). My previous analyses of lepidopteran wing colours using models of both types of avian visual system, violet-sensitive (VS) and ultraviolet-sensitive (UVS) (Hart *et al*. 1999), showed very similar results (see Chapters 3 and 4), so in this study I restricted the analysis to the UVS visual system. I converted reflectance data to avian cone catches with a polynomial mapping technique (Westland and Ripamonti, 2004; Stevens *et al.*, 2007a; Pike, 2011; Troscianko and Stevens, 2015), using data on the sensitivity of each cone type from the model species for this visual system, the blue tit, *Cyanistes caeruleus* (Hart *et al.*, 2000). The end result was a composite of five image layers for each wing, with predicted cone catch values for each photoreceptor type: ultraviolet (UV or V), short wavelength (SW), medium wavelength (MW) and long wavelength (LW) sensitive photoreceptors, as well as double cones. Wing markings and background areas on each photograph were selected using the freehand tool in ImageJ, following the same general rules as for *Z. filipendulae* (Chapters 2 and 4). I focused my analysis on the red markings of the forewings, as these are most often exposed to visual predators; in the Zygaenidae, the hindwings are covered at rest. However, several species did not present red markings on their forewings: for *Rhagades pruni*, the iridescent blue patch at the base of the forewing was selected as its markings, while the uniform forewing background colour was used in the unpatterned *Theresimima ampellophaga*.

Based on the average cone catch values for the wing markings of each individual, several coloration metrics were calculated, as described in Chapters 2, 3 and 4: luminance, saturation, hue, and both chromatic and luminance contrasts between markings and background colours in the wings. Luminance was taken as the double cone catch value (Jones and Osorio, 2004; Osorio and Vorobyev, 2005) and provides a measure of perceived lightness. Saturation, representing colour intensity, was determined as the Euclidean distance

between the colour of interest and the centre of a tetrahedral colour space, as in previous studies of animal coloration (Endler and Mielke, 2005; Stoddard and Prum, 2008). Hue was calculated using a technique broadly inspired by the concept of colour opponency, known to be important for colour vision in birds (Osorio, Vorobyev and Jones, 1999), whereby colour can be described in the form of a ratio. Following methods developed by Spottiswoode and Stevens (2011), principal component analysis (PCA) was performed on a covariance matrix of the standardised values of all colour patches for the four photoreceptor channels (UV, SW, MW, LW) for each visual system. The first principal component accounted for over 91% of variation in marking colour, so was used to derive a single ratio of cone catch values that captures the principal axis of variation in colour across samples. The specific equation used for hue values in this study is as follows:

$$Hue_{FWM\ (UVS)} = \frac{LW+UV}{SW+MW} \quad (5.1)$$

UV, SW, MW, LW = standardised cone catch values for the UV-, SW-, MW- and LW- sensitive photoreceptors respectively. FWM = forewing markings, UVS = ultraviolet-sensitive visual system.

High values of hue thus correspond to colours with relatively greater reflectance in the long wavelength (LW) and/or ultraviolet (UV) wavelength colour channel than in the short and medium wavelength channels (SW, MW), so represent redder colours, or higher ultraviolet reflectance, or both.

Chromatic and luminance contrasts between the markings, when present, and background colours of the wings were measured as just-noticeable differences (JNDs). Two colours can be perceived as distinct in optimal lighting conditions if JND>1, and are increasingly easy to distinguish when JND>3 (Siddiqi *et al.*, 2004). Chromatic contrast was calculated according to a log version of the receptor noise-limited Vorobyev-Osorio colour discrimination model (Vorobyev and Osorio, 1998), which accounts for the sensitivity and abundance of each cone type and the noise in the photoreceptors, computed here with a relatively conservative estimate of the Weber fraction, $\omega = 0.05$, for the most abundant cone type (Eaton, 2005; Håstad, Victorsson and Ödeen, 2005; Stevens, 2011; Stevens, Lown and Wood, 2014a). Following the same principle, luminance

contrast was taken as the natural logarithm of the ratio between mean double cone catch values of background and marking areas, divided by the same Weber fraction (Siddiqi *et al.*, 2004). *Theresimima ampellophaga*, which has no wing markings, was excluded from this analysis. Further details of the rationale behind the calculations of colour metrics can be found in Chapter 2.

5.3.3 Quantification of cyanogenic glucosides

Cyanogenic glucoside concentration in each sample of plant or insect tissue was obtained via liquid chromatography – mass spectrometry (LC-MS), using the same techniques as for *Z. filipendulae* samples (see Chapter 4). Specimens were prepared for analysis by breaking them up in 1ml ice-cold 55% MeOH, with 0.1% formic acid and 0.044mM amygdalin, a cyanogenic glycoside not found in the Zygaenidae which acts as an internal standard. Details of the procedure for LC-MS analysis are recorded in Chapter 4. The mass spectral data were analysed with the native data analysis software. Sodium adducts of linamarin were compared to authentic standards (Møller, Olsen and Motawia, 2016), and the total amount of each compound was estimated according to its Extracted Ion Chromatogram (EIC) peak areas and calibration curves for linamarin, lotaustralin, and amygdalin standards. The concentration of cyanogenic glucosides was determined by dividing the total amount of compounds in each sample by the specimen mass, recorded at the time of preservation. The LC-MS was run on samples taken in 2015 and 2016 separately; however, I verified that any differences in toxicity measurements between years were not due to differences in the sensitivity of the equipment by re-running a subset of samples from both years in 2017 (see Appendix 5.2).

5.3.4 Phylogenetic reconstruction

The phylogenetic tree used for analysis was based on available sequences for the Zygaenidae, as used in previous work on the evolutionary history of this family (Niehuis, Naumann and Misof, 2006a; Niehuis *et al.*, 2007): complete sequences of the mitochondrial genes for NADH dehydrogenase subunit 1 (ND1), tRNA-leucine (tRNA-Leu), the large subunit ribosomal RNA (16S rRNA), tRNA-valine (tRNA-Val) and a large fragment of the sequence for the mitochondrial small subunit of rRNA (12S rRNA), as well as two nuclear DNA

fragments, an almost complete sequence of the small subunit rRNA (18S rRNA) and the 5' end of the large subunit rRNA (28S rRNA). Following Niehuis, Naumann and Misof (2006a,b,c), the lunar hornet moth *Sesia bembeciformis* (Lepidoptera: Sesiidae) was used as an outgroup to root the tree. A set of sequences for each species photographed, and the outgroup, were acquired from GenBank (http://www.ncbi.nlm.nih.gov/; see Table 5.2 for taxa and accession numbers). The sequences for each gene were aligned using MUSCLE (Edgar, 2004), implemented by the 'ape' package (Paradis, Claude and Strimmer, 2004) in R 3.3.1 (R Development Core Team, 2015). These alignments were then concatenated to produce a final alignment (5697 base pairs [bp] long). Figure 5.2 provides a sense of the genetic distances between sequences in this alignment.

Table 5.2: Species names and EMBL accession numbers for the sequences used in this study.

Taxon	**Accession numbers**				
	ND1	16S rRNA*	12S rRNA	18S rRNA	28S rRNA
Sesiidae					
Sesia bembeciformis (Hübner,1806)	AJ844306	AJ831588	AJ785615	AJ830746	AJ844024
Zygaenidae – Chalcosiinae					
Aglaope infausta (Linnaeus, 1767)	AJ844314	AJ831596	AJ785623	AJ830754	AJ844032
Zygaenidae – Procridinae					
Rhagades pruni (Denis & Schiffermüller, 1775)	AJ844324	AJ831606	AJ785633	AJ830764	AJ844042
Theresimima ampellophaga (Bayle-Barelle, 1808)	AJ844325	AJ831607-8	AJ785634	AJ830765	AJ844043
Zygaenidae – Zygaeninae					
Zygaena cynarae samarensis (Holik, 1939)	AJ844389	AJ831677	AJ785698	AJ830829	AJ844107
Zygaena ephialtes albaflavens (Verity, 1920)	AJ844427	AJ831722	AJ785736	AJ830867	AJ844145
Zygaena erythrus actae (Burgeff, 1926)	AJ844390	AJ831678	AJ785699	AJ830830	AJ844108
Zygaena exulans exulans (Hohenwarth, 1792)	AJ844428	AJ831723	AJ785737	AJ830868	AJ844146
Zygaena filipendulae gemina (Burgeff, 1914)	AJ844429	AJ831724	AJ785738	AJ830869	AJ844147
Zygaena lonicerae leonensis (Tremewan, 1961)	AJ844433	AJ831728	AJ785742	AJ830873	AJ844151
Zygaena minos ingens (Burgeff, 1926)	AJ844407	AJ831698	AJ785716	AJ830847	AJ844125
Zygaena occitanica huescacola (Tremewan & Manley, 1965)	AJ844362	AJ831649	AJ785671	AJ830802	AJ844080
Zygaena sarpedon lusitanica (Reiss, 1936)	AJ844418	AJ831713	AJ785727	AJ830858	AJ844136
Zygaena transalpina hippocrepidis (Hübner, 1799)	AJ844442	AJ831737	AJ785751	AJ830882	AJ844160
Zygaena trifolii diffusemarginata (Rothschild, 1933)	AJ844444	AJ831739	AJ785753	AJ830884	AJ844162

*: Including tRNA-Leu and tRNA-Val

Phylogenetic relationships were assessed with maximum likelihood (ML) methods, using the 'phangorn' package (Schliep, 2011) in R. ML estimates calculated with the modelTest function in 'phangorn' identified a GTR+G+I

model, allowing for variation in mutation rates between sites and the presence of invariant sites, as the most appropriate model of evolution. Tree topology was then optimised by nearest-neighbour interchange (NNI), using the optim.pml function; no further improvement was seen with stochastic rearrangement. Finally, partitions allowing different rates of evolution for nuclear and mitochondrial sequences (3263 and 2434 bp respectively) and for each gene (ND1 = 1013 bp, 12S rRNA = 626 bp, 16SrRNA [+tRNA-Leu, tRNA-Val] = 1624 bp, 18S rRNA = 1881 bp, 28SrRNA = 553 bp) were tested with the pmlPart function. The highest likelihood was found for a partitioned model considering each gene separately ($logLik_{no\ partition}$=-19987.92, $logLik_{nuclear/mitochondrial\ partition}$=-19749.85, $logLik_{partition\ by\ gene}$=-19661.71; $AIC_{no\ partition}$=40049.83, $AIC_{nuclear/mitochondrial\ partition}$=39575.70, $AIC_{partition\ by\ gene}$=39405.41). The final rooted tree (Figure 5.3) was bootstrapped with 1000 replicates, and nodes with less than 70% support were collapsed into polytomies.

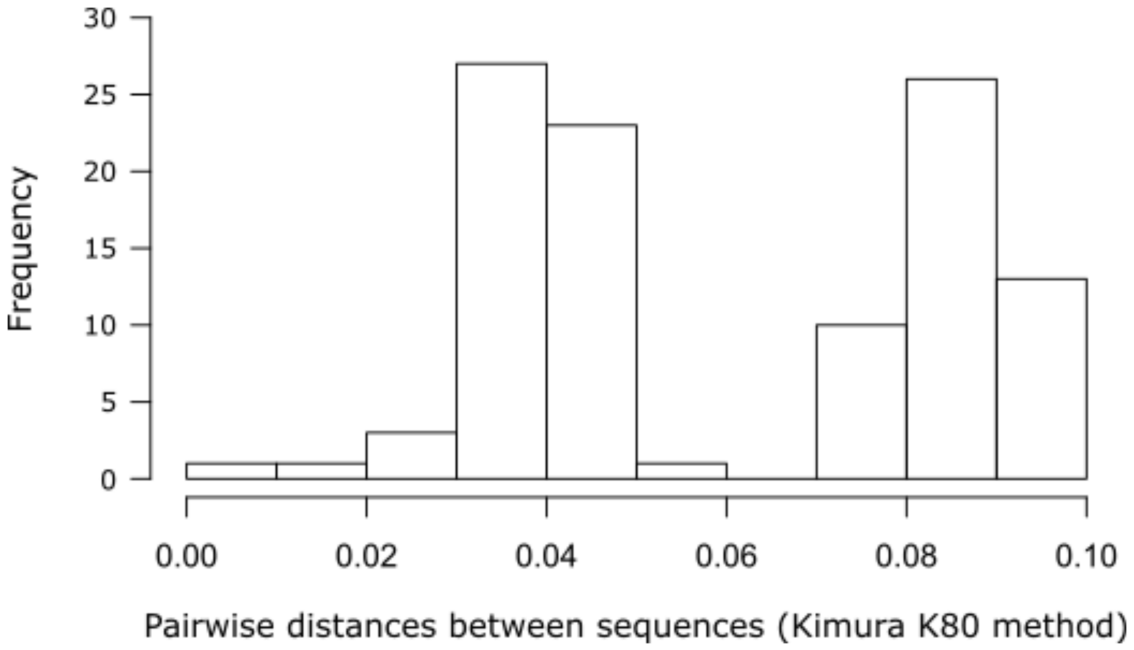


Figure 5.2: Distribution of pairwise differences between the concatenated sequences in the final alignment used for phylogenetic reconstruction, calculated following the K80 model, allowing for different rates of transitions and transversions (Kimura, 1980).

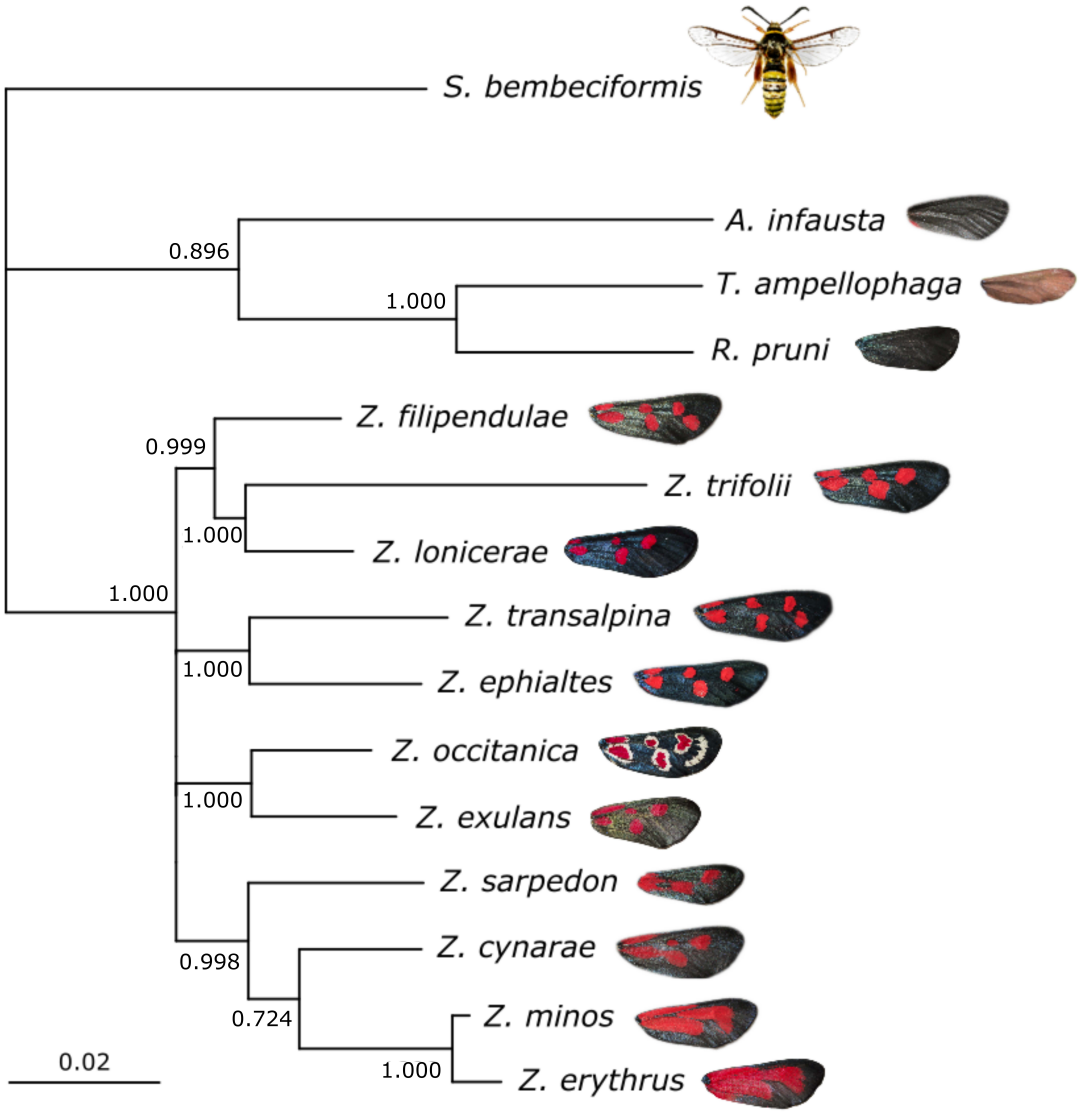


Figure 5.3: Phylogenetic tree of the Zygaenidae species used in this study. Branch labels represent bootstrap values for 1000 replicates. The scale bar corresponds to genetic distances between sequences, represented by the length of the branches. Image credits: *S. bembeciformis*, www.biolib.cz/en/image/id129534, ©Josef Dvořák; *T. amphellophaga,* adapted from www.lepinet.fr/especes/nation/lep/index.php?id=02140, ©Daniel Morel; all other images E. S. Briolat.

5.3.5 Weather data

Climatic data (monthly mean, minimum, and maximal temperature, total rainfall and total hours of sunshine in 2015 and 2016) were collated from publicly-available historical observations at the Met Office and Météo France. Eight weather stations, closest to all field collection sites, were selected for analysis

(Table 5.3). Weather conditions in Denmark were not recorded, as samples of *Z. filipendulae* from Denmark were only collected in 2015 from a single field site. Data from the three coldest months of the year (December, January, and February) and the three months prior to specimen collection (March, April, and May) were examined to provide a sense of winter and growing season conditions respectively. Records of hours of sunshine were not available for Cap Cépet station.

Table 5.3: Locations of selected weather stations

Station name	**Country (Region)**	**Latitude**	**Longitude**	**Altitude (m)**
Camborne	United Kingdom (Cornwall)	50.218	-5.327	87
Cap Cépet – 7661	France (Provence Alpes Côte d'Azur)	43.079	5.941	115
Dijon – 7280	France (Bourgogne Franche-Comté)	47.268	5.088	219
Embrun – 7591	France (Provence Alpes Côte d'Azur)	44.566	6.502	871
Montpellier – 7643	France (Languedoc-Roussillon Midi-Pyrenées)	43.577	3.963	2
Nice – 7690	France (Provence Alpes Côte d'Azur)	43.649	7.209	2
St Girons – 7627	France (Languedoc-Roussillon Midi-Pyrenées)	43.005	1.107	414
Tours – 7240	France (Centre – Val de Loire)	47.445	0.727	108

5.3.6 Statistical analyses

All analyses were carried out in R 3.3.1 (R Development Core Team, 2015). Restricting the data to the seven species collected in both years (see Table 5.1), differences in cyanogenic glucoside concentration and colour metrics between years were analysed with linear models. As results of previous work on *Z. filipendulae* suggested that there may be differences between sexes (see Chapter 4), I included sex in these models, and allowed year, sex and species to interact. Luminance, hue and chromatic contrast were log-transformed to fit model assumptions. Winter and growing season weather data (temperature, hours of sunshine and rainfall) were analysed using linear mixed effects models (LMEs), with year as a fixed effect and month and location as random effects, using the package 'lme4' (Bates *et al.*, 2014). Model assumptions were checked with the mcp.fnc function in the 'LMERConvenienceFunctions' package (Tremblay and Ransijn, 2014).

Due to the significant effects of year and sex on both toxicity and colour metrics, the relationship between colour metrics and cyanogenic glucoside levels across species were analysed for each year separately. In each case, the data were also analysed over both sexes, and for males and females separately. Phylogenetic generalised least squares (PGLS) models were applied to account for evolutionary relatedness between species (see Figure 5.3), using the package 'caper' (Orme, 2013) and allowing λ to be fitted by maximum likelihood, following methods presented in Mundry (2014). My basic approach was to test the relationship between cyanogenic glucoside concentration and all available colour metrics in a single model. However, to deal with the problem of collinearity, several models had to be run in each case, swapping out highly-correlated variables. Variance Inflation Factors (VIFs) were calculated using the vif function in the 'car' package (Fox and Weisberg, 2011). Appropriate models were then chosen by a combination of a commonly-used "rule-of-thumb", whereby VIFs should not exceed 10, and logical expectations of correlations (O'Brien, 2007; Dormann *et al.*, 2013): for example, colour measures such as saturation, hue and chromatic contrast are calculated from the same cone catch values, so are expected to be correlated, while marking size is not tied to these variables. To fit model assumptions, for the dataset of females in 2015, saturation was transformed using the square-root function, and chromatic contrast was log-transformed. Cyanogenic glucoside concentration was log-transformed for all the 2016 datasets.

5.4 Results

5.4.1 Patterns of coloration and toxicity across species, sex and years

Both the year the samples were collected in and the sex of the individuals had an impact on the variables of interest, as demonstrated by significant interactions between sex, year and species (Table 5.4). Differences in cyanogenic glucoside concentration between years varied across species and between sexes: cyanogenic glucoside levels in females increased between 2015 and 2016 in most species, with the exception of *Z. sarpedon*, whereas the picture was more complex in males (Figure 5.4). Clearer trends emerged from measures of coloration, with individuals from 2016 having consistently more salient markings (Figure 5.5). In terms of luminance, individuals of all species

with red wing markings collected in 2015 had paler wing markings than in 2016, although the extent of the difference varied between species and sexes (Figure 5.5a; Table 5.4). Conversely, for all other colour metrics, values for these species were overall higher in 2016 than 2015, despite differences between sexes (Figures 5.5b-5.5f). In *Rhagades pruni*, which displays iridescent blue markings, trends for luminance and hue were opposite to those seen in all other species (Figures 5.5a and 5.5d). Nevertheless, this led to similar effects on marking saturation and internal contrasts in the forewings, which were all higher in 2016 than 2015. As sex and year do strongly influence both colour metrics and cyanogenic glucoside levels, subsequent analyses of the relationship between colour and toxicity were carried out separately for each year and each sex.

Table 5.4: Results of linear models testing differences in cyanogenic glucoside (CNGlc) concentration and colour metrics between 2015 and 2016. Significance levels: *:p<0.05, **:p<0.01, ***:p<0.001.

Metric	Factor	F	df	P	Significance
CNGlc concentration	Sex:Species:Year	3.213	5, 192	0.00826	**
Luminance	Sex:Species:Year	2.354	5, 192	0.0422	*
Saturation	Sex:Species:Year	1.424	5, 192	0.217	-
	Sex:Year	0.171	1, 197	0.680	-
	Sex:Species	1.488	5, 198	0.195	-
	Species:Year	4.166	6, 203	<0.001	***
	Sex	5.869	1, 203	0.0163	*
Hue	Sex:Species:Year	0.821	5, 192	0.536	-
	Sex:Year	0.0614	1, 197	0.805	-
	Sex:Species	1.525	5, 198	0.184	-
	Species:Year	27.948	6, 203	<0.001	***
	Sex	4.995	1, 203	0.0265	*
Chromatic contrast	Sex:Species:Year	0.472	5, 192	0.797	-
	Sex:Year	0.0056	1, 197	0.940	-
	Sex:Species	3.080	5, 198	0.0106	*
	Species:Year	3.320	6, 198	0.00389	**
Luminance contrast	Sex:Species:Year	1.123	5, 192	0.350	-
	Sex:Year	2.059	1, 197	0.153	-
	Sex:Species	5.571	5, 198	<0.001	***
	Species:Year	10.674	6, 198	<0.001	***
Relative marking size	Sex:Species:Year	0.84	5, 192	0.350	-
	Sex:Year	0.0013	1, 197	0.971	-
	Sex:Species	5.455	5, 198	<0.001	***
	Species:Year	2.970	6, 198	0.00846	**

Figure 5.4: Mean concentration of cyanogenic glucosides (CNGlc) in males and females of each species, collected in 2015 and 2016. Error bars represent standard errors. Filled circles = samples collected in 2015, Open circles = samples collected in 2016.

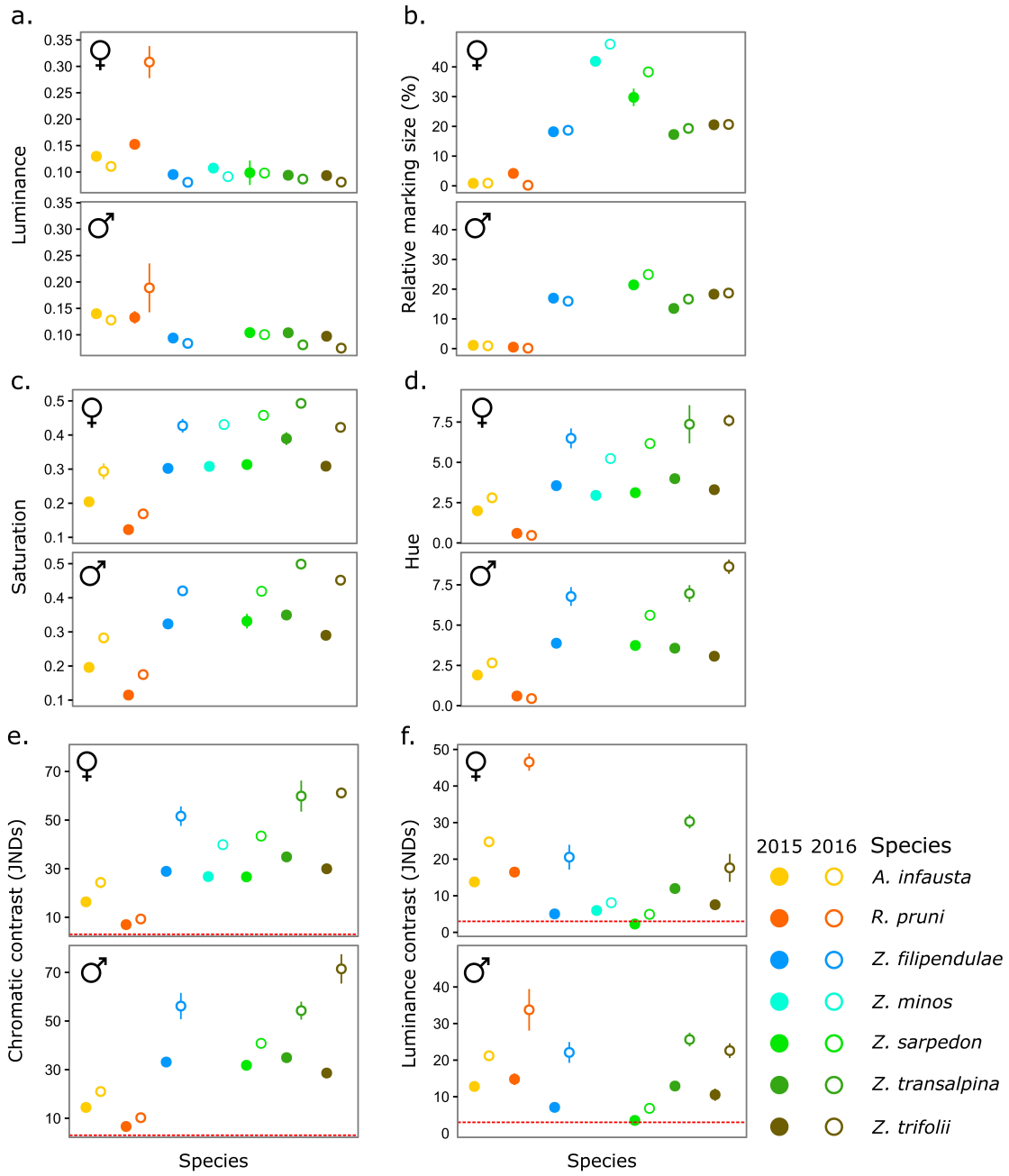


Figure 5.5: Mean values of coloration for males and females of seven species collected in 2015 and 2016. Error bars represent standard errors. Filled circles = samples collected in 2015, Open circles = samples collected in 2016. In (b), relative marking size is measured as the percentage of the forewing area occupied by contrasting markings. In (e) and (f), the red dashed line represents the threshold for discrimination, JND = 3. All contrasts exceed this threshold, with the exception of *Z. sarpedon* luminance contrast in 2015, suggesting that the internal pattern of the wings is visible to avian predators.

5.4.2 Relevant differences in climatic conditions between years

Although collection sites were widely distributed across France and in Cornwall (UK), in a range of habitat types, there were broad climatic differences between the two years across all localities. The winter preceding the first set of field collections (December 2014 – February 2015) was significantly colder than the second (December 2015 – February 2016), with lower mean minimum, mean, and maximum temperatures (LME, minimum temperature, $(\chi^2)_1$=38.714, p<0.001; mean temperature, $(\chi^2)_1$=42.638, p<0.001; maximum temperature, $(\chi^2)_1$=24.919, p<0.001; Figure 5.6). By contrast, spring 2015 was warmer, sunnier and drier than spring 2016 (LME, minimum temperature, $(\chi^2)_1$=15.312, p<0.001; mean temperature, $(\chi^2)_1$=22.472, p<0.001; maximum temperature, $(\chi^2)_1$=19.436, p<0.001; hours of sun, $(\chi^2)_1$=8.3144, p<0.001; rainfall, $(\chi^2)_1$=6.662, p<0.001; Figure 5.7).

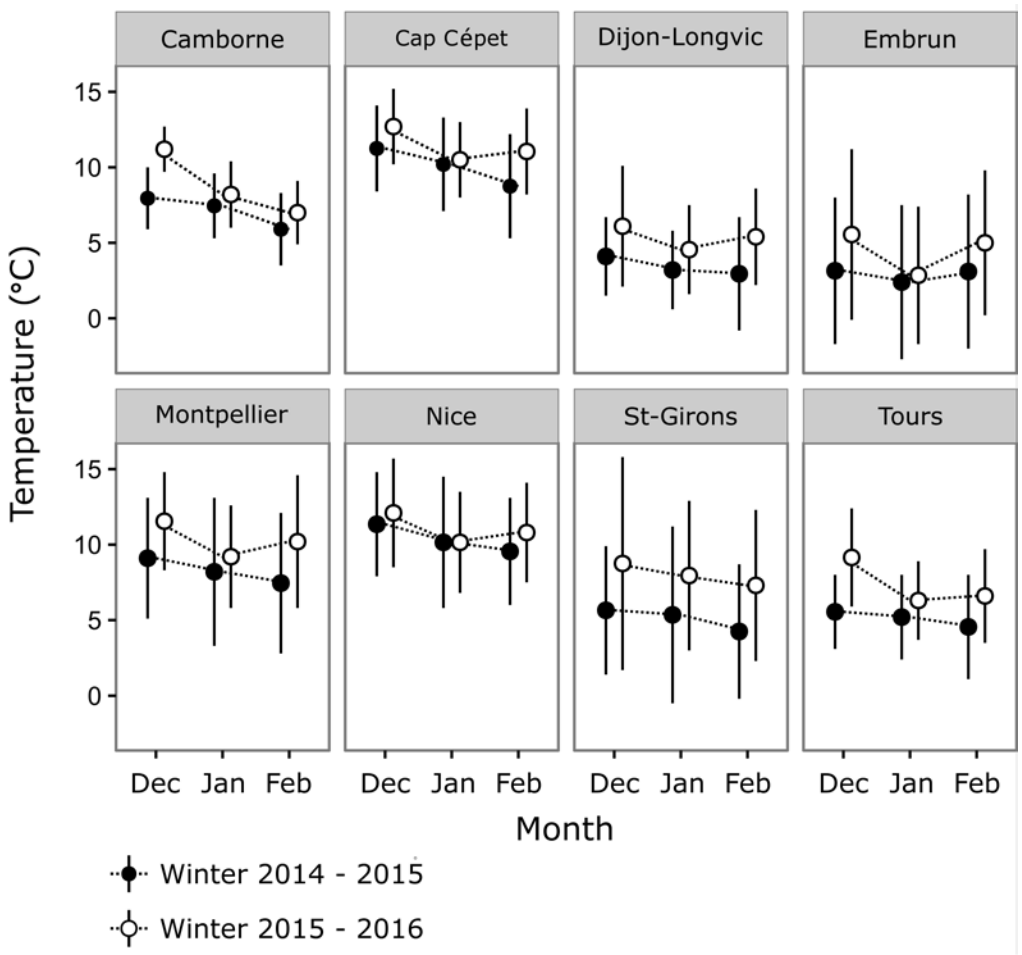


Figure 5.6: Temperature ranges in the winters preceding the 2015 and 2016 collections. Circles indicate monthly mean temperature and solid lines represent mean minimum and maximum temperatures.

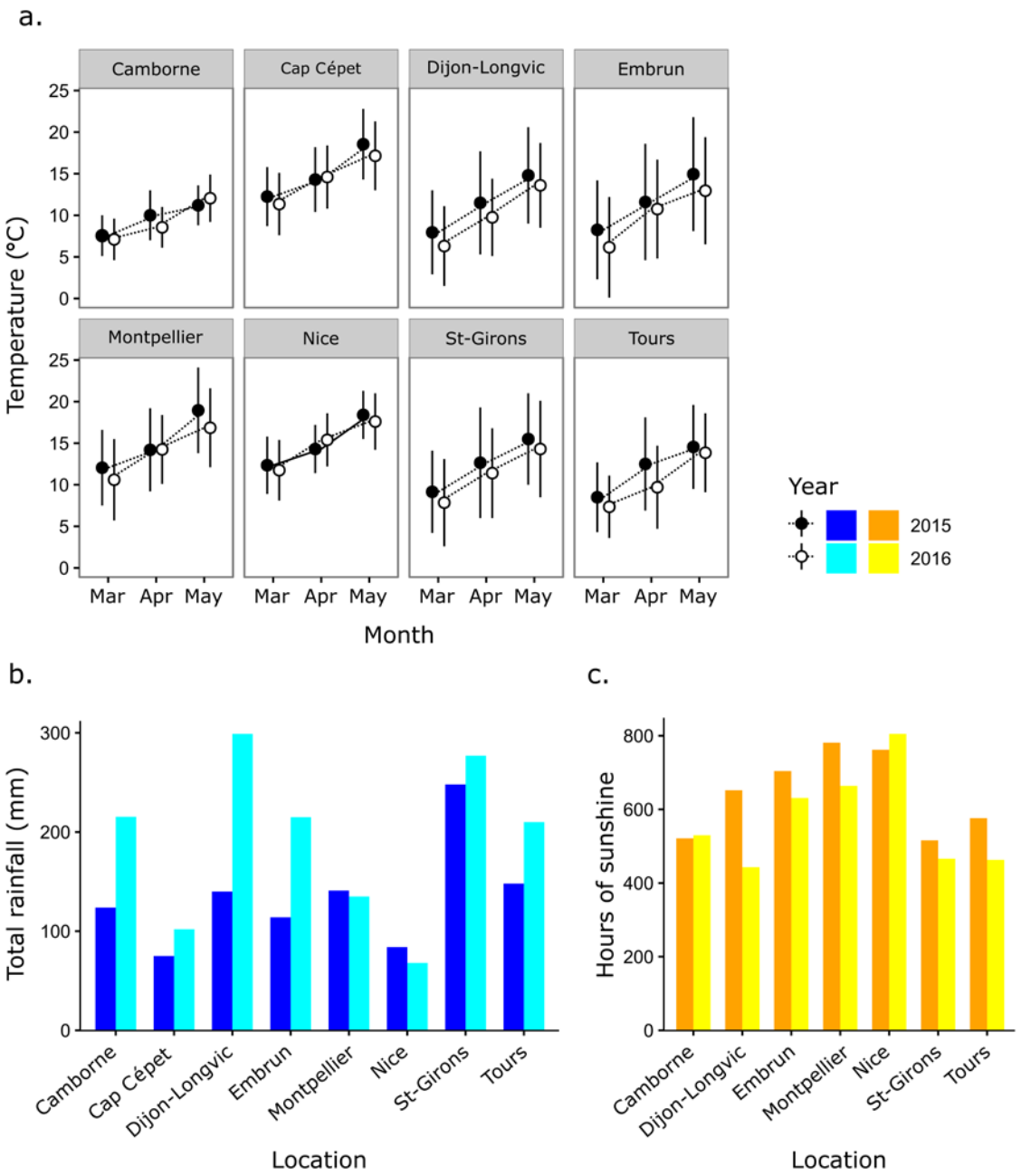


Figure 5.7: Weather conditions in the growing season in 2015 and 2016. In (a), circles indicate monthly mean temperature and solid lines represent mean minimum and maximum temperatures, while bars in (b) and (c) correspond to total rainfall and hours of sunshine respectively.

These differences in the winter and growing conditions are likely to have affected the growth, nutritional value and toxicity of zygaenid hostplants, and may have had a direct impact on the larvae themselves. For example, harsher diapause conditions during the first winter (2014-2015) may have reduced larval survival and caused surviving larvae to use up more resources, while warmer

and sunnier conditions may have altered the costs and benefits of pigmentation in the larval stages, potentially shaping the allocation of pigments in adults.

5.4.3 Relationship between colour and toxicity across species, within years

There were few significant correlations between measures of coloration and cyanogenic glucoside concentration across species (see Appendix 5.3 for full model tables). The trends that did emerge were similar whether males, females, or all specimens were considered, although these relationships were not always significant. For samples collected in 2015, there was a positive correlation between luminance and cyanogenic glucoside concentration (PGLS; across both sexes, $F_{1,7}$=13.409, p=0.00954; for females, $F_{1,6}$=14.975, p=0.00827, and close to reaching significance for males, $F_{1,7}$=5.916, p=0.051; Figure 5.8a). There was also a trend towards a negative relationship between measures of colour (saturation, hue, and chromatic contrast between markings and background colours) and cyanogenic glucoside levels, but this was only significant in females (PGLS; saturation, $F_{1,6}$=11.78, p=0.0139; hue, $F_{1,6}$=15.68, p=0.00745; chromatic contrast, $F_{1,6}$=13.713, p=0.0101; Figure 5.8b).

In addition, the relationships emerging between coloration and toxicity were not the same in both collection years. In 2016, there was a positive correlation between luminance contrast and cyanogenic glucoside concentration, although this was not significant in females (PGLS; across both sexes, $F_{1,9}$=6.803, p=0.00285; in males, $F_{1,8}$=11.474, p=0.00954; in females, $F_{1,6}$=3.957, p=0.0938; Figure 5.9). This relationship between internal luminance contrast and the level of chemical defences could not be attributed to trends in marking luminance. Unlike in 2015, there was no trend between luminance and cyanogenic glucoside concentration in either sex, or overall, in 2016 (Figure 5.10). There were no significant relationships between any other colour metrics and the levels of defensive chemicals in 2016.

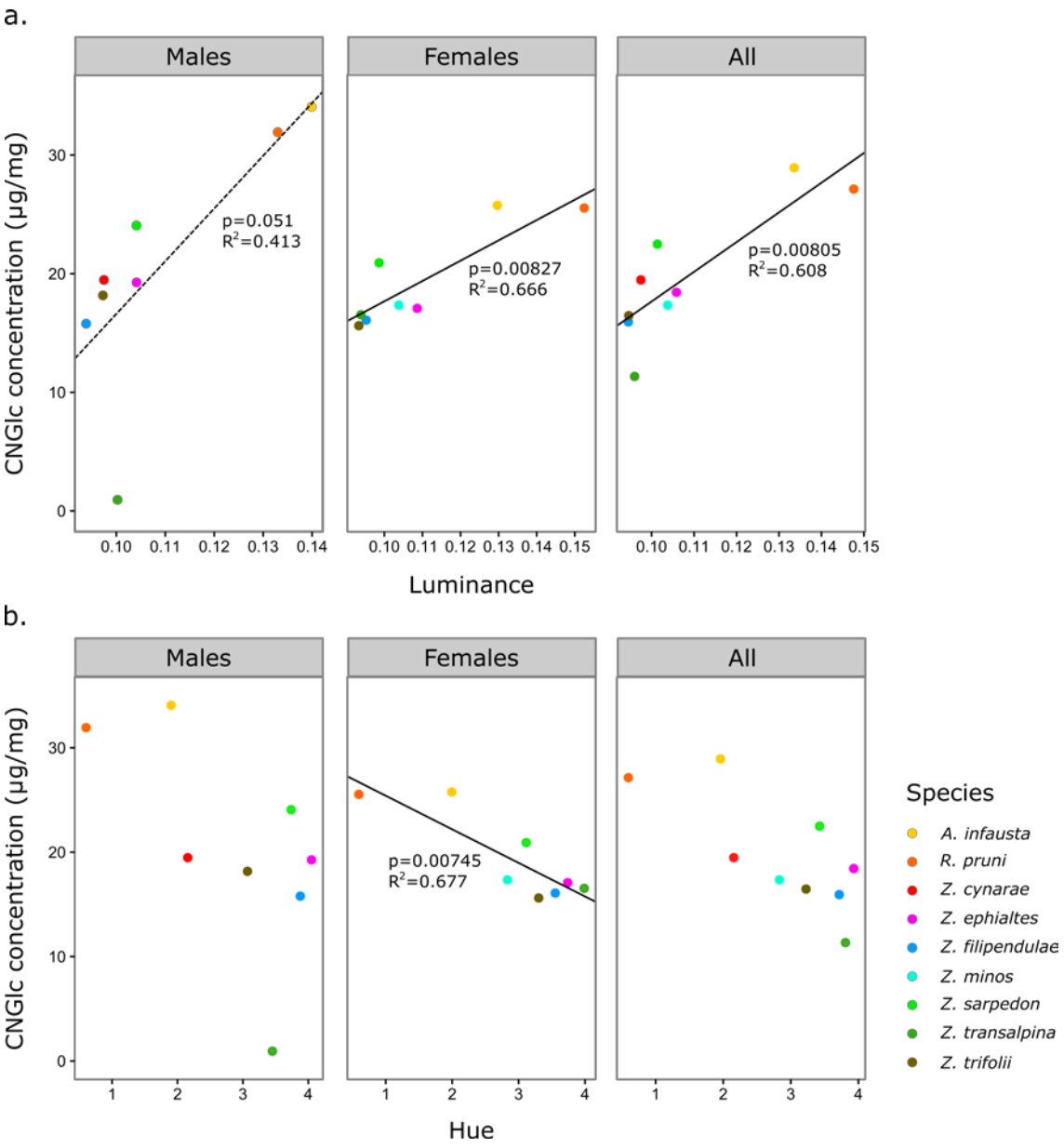


Figure 5.8: Mean cyanogenic glucoside (CNGlc) concentration and (a) luminance and (b) hue in species sampled in 2015, calculated in males, females and across both sexes. Lines represent the results of PGLS models: solid lines correspond to significant results, dashed lines to results close to significance.

Finally, maximum likelihood estimates found very little phylogenetic signal in the residuals of the regressions between colour metrics and cyanogenic glucoside levels ($\lambda=1*10^{-6}$ in each case). However, λ is difficult to estimate with small sample sizes (Symonds and Blomberg, 2014; Arenas, Walter and Stevens, 2015). This limitation should be taken into account, as most of the significant relationships between colour and toxin levels disappeared if λ was set to 1, corresponding to a Brownian motion model of evolution (Table 5.5).

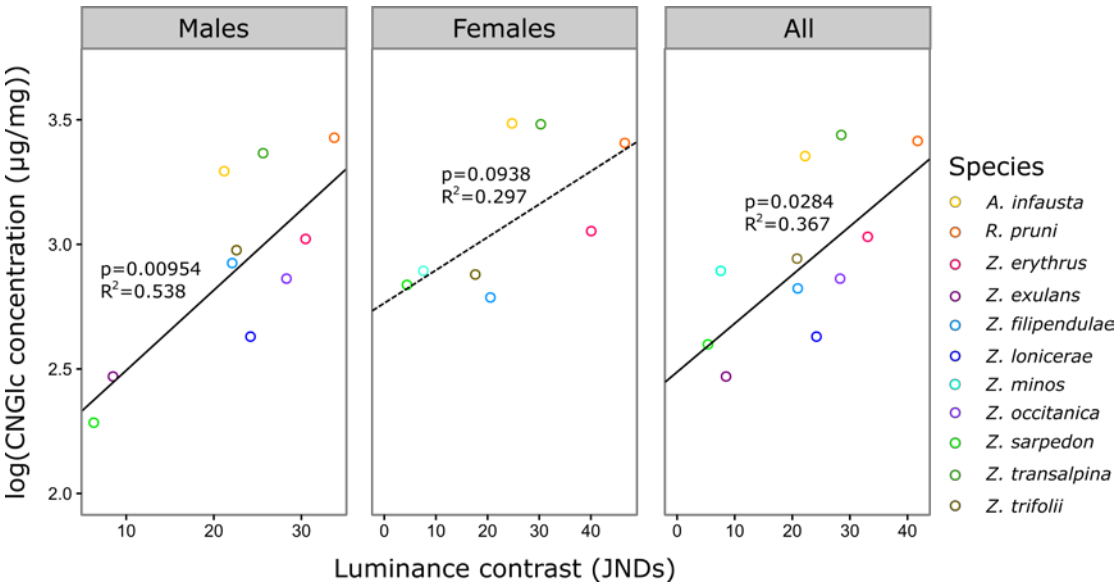


Figure 5.9: Mean log-transformed cyanogenic glucoside (CNGlc) concentration and luminance contrast in species sampled in 2016, calculated in males, females and across both sexes. Lines represent the results of PGLS models: solid lines correspond to significant results, dashed lines to results close to significance.

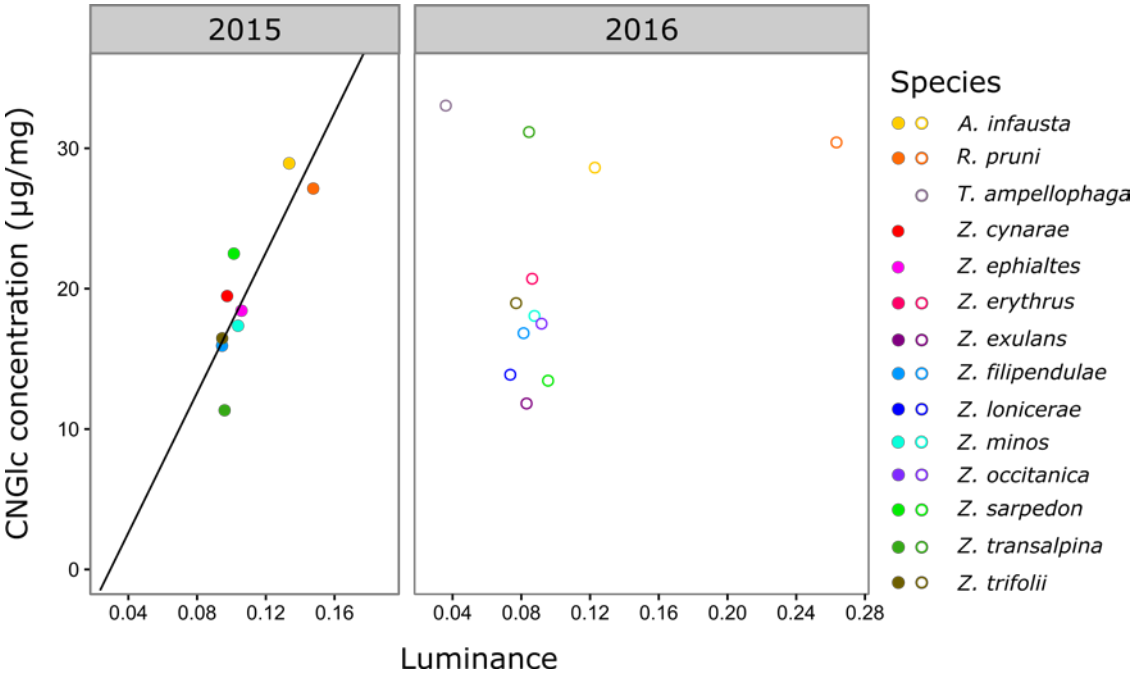


Figure 5.10: Mean value of luminance and cyanogenic glucoside (CNGlc) concentration per species, across both sexes, in specimens from 2015 and 2016. Filled circles=2015, Open circles=2016.

Table 5.5: Results of PGLS models testing the relationship between cyanogenic glucoside concentration ([CNGlc]) and colour metrics, with λ estimated by maximum likelihood ($\lambda=1*10^{-6}$) and λ=1 (Brownian motion model of evolution).

Dataset	Model	Results with $\lambda=1*10^{-6}$	Results with λ=1
2015, overall	[CNGlc] ~ luminance	$F_{1,7}$=13.409, p=0.00805	$F_{1,7}$=5.454, p=0.0522
2015, males	[CNGlc] ~ luminance	$F_{1,6}$=5.916, p=0.051	$F_{1,6}$=2.669, p=0.153
2015, females	[CNGlc] ~ luminance	$F_{1,6}$=14.975, p=0.00827	$F_{1,6}$=4.368, p=0.0816
2015, females	[CNGlc] ~ saturation	$F_{1,6}$=11.78, p=0.0139	$F_{1,6}$=3.563, p=0.0108
2015, females	[CNGlc] ~ hue	$F_{1,6}$=15.68, p=0.00745	$F_{1,6}$=5.277, p=0.0613
2015, females	[CNGlc] ~ chromatic contrast	$F_{1,6}$=13.713, p=0.0101	$F_{1,6}$=4.583, p=0.0761
2016, overall	[CNGlc] ~ luminance contrast	$F_{1,9}$=6.803, p=0.0285	$F_{1,9}$=4.241, p=0.0696
2016, males	[CNGlc] ~ luminance contrast	$F_{1,8}$=11.474, p=0.00954	$F_{1,8}$=11.61 p=0.00926
2016, females	[CNGlc] ~ luminance contrast	$F_{1,6}$=3.957, p=0.0938	$F_{1,6}$=3.637, p=0.105

5.5 Discussion

The primary aim of this study was to assess signal honesty across closely-related species in the Zygaenidae. However, collecting specimens over two field seasons uncovered some unexpected complications, which highlight some of the difficulties associated with studying signal honesty across species or populations. I chose to analyse specimens collected in 2015 and 2016 separately, as there were important differences in the measurements of colour and toxicity in these two years, across all species. In addition, my previous work on *Z. filipendulae* (see Chapter 4) suggested that differences between the sexes could be relevant, and relationships across species were indeed variable between sexes in this study.

5.5.1 Signal honesty across species

In terms of the original question, I found little evidence of signal honesty across these species. Luminance was positively correlated with the concentration of cyanogenic glucosides across species in 2015, but paler markings seem

unlikely to constitute more salient markings. In terms of other colour metrics, the only relationships that emerged in 2015 were negative correlations, suggesting dishonesty in signalling: saturation, hue and chromatic contrast were all negatively correlated with cyanogenic glucoside levels, especially in females. In 2016, the only relationship between coloration and toxin levels was a positive correlation between luminance contrast and cyanogenic glucoside concentration. This could potentially be a useful cue for predators, but there were no other significant correlations between coloration and toxicity. Achromatic information is generally considered less important than chromatic cues for avoidance learning, especially in birds, but could still be relevant to them, in particular in terms of distinguishing small pattern elements (Stevens, 2007) or triggering initial avoidance of aposematic patterns (Sandre, Stevens and Mappes, 2010). Luminance contrast in the pattern of prey items also facilitated detection and avoidance learning in experiments with mantids (Prudic, Skemp and Papaj, 2007), suggesting that it could be a useful cue for invertebrate predators, to which burnet moths are also exposed. To interpret the results of this present study with confidence, it would be useful to test which visual cues predators attend to when faced with a burnet moth-like pattern, and especially gauge their response to variation in luminance or chromatic features of the wing markings. It is also important to note that the trend in luminance contrast observed in 2016 was not due to differences in marking luminance, so was likely to be driven by changes in the luminance of the moth wings' dark background scales. As the dark pigment melanin is involved in many other functions, from immune defences to thermoregulation (Solano, 2014), other selective pressures besides avoiding predation could be responsible for the trends in wing background luminance, and hence the relationship between luminance contrast and toxin levels.

The absence of signal honesty in the Zygaenidae is contrary to the results of other recent studies of signal honesty across species, in ladybirds (Arenas, Walter and Stevens, 2015) and nudibranchs (Cortesi and Cheney, 2010), and demonstrates that quantitative signal honesty is not ubiquitous across families of aposematic species. Across species, a range of factors, including different habitat or microhabitat features (Endler, 1993), predator communities (Endler and Mappes, 2004; Nokelainen *et al.*, 2014) and life-history traits (Longson and

Joss, 2006), are likely to impose different fitness costs and benefits on the production of both signals and defences. If these costs and benefits do not change in parallel, honest signalling may not evolve (Speed and Ruxton, 2007). In the Zygaenidae, the economics of signals and defences are likely to differ between species, as they vary in their means of acquiring toxins, as well as in their behaviour. The Procridinae behave more like cryptic species, flying rapidly and seeking to evade capture, while red-spotted burnet moths are much more sluggish (Hofmann and Tremewan, 2017), affecting their exposure to predators. Nevertheless, it is important to note that many studies of signal honesty across aposematic species have used conspicuousness of the prey pattern to natural backgrounds as their measure of signal strength, while this study concentrates on measures of coloration inherent in the moth wings themselves. For logistical reasons, it was not possible to photograph all the host plants of the different species. Some polyphagous species were collected as cocoons, so the host plant was unclear (e.g. *Z. exulans*), and, in any case, burnet moths are often found at rest on plants other than their hosts, and especially on flowers, which were not in bloom at the time when larvae and pupae were collected. Considering conspicuousness against natural backgrounds would be an important next step in this analysis, so as to make this data more comparable to previous studies.

In addition to establishing which measures of colour are more relevant to predators, it would also be useful to gain a better understanding of how avian predators may experience the chemical defences of the Zygaenidae. Toxin levels may not always be the best indicator of a species' defences. For example, *Aglaope infausta* and *Rhagades pruni* have the highest concentration of toxins across the studied species, but they are also much smaller, and so hold a smaller total amount of cyanogenic glucosides. It is reasonable to expect that the concentration of cyanogenic glucosides is most relevant, as this will determine the aversiveness of defensive secretions emitted by adults (Rothschild, 1985) and of the haemolymph if the moth is wounded. Size will also affect how attractive a prey item will appear to predators, as they balance the relative nutritional gain from consuming prey against the noxious effects of their defences to make educated decisions while foraging; in this case, smaller toxic prey, such as *A. infausta* or *R. pruni,* may be more likely to be avoided (Smith,

Halpin and Rowe, 2014, 2016). Alternatively, small species could be more likely to be completely ingested, suggesting that the total amount of toxins, or how nasty the experience of consuming a specimen would be, might be more relevant to predator learning. Most of the observations of birds attacking burnet moths are anecdotal (Tremewan, 2006), so more rigorous investigations of how birds respond to different zygaenid species, for example whether or not they can taste and reject them, would be helpful in resolving this issue.

Relatively few studies have explored the relationship between coloration and the levels of chemical defences across species while accounting for phylogeny (Summers and Clough, 2001; Cortesi and Cheney, 2010; Santos and Cannatella, 2011), so this present study makes a rare and valuable contribution to the field. There are of course limitations to this dataset, primarily due to very small sample sizes for certain species (N=1 or N=2), but these were included in the analysis as increasing the number of species in phylogenetic analyses is crucial to more reliable results. It would in fact be preferable to include even more species, especially when attempting to separately analyse specimens of each sex, or collected in different years. Small phylogenies suffer from a lack of power (Freckleton, Harvey and Pagel, 2002), making it difficult to accurately estimate parameters of phylogenetic signal, such as λ (Symonds and Blomberg, 2014). My results demonstrate that the value of λ does alter the significance of the relationships between colour metrics and measures of toxin levels, although it does not dramatically change the overall conclusions. To strengthen this analysis, it would nevertheless be useful to sample more species and include more specimens from each species. As this is difficult to do using only field collections, especially when attempting to account for fluctuations in resource levels between seasons and differences between populations, raising individuals of each species from wild-caught gravid females could be a useful approach.

5.5.2 Sex differences in signal honesty

In this dataset, trends were broadly similar across both sexes, but the significance of the relationships did differ. For example, the negative relationship between measures of colour (hue, saturation and chromatic contrast) and cyanogenic glucoside levels across species was only significant

for females, and not when averages per species across both sexes were considered. This suggests that ignoring differences between the sexes could mask sex-specific trends or lead to significant relationships being overlooked, an important consideration as no existing studies of quantitative honesty across aposematic species and populations analyse males and females separately, even in taxa in which males and females are known to differ (e.g. in ladybirds; Arenas, Walter and Stevens, 2015). Sex differences in the relationship between coloration and defences have previously been found in seven-spot ladybirds (*Coccinella septempunctata*; Blount *et al.*, 2012): larger females, thought to be more resource-limited than males, were found to have an honest relationship between elytra carotenoids and coccinelline levels, while males displayed a dishonest relationship, matching the predictions of the resource allocation theory (Blount *et al.*, 2009). Female ladybirds may also benefit more from honest signalling as their larger size makes them a more attractive and conspicuous prey item to predators (Blount *et al.*, 2012). Similar considerations could apply to burnet moths, which are also sexually dimorphic in size, with females much larger than males.

Males and females will also experience different trade-offs of coloration as an aposematic signal with other functions, such as sexual selection. In burnet moths, little is known about the potential for mate choice based on coloration, but visual cues could be relevant to intra-specific communication, and this would likely differ between sexes (Zagatti and Renou, 1984; Toshova, Subchev and Toth, 2007; see Chapter 4). They also behave differently, with males flying to locate perched females calling with pheromones (Naumann, Tarmann and Tremewan, 1999). This could mean that the sexes are differentially exposed to predators, although it is unclear whether the more active sex would actually be more visible to predators or benefit more from quantitative signal honesty, as accurately assessing the colour of flying males may be more difficult than for females at rest.

5.5.3 Differences between years and the effect of environmental conditions

As well as analysing males and females together, existing studies of the relationship between signals and defences in aposematic species are typically restricted to specimens collected within a relatively short time frame, and do not

consider temporal variation in signals and defences (see Chapter 1). This present study suggests that, on the contrary, seasonal differences could be relevant, as I found significant variation in coloration and toxicity across two years of sampling. I made every effort to ensure that these between-year differences were not caused by inconsistencies in the experimental procedures, but rather reflected real effects seen in natural populations. Rearing conditions were kept as consistent as possible between specimens collected in 2015 and 2016: although caterpillars were held in natural conditions during field trips, I regularly sent new pupae away to be housed in an incubator, with the same settings in both years. In addition, there were differences in coloration and cyanogenic glucoside between years in *Z. trifolii* specimens, collected at the pupal stage in the same location and immediately placed in the incubator, suggesting that any variation in larval rearing conditions was not responsible for the differences between field seasons. The time spent in the -80°C freezer between termination and photography was shown not to impact coloration (see Chapter 2), while the methods and equipment used for image capture did not vary between years. All images from both seasons were processed and analysed together. In terms of differences in cyanogenic glucosides, running a subset of samples from both years again together suggested that the differences observed between years could not be attributed to variation in the sensitivity of the LC-MS equipment (see Appendix 5.2).

With only two years of data, it is difficult to determine the causes of the variation I found between years, especially the complex trends in cyanogenic glucoside levels. Nevertheless, there were general differences in climatic variables between years, across all the diverse areas in which samples were collected, suggesting that environmental conditions and available resources broadly differed between 2015 and 2016. Environmental variation is likely to impact investment in coloration and chemical defences in burnet moths, although predicting these effects is not straightforward. Variation in temperature, for example, encompasses a range of measures (such as the overall mean temperature, extreme temperatures or seasonality) which may have different impacts on specific species (Seabra *et al.*, 2015). In terms of coloration, temperature is known to affect both adult and larval colour patterns in aposematic tiger moths (Erebidae; Goulson and Owen, 1997; Lindstedt,

Lindström and Mappes, 2009). Fluctuations in the colour of adult warning signals across years in the wood tiger moth (*Arctia plantaginis*) have also been linked to variation in local ecological conditions (Galarza *et al.*, 2014). The increased salience of red markings in burnet moths in 2016 compared to 2015 could therefore be linked to temperature differences during overwintering or larval development. For example, colder conditions during the winter diapause of zygaenid larvae in 2014/2015 may have been more costly in terms of resources, reducing their ability to later produce as much pigment as in more favourable years. However, data would have to be collected over many more field seasons to properly test such speculations.

Environmental conditions could also affect the moths' levels of chemical defences, by influencing the cyanogenic glucoside content of host plants, or more generally impacting the amount of resources available for developing larvae. Cyanogenic glucoside levels in plant tissue are highly variable and strongly affected by environmental conditions (Gleadow and Woodrow, 2002), including temperature, nitrogen and phosphorus availability, carbon dioxide levels and water stress (Patel and Wright, 1958; Gleadow, Foley and Woodrow, 1998; Woodrow, Slocum and Gleadow, 2002). Research on white clover (*Trifolium repens*) across gradients of latitude and altitude suggests that colder temperatures are associated with lower frequencies of cyanogenic plants (Daday, 1954a, 1954b, 1958; De Aráujo, 1976; Richards and Fletcher, 2002) although there can be contradictory effects within seasons (Stochmal and Oleszek, 1997). The effect of temperature on bird's foot trefoil (*L. corniculatus*), the main host plant of *Z. filipendulae*, is less clear-cut (Ellis, Keymer and Jones, 1977; Jones, 1977) and some studies have revealed opposite trends to *T. repens*, finding greater levels of cyanogenic glucosides at higher altitudes (Salgado *et al.*, 2016).

Moreover, the zygaenid species included in this study feed on a range of plants, which do not all possess cyanogenic glucosides. It is difficult to predict the effect of the differing conditions between the two years on all these plants, and more work is needed to determine how temperature changes over winter and during the growing season may impact the nutritional content of potential host plants, including the toxin load of cyanogenic plants. For the zygaenid

larvae able to sequester defences from their host plants, the cyanogenic glucoside content of those plants may affect their chemical defences under natural conditions, even though they are capable of *de novo* synthesis, and may be able to compensate for an acyanogenic diet in the laboratory (e.g. *Z. filipendulae*, Zagrobelny *et al.*, 2007a). Unfortunately, I did not collect plant samples from study sites in both years, so the relationship between host plant and moth toxin content cannot be elucidated here. For the species relying completely on *de novo* synthesis of cyanogenic glucosides, the quality of host plants may still be important. Plant productivity will impact the availability of host plants for larvae to feed on, while nitrogen limitation will lead to reduced investment in cyanogenic glucosides due to trade-offs with other products, as suggested by the breakdown of cyanogenic glucosides during pupation to fuel chitin synthesis (Zagrobelny *et al.*, 2007b). Interestingly, the species in which cyanogenic glucoside levels decreased between years in males (*A. infausta*, *R. pruni* and *Z. sarpedon*) all feed on acyanogenic host plants, suggesting that resource allocation trade-offs may broadly differ between species able to sequester cyanogenic glucosides from their host plants and those who cannot. Investigating coloration and chemical defences within populations across multiple seasons could be a valuable means of testing for quantitative honesty in aposematic signalling, providing the opportunity to study how resources are allocated to these two elements of aposematism in response to environmental conditions, and as the communities of predators and prey co-evolve.

Overall, this study suggests that the relationship between signals and defences in and across aposematic species is more complicated and dynamic than may have been previously appreciated. Although the sample sizes for certain species are small, this dataset provides cross-species information on colour and defences with a rare level of detail, using sophisticated measurements of well-understood chemical defences and multiple measures of coloration, including marking size, saturation, hue, luminance and internal contrasts, while taking into account predator perception. The results indicate that the relationship between coloration and defence may not be consistent between sexes or between seasons, highlighting the potential pitfalls of ignoring these complicating factors. It is clearly difficult to obtain sufficient data to adequately account for sex, population and seasonal differences when testing for a relationship between

colour and toxicity in wild-caught animals, yet this could be crucial to capturing the complexity of aposematic signalling strategies. One slightly less ambitious step towards this more detailed approach would be to explore the effects of variation in resources across seasons in a single population. Conversely, measuring the coloration and toxicity of several species in a single location would help resolve the question of mimicry across closely-related species exposed to the same predator community. Moreover, in order to make inferences about signal honesty, it is of paramount importance to gain a better understanding of which aspects of aposematic signals are most relevant to predators, and may be used in their assessment of prey profitability, as well as how predators respond to variation in the concentration versus the total amount of toxins in a prey item.

Chapter 6

Effect of spot presence, size, colour, and wing iridescence on predation rates of artificial burnet moths in the wild

Real and artificial six-spot burnet moths on Penhale Sands. Photograph: E. S. Briolat

6.1 Abstract

Aposematic species advertise their defences using conspicuous visual signals, often with multiple components and properties such as colour, brightness and pattern. The relative importance of each of these traits in generating predator avoidance is difficult to ascertain, yet is crucial to resolving the question of quantitative honesty in aposematism. Studies of signal honesty record differences in visual traits and variation in the potency of chemical defences, but the relationship between these two elements will only affect prey survival if their natural predators pay attention to these specific features and take them into consideration when making foraging decisions. To explore the relevance of multiple aspects of the six-spot burnet moth's warning signals, artificial predation trials were carried out using burnet moth-like prey made of wire, paper, and plasticine. Separate experiments were designed to test firstly the effect of forewing spot colour and wing iridescence, and secondly the impact of spot presence and size, on attack rates by avian predators. To maximise the relevance of these experiments to burnet moths in the wild, the artificial prey were designed to mimic the colours of real six-spot burnets as perceived by avian predators, and were deployed in a natural habitat for this species. In addition, predation experiments were run during three distinct field seasons in 2016 and 2017, enabling an investigation of the effect of seasonality on predation rates. Spot colour appeared to have a significant effect on predation risk for the moth-like baits, but, contrary to expectations that more conspicuous and redder signals would be more effectively avoided, prey with redder spots incurred higher predation rates. In support of previous work in Lepidoptera, the presence of an iridescent sheen appeared to have no impact on survival. Predation rates of moths with and without spots differed greatly between field seasons, yet there was no significant difference in predation rates of prey with red spots, either large or small, versus prey with a uniform brown wing colour, in any of the three field seasons. These results suggest that spot colour rather than pattern may be the most important aspect of burnet moth warning signals, and also highlight some strengths and limitations of artificial predation experiments in assessing the effectiveness of aposematic signals.

6.2 Introduction

While most animals attempt to avoid catching the eye of a predator, defended species can benefit from a conspicuous appearance. These warningly-coloured, or aposematic, animals use bright or otherwise highly visible visual signals to warn predators of their unprofitability and deter attack (Ruxton, Sherratt and Speed, 2004). Conspicuousness itself is an advantageous feature, in terms of both aspects of signal function: the strategic, or informational, component, relating to the message carried by the signal, and the efficacy component, concerned with communicating this message most effectively (Guilford and Dawkins, 1991). By attracting the attention of predators, conspicuousness is an inherently costly trait: while defended prey may be rejected or survive an attack, being so obvious to predators is too risky for profitable prey. From a strategic perspective, conspicuousness is thus well-suited to conveying reliable information about prey defences (Sherratt and Beatty, 2003). Conspicuous signals are also considered to stimulate predator learning in multiple ways, from increased detectability to improved memorability (reviewed in Speed, 2000), thereby enhancing signal efficacy. Nevertheless, it is difficult to disentangle the effects of conspicuousness from those of novelty or distinctiveness (Ruxton, Sherratt and Speed, 2004). For example, experiments with domestic chicks (*Gallus gallus domesticus*) suggest that avoidance of distasteful water can be stimulated by unfamiliarity, regardless of the specific colour used (Shettleworth, 1972). Adding to this complexity, conspicuous warning signals are often composed of multiple pattern elements, or components (Rowe, 1999), and can be described by several visual properties, such as brightness, colour and pattern, as well as by their contrast to natural backgrounds. Teasing apart the relative contributions of these different signal features is a complex task, and a key area of future research into warning signals (Stevens and Ruxton, 2012; see Chapter 3).

Many experiments have attempted to unpick the roles of colour and pattern in aposematic signals, yet this debate remains somewhat unresolved. Tests with artificial patterns in the laboratory have suggested that domestic chicks pay more attention to colour than pattern elements (Gamberale-Stille and Guilford, 2003; Aronsson and Gamberale-Stille, 2008), yet other studies have shown that highly contrasting markings can also attract their attention (Osorio, Jones and Vorobyev, 1999). In similar experiments with blue tits (*Cyanistes caeruleus*),

colour again appeared more important than pattern, but the presence of contrasting markings did contribute to avoidance learning of distasteful prey, as striped prey benefitted from faster learning by predators (Aronsson and Gamberale-Stille, 2012b). Achromatic information may be most important for generating initial avoidance, by making the signals more salient, while colour plays a larger role in subsequent learning in birds, although perhaps not in invertebrates (Stevens and Ruxton, 2012; Prudic, Skemp and Papaj, 2007). In the field, work on *Heliconius* wing patterns and ladybirds supports the idea that colour may be the most important feature of warning signals (Finkbeiner, Briscoe and Reed, 2014; Arenas, 2015), yet other studies have demonstrated that the extent of melanisation (Hegna *et al*., 2013), distinctive markings (Wüster *et al*., 2004), achromatic contrast (Prudic, Skemp and Papaj, 2007) and overall pattern (Tan, Reid and Elgar, 2016) can have an impact on survival in predation trials. For colour itself, the relative importance of novelty, conspicuousness against the background, and specific colours *per se*, is also unclear. Long wavelength colours, such as red and yellow, may be more effective warning signals as they present more consistent chromatic and achromatic contrasts across different illuminations (Arenas, Troscianko and Stevens, 2014), making it difficult to separate the role of conspicuousness from any inherent quality of red and yellow signals. Further studies of the impact of signal features on predation risk in other systems would thus be helpful to clarify the situation.

As reported in Chapters 2, 4 and 5, there is variation in wing colour and pattern in burnet moths (Zygaenidae), both within the six-spot burnet (*Z. filipendulae*) and across species. Establishing how these features contribute to protecting these insects will help to further our understanding of the roles of colour and pattern in aposematic species, and is also relevant to the question of signal honesty. Measuring variation in coloration among aposematic individuals, populations, and species, and relating these differences to variation in toxicity (Chapters 4 and 5), are only the first steps in investigating the potential for quantitative honesty in defended animals. For the relationship between signals and defences to have an impact on prey survival in the wild, variation in signal form must have a measurable effect on predator behaviour, affecting their foraging choices. Testing predator responses to variation in burnet moth signals will help clarify the relevance of any relationships, or absence thereof, between

signal characteristics and chemical defences in these species. The following experiments were designed to test the importance of variation in several features of the forewing pattern of *Z. filipendulae*: spot presence, size and colour. According to efficacy theory, and to the results of similar studies with ladybird models (Arenas, Walter and Stevens, 2015), redder and larger spots were expected to increase predator avoidance, and thus have a beneficial impact on prey survival.

In addition, the forewings of the Zygaeninae are iridescent, displaying a striking green or blue sheen at certain angles of incident light. Iridescence is thought to prevent predation in a number of ways, by enhancing camouflage and hindering prey capture, especially when in motion (Pike, 2015), or deterring predators by heightening startle displays and functioning as an aposematic signal (Doucet and Meadows, 2009). Aposematism involving iridescence has often been proposed to exist in beetles, butterflies and true bugs (Bowers and Larin, 1989; Vulinec, 2015; Seago *et al*., 2009; Rutowski, Nahm and Macedonia, 2010; Pegram, Han and Rutowski, 2015; Fabricant *et al*., 2014), but very few studies have tested whether iridescence actually promotes avoidance of prey by natural predators (Fabricant *et al*., 2014; Pegram, Han and Rutowski, 2015). In learning trials in captivity, iridescent patches resulted in greater initial avoidance of hibiscus harlequin (*Tectocoris diophthalmus*) baits and affected the ability of great tits to generalise to similar stimuli (Fabricant *et al*., 2014). However, field experiments with blue and orange pipevine swallowtail butterflies (*Battus philenor*) found no effect of iridescence on prey survival (Pegram, Han and Rutowski, 2015). My study aimed to test whether iridescence could convey an additional protective effect on red and black patterned moth-mimicking prey, and thus might play a role in aposematism in the Zygaenidae.

One of the most common and versatile techniques to investigate predatory behaviour in the wild is the use of predation experiments with artificial prey. In the field of visual signals, this tool has been successfully used to test the impact of a wide range of features, such as colour (Arenas, Walter and Stevens, 2015), luminance contrast (Flores *et al*., 2015), pattern complexity and other pattern features (Easley and Hassall, 2014; Rojas, Devillechabrolle and Endler, 2014; Barnett, Scott-Samuel and Cuthill, 2016), as well as predator preference for

local or novel morphs (e.g. Chouteau, Arias and Joron, 2016; Hegna, Saporito and Donnelly, 2013). More recently, artificial predation experiments have been refined to test more complex and dynamic factors affecting prey survival, such as the composition of the predator community and the frequency of different morphs in the prey community (Nokelainen *et al*., 2014; Wennersten and Forsman 2009), seasonality (Mappes *et al*., 2014), predator learning (Dell'Aglio, Stevens and Jiggins, 2016) and microhabitat structure (Willmott *et al*., 2017). Learning from these studies, my work was designed to comprehensively test the impacts of burnet moth signal features, in as naturalistic a setting as possible. The artificial prey were deployed in a natural habitat of *Z. filipendulae* in Cornwall (UK), ensuring that they were exposed to a relevant predator community, and camera traps were used to determine the key predator species attacking the artificial prey. One experiment was repeated across multiple seasons, to test the effect of seasonality on predation rates. Unlike most predation experiments with stimuli mimicking Lepidoptera, in which baits are pinned to trees or plants, the artificial moths used here were stuck into the ground, to closely resemble adult *Z. filipendulae* later seen on low-growing flowers in this same location. Finally, a key advantage of using artificial prey in studies of visual signals is the ability to carefully manipulate the stimuli, to test the effect of specific signal characteristics. With modern visual modelling techniques (Stevens *et al*., 2007a; Endler and Mielke, 2005), stimuli can even be designed to match colours as perceived by the visual system of relevant predator species. Here, the prey colours were chosen to mimic those of real burnet moths collected in Cornwall, as perceived by avian predators. Altogether, these precautions and technical details ensure that the effects of spot colour, pattern and iridescence measured here are as relevant as possible to the predation risks experienced by wild burnet moths.

6.3 Methods

6.3.1 Colour measurements from wild burnet moths

I collected *Z. filipendulae* caterpillars and cocoons (n=50; n_{male}=23, n_{female}=27) from three sites in Cornwall, UK: Holywell Bay (50° 23' 22.53'' N, 5° 40' 13.56'' W), Lamorna Cove (50° 3' 40.50'' N, 5° 33' 16.70'' W) and Pendeen Watch (50° 9' 50.42'' N, 5° 40' 13.56'' W) in May and June 2015. The moths were reared to

adulthood inside an incubator (16:8h day:night cycle, temperature at 21°C), conditions similar to those used in previous projects with this species (Zagrobelny *et al*., 2007a), then euthanised in a -80°C freezer immediately after eclosion. As described in Chapters 2, 4 and 5, I then cut their wings off and photographed them on a background of grey ethylene-vinyl acetate (EVA), using a calibrated Nikon D7000 camera. Photographs were taken in controlled lighting conditions inside a dark room, illuminated by an EYE Color Arc Lamp MT70 bulb (Iwasaki Electric Co. Ltd.), emitting a spectrum of light similar to D65 daylight conditions. All images included a scale bar and a set of 7% and 93% reflectance standards, which reflect light equally at every wavelength, so as to control for any variation in light conditions.

Each specimen was photographed twice, using different filters (a UV/infrared blocking filter [Baader UV/IR Cut Filter], transmitting between 400 and 700nm, and a UV pass and IR blocking filter [Baader U filter], transmitting between 300 and 400nm). Merging these photographs yields a set of four image layers, corresponding to different parts of the visual spectrum: long wavelength (LW), medium wavelength (MW), short wavelength (SW) and ultraviolet (UV). Subsequent image analysis was performed using a dedicated image calibration and analysis toolbox in ImageJ (Troscianko and Stevens, 2015); images were linearised and normalised (Stevens *et al*., 2007a) as per the methods described in the software guide. The wing colours were then analysed from the perspective of potential predators, which in this case are most likely to be birds, with anecdotal reports of burnet moth predation attributed to many different species, including blackbirds and skylarks (Tremewan, 2006). In order to do this, the moth wing images were mapped to the two known categories of avian visual system, which differ in the sensitivity of their most shortwave-sensitive cone type (the violet-sensitive [VS] and ultraviolet-sensitive [UVS] groups; Hart, Partridge and Cuthill, 1999), using data from their respective model species, the blue tit *Cyanistes caeruleus* (Hart *et al*., 2000) and the peafowl *Pavo cristatus* (Hart, 2002). Using the same software package (Troscianko and Stevens, 2015), linearised and normalised images were transformed via a polynomial mapping technique with a D65 irradiance spectrum (Westland and Ripamonti, 2004; Stevens *et al*., 2007a; Pike, 2011), yielding five image layers, with predicted cone catch values for each photoreceptor type: ultraviolet- (UV or

VS), short wavelength- (SW), medium wavelength- (MW) and long wavelength- (LW) sensitive photoreceptors, as well as double cones. Though potential predators of burnet moths include species falling in both avian groups (see Chapters 4 and 5), all subsequent analysis and stimulus design was based on the ultraviolet-sensitive visual system alone.

Only the right forewing of each individual was measured, as the wings are iridescent, so the direction of the light source and wing scales affects their colour. Wing markings and background areas were selected using the freehand tool in ImageJ, and cone catch values for every photoreceptor type were measured. After viewing the resulting data, one outlier was removed from the final dataset (n=49; n_{male}=22, n_{female}=27). I used these values, along with size measurements of the same individuals, to design the artificial prey for the following experiments.

6.3.2 General design of artificial prey

The overall shape of the artificial prey was designed to mimic a burnet moth at rest, with two triangular forewings either side of a black body. I drew the wings in Inkscape, then printed them onto waterproof paper (Xerox Premium NeverTear 120µm) using a laser printer (HP Color Laser JetPro M252dw). Experiment 1 was designed to test the effect of variation in the red forewing spot colour, and of the presence or absence of iridescence, on predation rates for artificial moths in the wild: each artificial moth had a unique spot colour, and was either iridescent (I) or matte (M). For Experiment 2, four treatments were devised to test the effect of spot size on predation rate: large spots (LS), small spots (SS), and two uniform treatments (ULS and USS, matching the overall colour of the LS and SS stimuli respectively; Figure 6.1). Spot size was based on measurements of red spot area in the wild burnet moth dataset described above, with the large and small spots corresponding to the 5th and 95th percentiles respectively (Table 6.1). To maximise detectability while remaining within the natural size range of *Z. filipendulae*, total wing area of stimuli in both experiments was based on the 95th percentile of the wild burnet moth dataset (Table 6.1).

I assembled the printed wings into artificial moths by threading them through the middle onto lengths of gauge 19 green floral wire; the wire was then folded back 90°, so that the moth would lie parallel to the surface when the wire was inserted into the ground. The body was represented by a cylindrical piece of non-toxic black modelling clay (Newplast^{TM}), 15 mm long and 4 mm in diameter, crafted with a clay extruder to obtain a consistent size. This was glued to the middle of the moth with superglue, covering the wire, and hooked over the tip of the wire at the front of the moth for additional security (Figure 6.4b).

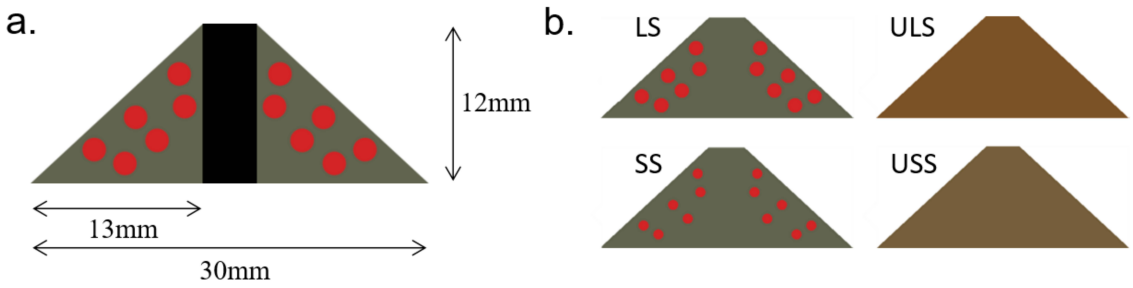


Figure 6.1: General design and dimensions of all the artificial prey (a) and wing patterns used in Experiment 2 (b). Moth wings in Experiment 2 are the same size as those in experiment 1. LS = Large spots, SS = Small Spots, ULS = Uniform equivalent to large spot treatment, USS = Uniform equivalent to small spot treatment

Table 6.1: Size of forewings and red spots in the wild moth dataset and the experimental stimuli. All artificial prey in Experiments 1 and 2 were the same size; spot size in Experiment 1 was identical to spot size in the LS treatment of Experiment 2 (LS = large spots, SS = small spots). r = spot radius

	Wild burnet moth dataset			**Artificial prey**	
	5th percentile	Mean ± s.d.	95th percentile	SS stimulus	LS stimulus
Forewing area (mm^2)	54.18	65.65 ± 8.26	79.19	78	78
Total spot area (mm^2)	7.99	11.90 ± 2.44	15.28	7.8 (6 spots with r = 0.64mm)	14.94 (6 spots with r = 0.89mm)

6.3.3 Colour matches between wild burnet moths and artificial prey

Following Arenas, Walter and Stevens (2015), artificial prey colours were chosen based on the visible spectrum of reflectance of natural moth wings (wavelengths between 400 and 700 nm). I selected printed colours to match those of real burnet moths from the dataset described above, according to a trichromatic model of bird vision. As photoreceptor cone catches in the wild burnet moth dataset did fall into normal distributions, median values were used to define "ideal" black and red colours to which the artificial prey colours should be matched. Colour matches were found via a combination of feed-forward and feed-back processes, using custom-made plugins in ImageJ. All colours were printed onto waterproof paper (Xerox Premium NeverTear 120µm) using a laser printer (HP Color Laser JetPro M252dw). Initially, a calibration sheet of 1026 randomly-generated colours was produced (Figure 6.2a), photographed in the same conditions and with the same equipment as the moth wings, then transformed to a trichromatic model of bird vision as described above. This was used to generate a polynomial model relating printer colours to avian cone catches, from which I could estimate the RGB values corresponding to the "ideal" black colour. To obtain the best possible match, I then generated a second sheet of colour swatches (Figure 6.2b), with colours varying randomly around the model's best RGB estimate. Similarity between the "ideal" and printed colours was then assessed by calculating just-noticeable differences (JNDs, following the receptor-noise limited Vorobyev-Osorio model [Vorobyev and Osorio, 1998]; see details in Chapter 2). I chose a colour differing from the "ideal" black by less than 1 JND, at which point the colours are not distinguishable by avian vision, even in perfect lighting conditions (Siddiqi *et al*., 2004). This colour formed the black background colour of every artificial moth, with the exception of iridescent moths (treatment I) in Experiment 1. The same technique was used to find the closest match to the colour of the red forewing spots, used in the LS and SS treatments, as well as to create the uniform treatments, ULS and USS, in Experiment 2 (Figure 6.2c-e; Table 6.2). For these uniform treatments, the "ideal" colours were based on measurements of the overall colour of wings in the LS and SS treatments respectively, printed to size and photographed using the same protocols as the colour calibration sheets.

Table 6.2: Photoreceptor cone catches and contrasts between burnet moth colours and printed colours for the artificial prey. LS = Large spots, SS = Small Spots, ULS = Uniform equivalent to large spot treatment, USS = Uniform equivalent to small spot treatment.

	Median cone catch values for natural colours	**Cone catch values for chosen colours**	**Difference between natural and matched colours**	**Corresponding RGB values for printed colours**
Black background (all treatments except iridescent moths in Expt. 1)	lw = 0.0689 mw = 0.0798 sw = 0.0707	lw = 0.0694 mw = 0.0785 sw = 0.0719	JND = 0.454	R = 97 G = 99 B = 78
Black background with iridescent paint (Expt. 1)	lw = 0.0689 mw = 0.0798 sw = 0.0707	lw = 0.0716 mw = 0.0806 sw = 0.0714	JND = 0.443	R = 116 G = 113 B = 87
Red spot colour (LS and SS treatments)	lw = 0.211 mw = 0.0310 sw = 0.0540	lw = 0.207 mw = 0.0314 sw = 0.0557	JND = 0.687	R = 211 G = 36 B = 37
ULS colour	lw = 0.0900 mw = 0.0597 sw = 0.0535	lw = 0.0927 mw = 0.0615 sw = 0.0539	JND = 0.330	R = 124 G = 82 B = 37
USS colour	lw = 0.0843 mw = 0.0697 sw = 0.0594	lw = 0.0790 mw = 0.0615 sw = 0.0558	JND = 0.0264	R = 116 G = 94 B = 58

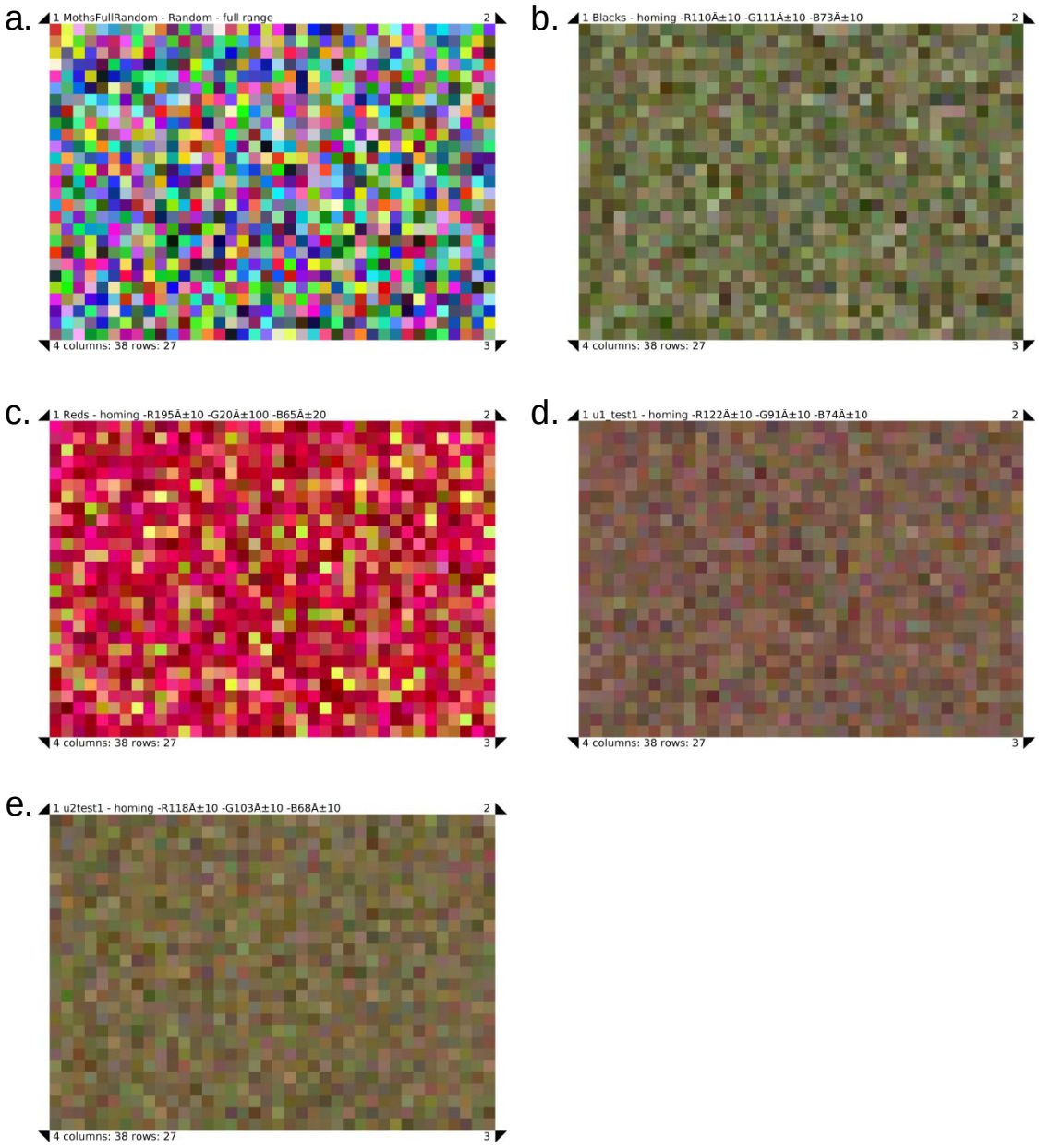


Figure 6.2: Calibration sheets used to find colour matches for the artificial prey. (a) Initial set of randomly-generated colours. (b-e) Set of random colours generated from the model estimate to find the closest colour matches used in Experiment 2: for the black background (b), forewing spots in LS and SS treatments (c) and the ULS (d) and USS treatments (e)

6.3.4 Red forewing spot colours for Experiment 1

For Experiment 1, to test the effect of variation in the colour of the red forewing spots on predation rates, I generated 1000 artificial prey, each with a unique red forewing spot colour. These colours were created using custom-made plugins in

ImageJ. Starting from the colour chosen to match the median red colour of the wild burnet moth dataset (and also used in the LS and SS treatments of Experiment 2), I generated new red colours by allowing each colour channel (R, G, B) to follow its own independent distribution, similarly to the pattern of natural variation. To assess this variation, the colours of the wild burnet moth dataset were converted to RGB values in ImageJ, using the polynomial model produced earlier for colour-matching and the standard deviations in each RGB channel were calculated ($sd_R = 4.17$, $sd_G = 20.09$, $sd_B = 5.92$). The final 1000 RGB values were drawn from normal distributions centred on the value for the median red colour, with a standard deviation equal to 2 standard deviations as calculated above for each channel. The resulting colour set (Figure 6.3a) included 735 colours (73.5%) that fall within 3 JNDs of at least one of the median, 5th and 95th percentile values of red colours in the burnet moth dataset; these were considered to be within the natural range of colours for this species. A further 265 colours (26.5%) had a JND greater than 3 from all of these natural colours, making them distinguishable from real burnet moth colours; these were taken to represent colours outside the six-spot burnet's natural range.

In addition, to test the effect of iridescence on predation risk, a second set of 1000 moths were produced. Previous experiments testing the effect of iridescence have relied on removing it from certain individuals, for example by painting over iridescent patches (Fabricant *et al*., 2014; Pegram, Nahm and Rutowski, 2015), but the prey used here are entirely artificial. Iridescence was re-created by painting prey items with interference paint (C.T. Interference Green-Blue #2484-1, Golden Fluid Acrylics ®, Golden Artist Colours Ltd.). This changes colour as the light angle varies, the key characteristic of iridescent surfaces (Doucet and Meadows, 2009). A realistic green iridescence was produced, mimicking the sheen of natural burnet moth wings, at least to human observers. Colours for the iridescent moths were obtained by measuring calibration sheets coated in the interference paint. The 1000 red spot colours for this iridescent (I) group (Figure 6.3b) were drawn from the same distributions as the previous, non-iridescent (N), set. When painted with the interference paint, photographed and measured as per the above methods, the colour sheet thus produced includes a similar number of colours falling within the natural range of burnet forewing colours (70%) and without (30%). The presence of the

interference paint also altered the colour match for the black background colour, so a new colour was chosen to best match the “ideal” black colour, based on photographs of colour calibration sheets coated with interference paint (Table 6.2). As such, the effect of iridescence on predation risk could be tested by comparing the survival of moths with a black background colour matched to natural burnet moths, and red colours drawn from the same distribution, but differing in the presence or absence of an iridescent coating of interference paint. All photographs involving interference paint were taken with the light source placed at 0° from the wings, an angle at which no iridescence was produced.

6.3.5 Colour measurements used in the analysis of Experiment 1

For each red colour used in the experiment, a number of metrics were calculated, to be used in the final analysis of the effects of coloration on prey survival. As the perception of brightness differences in birds is thought to be mediated by their double cones (Jones and Osorio, 2004), luminance was measured as the cone catch values of each colour for blue tit double cones. Measurements of hue were based on cone catch values for the LW, MW and SW channels alone. Hue was calculated on the basis of colour opponency, following methods previously used in a number of studies of animal coloration (e.g. Spottiswoode and Stevens, 2011; Stevens, Lown and Wood, 2014a,b, see Chapters 2, 3, 4 and 5). Though the exact opponent channels operating in avian vision have yet to be determined, it is possible to construct ratios approximating a measure of hue by performing principal component analysis (PCA) on a covariance matrix of the standardised values for each colour channel, as in Spottiswoode and Stevens (2011). The first two principal components thus obtained were used to calculate two ratios forming logical colour channels (Hue1 and Hue2). Only Hue1, accounting for over 76% of the variance in colour, was included in subsequent analyses (hereafter referred to as hue). The specific equations for hue are as follows:

$$Hue1 = \frac{LW}{(SW+MW)/2} \quad (6.1)$$

$$Hue2 = \frac{(LM+MW)/2}{SW} \quad (6.2)$$

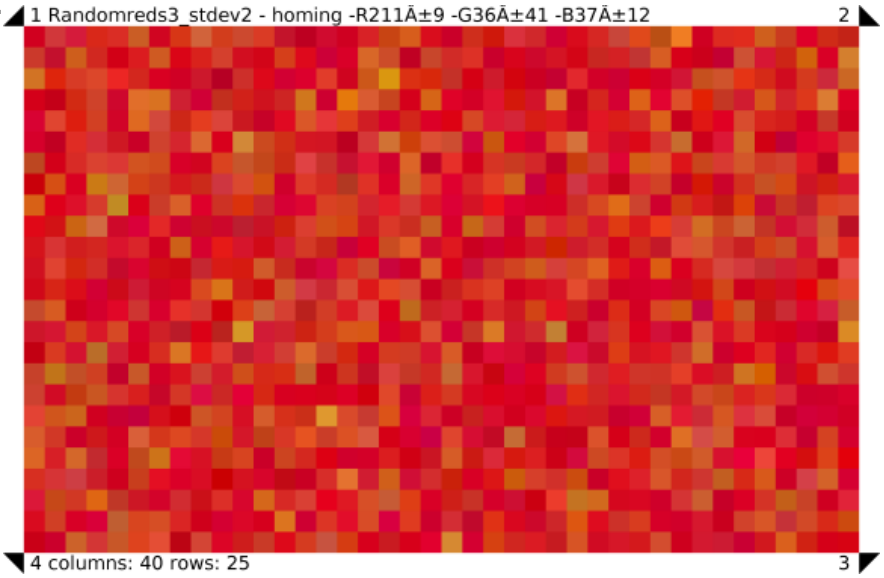
SW, MW, LW = Standardised cone catch values for the SW-, MW- and LW-sensitive photoreceptors respectively.

Higher hue values therefore represent colours with relatively higher reflectance in the long wavelength colour channel, indicating redder colours.

Internal chromatic contrasts were calculated between the red marking and black background colours for each prey. Chromatic contrasts were based on trichromatic JNDs following the Vorobyev-Osorio model (Vorobyev and Osorio 1998), with blue tit cone ratios (Hart, 2001b) and a Weber fraction of 0.05. Luminance contrasts between colours were taken as the natural logarithm of the ratio of cone catches for the double cones, based on methods in Siddiqi e*t al*. (2004).

Finally, to gain a sense of the conspicuousness of the artificial prey against the natural backgrounds on which they were placed, 100 randomly selected prey from Experiment 1 ($n_{iridescent}$=n_{matte}=50) were photographed *in situ* in the field, with a calibrated Nikon D7000 camera and filters for both human-visible and UV wavelengths of light, as described above for photography of the model *Z. filipendulae* specimens. When estimating conspicuousness in the field, ultraviolet information was considered (Arenas, Walter and Stevens, 2015). Images were aligned, normalised and transformed to a tetrachromatic blue tit visual model using the same tools and techniques described above. On each prey item analysed, six red spots were randomly selected for measurements of spot colour and six equivalent circles, randomly placed on the black background, were used to measure the black colour in ImageJ. The natural background was measured by four rectangles (each the same width and length as the moth prey), randomly placed within three body lengths of the prey item. For each colour region (spot, black background, natural background), the measurements from each selected area were averaged to obtain one value per region and per prey item, and these were used to calculate chromatic and luminance JNDs between either the prey spots or black background colours and the natural background (Vorobyev and Osorio, 1998; Siddiqi *et al*., 2004; Troscianko and Stevens, 2015).

a.



b.

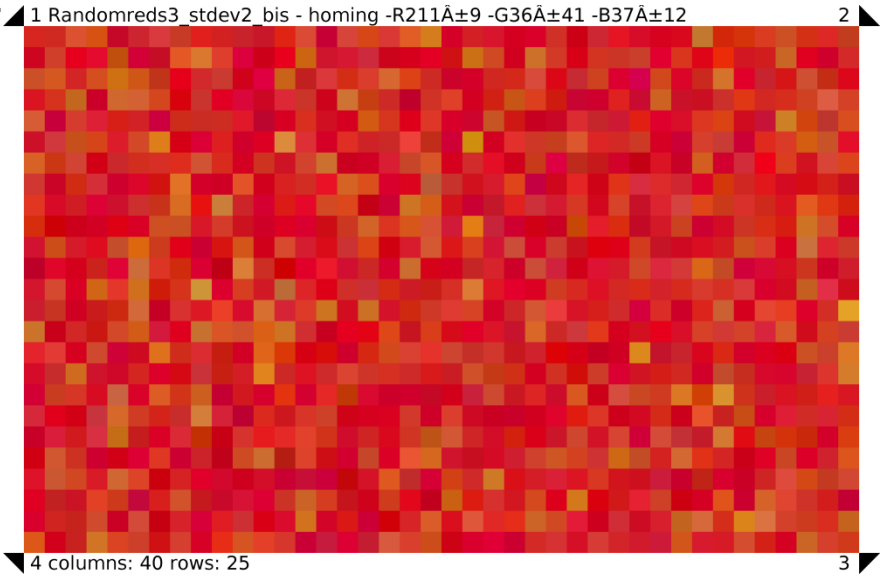


Figure 6.3: Sheets with 1000 colours used for the red forewing spots of artificial prey in Experiment 1, for the normal (a) and iridescent (b) stimuli.

6.3.6 Field site and experimental procedures

Experiments were carried out on Penhale camp (50° 22’ 38.97’’ N, 5° 8’ 27.99 W), a 350 ha site owned by the Ministry of Defence, within Penhale Dunes SSSI, on the north coast of Cornwall. The area is characterised by fixed grey dunes with marram, humid dune slacks, dune grassland and shifting sand dunes on the shoreline (English Nature, 2005; Figure 6.4a). The six-spot burnet (*Zygaena filipendulae*) is commonly found in these habitats along the Cornish coast, and numerous caterpillars, cocoons and adults were observed on site

over the course of the experiments. In addition, restrictions to public access in this area enabled the experiments to proceed with minimal disturbance from humans or domestic animals. Experiments 1 and 2 were carried out between 13th March and 27th April 2016, and Experiment 2 was then repeated from 14th April to 6th May 2017 and from 22nd June to 5th July 2017. These repeats were designed to test whether predation rates in spring were consistent across years, and whether the emergence of real burnet moths on site in summer had an impact on predator responses to the artificial prey. Experiment 1 and the first two runs of Experiment 2 took place before any adult moths emerged, ensuring that, while the birds on site were likely to have had previous experience of toxic burnet moths, none of them would have recently encountered this highly aversive stimulus. The final repeat of Experiment 2 was carried out while adult burnet moths were in flight on site; the first adults were observed on site 2 weeks before the start of the experiment, allowing potential predators to experience these distasteful prey prior to being exposed to the artificial mimics.

a. b.

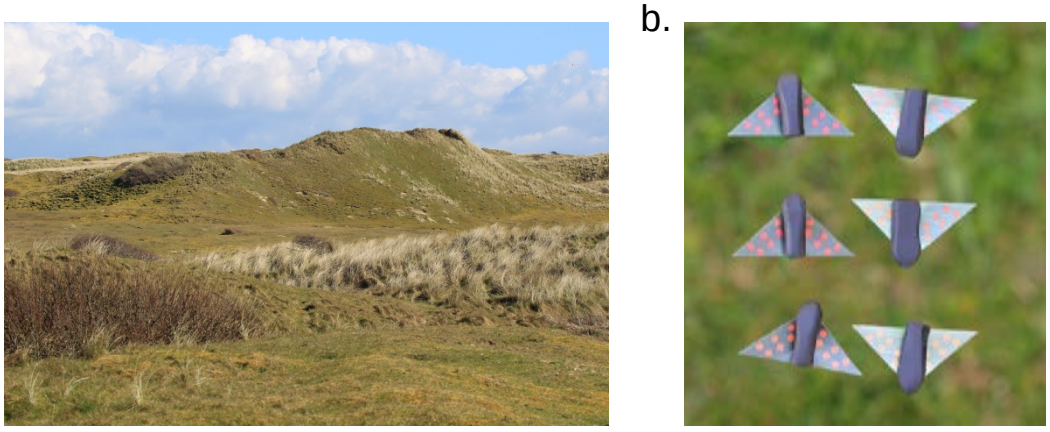


Figure 6.4: View of the field site (a) and examples of artificial prey used in Experiment 1 (b): matte on left, iridescent on right.

Artificial prey were set out in non-overlapping transects (blocks; Cuthill *et al*., 2005) 500m long, following paths and the contours of the landscape, such as dune ridges, streams and lakes. Along each transect, 100 prey items were placed 5m apart, for a total of 60 transects, or 6000 artificial moths (2000 for Experiment 1 and 1000, 1500 and 1500 for Experiment 2). Equal numbers of moths from each treatment were placed along every transect in a randomised

order (n_I=n_M=50 in Experiment 1, n_{LS}=n_{SS}=n_{ULS}=n_{USS}=25 in Experiment 2). In Experiment 1, the order of spot colours was also randomised. The moths were pushed into the ground so that their wings lay 3-5 cm above the surrounding vegetation, a height designed to mimic a moth resting on the nearby plants, while remaining sufficiently visible for transect checks. Each transect was put out at dawn, checked every 24 hours, and remained in the field for 72 hours in total.

With regard to potential predators of the artificial prey, a total of 42 bird species were identified on site across the experimental periods, including 20 insectivores or generalists which could attack the artificial prey. Camera traps placed on site in 2017 (Visortech® NX-4095, VicTsing® Trail Camera GEOD032AB and Sumikon® DSC-36.hd) recorded predation events by multiple species: carrion crow (*Corvus corone*), jackdaw (*Corvus monedula*), magpie (*Pica pica*), mistle thrush (*Turdus viscivorus*), meadow pipit (*Anthus pratensis*), stonechat (*Saxicola torquatus*), robin (*Erithacus rubecula*) and chaffinch (*Fringilla coelebs*) (Figure 6.5). Prey items were considered to have been attacked by birds if the plasticine bodies showed evidence of sharp v- or u-shaped marks, characteristic of bird beaks, while shallow marks that could have resulted from rain and hailstorms or contact with nearby vegetation were ignored. Predation by rodents (Figure 6.6a) was clearly identified by the presence of bite marks, while tiny holes and regular scuff marks were considered to have been caused by insects and snails. During each daily check, attacked items and prey severely damaged by rodents or insects were photographed and removed. Birds were filmed consuming the entirety of the plasticine body (see Figure 6.5d), so baits on which the body was missing were also considered attacked. However, red foxes (*Vulpes vulpes*) were also observed attacking the artificial prey (Figure 6.6b), as reported in previous artificial predation experiments (eg. Valkonen *et al*., 2012). Mammals such as rodents and foxes appear to be attracted to plasticine (Rangen, Clark and Hobson, 2000), whether out of curiosity or as a source of minerals, but as they rely primarily on olfactory cues, it is important to separate these mammalian attacks from avian predation (Valkonen and Mappes, 2012). Foxes were recorded pulling the moths out of the ground and chewing them on three separate occasions, usually bending the wings, so severely damaged moths

with no clear evidence of peck marks were excluded from the analysis. I scored the items as predated or not while checking them in the field, so this was not carried out blind to treatment (although the specific hue of red spots in experiment 1 could not be determined in the field). However, I also photographed all prey items with marks on, and later used these images to check that my scoring was consistent across the experimental period, to achieve as objective an assessment of predation as possible.

Figure 6.5: Examples of attacks filmed by camera traps. (a) Carrion crow (*Corvus corone*), (b) Magpie (*Pica pica*), (c) Jackdaw (*Corvus monedula*), (d) Mistle thrush (*Turdus viscivorus*), (e) Robin (*Erithacus rubecula*), (f) Stonechat (*Saxicola torquatus*).

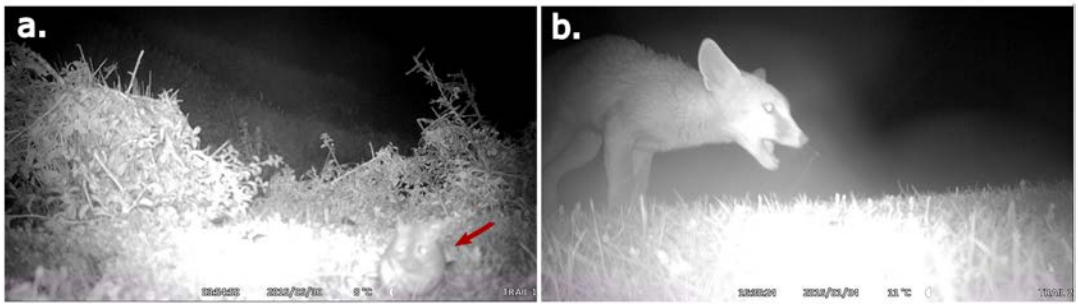


Figure 6.6: Mammalian predators of artificial prey on site. (a) Mouse (likely wood mouse, *Apodemus sylvaticus*) and (b) Red fox (*Vulpes vulpes*).

6.3.7 Statistical analyses

All statistical tests were carried out in R versions 3.3.1 (R Development Core Team 2015). Survival data were analysed using Cox mixed effects survival analyses with the packages “survival” (Therneau, 2015a) and “coxme” (Therneau, 2015b). For each experiment, the transect number was classed as a random effect (Arenas, Walter and Stevens, 2015). Prey items attacked by rodents and foxes or damaged by insects were censored (Cuthill *et al*., 2005), as were items that could not be recovered, unless their exact location was known and nearby prey had been pulled out of the ground, in which case they were considered to have been attacked. For Experiment 1, spot hue, spot luminance and iridescence treatment (iridescent or matte) were included in the full model and allowed to interact; the best model was then identified via stepwise model simplification. As hue and chromatic contrast were highly correlated (Spearman’s rank correlation=0.9370537), the effects of chromatic and luminance contrasts and the iridescence treatment were tested in a separate model. These two models were subsequently compared using Akaike’s Information Criterion (AIC). Similarly, chromatic and luminance contrasts between the prey spots and the median red colour of the wild *Z. filipendulae* specimens were analysed in a separate model. For Experiment 2, season and treatment (LS, SS, ULS and USS) were the only fixed effects tested.

In addition, to further validate the results, odds ratios and likelihood ratios, or G-tests, were computed. The odds ratio (OR), the ratio of the probability of predation for one treatment to the probability of predation for another (Stevens *et al*., 2007b), was calculated to compare iridescent and non-iridescent prey in

Experiment 1, and for planned comparisons between spotted and uniform treatments (SS and LS versus ULS and USS), and treatments with the same average colour (LS and ULS versus SS and USS) in Experiment 2. G-tests were run for the same comparisons and to compare every treatment in Experiment 2, relative to a null hypothesis of equal predation rates for each group, using the "RVAideMemoire" package (Hervé, 2016) in R; results are reported for tests without a Williams correction as the dataset is sufficiently large (>200 datapoints), and including the correction did not alter the results (data not shown).

Luminance and chromatic contrasts between a subset of artificial prey from Experiment 1 and their natural backgrounds were analysed with simple linear models, testing the effect of treatment (matte or iridescent) on contrast to the background; JNDs were log-transformed to fit the model assumptions. One-tailed t-tests were used to verify that contrasts between the prey and backgrounds exceeded the threshold for discrimination (JND=3).

6.4 Results

In Experiment 1, run in March-April 2016, 7.15% of artificial prey were considered to have been attacked by birds. For Experiment 2, 8%, 12.13% and 28.13% predation rates were recorded in March-April 2016, April-May 2017 and June-July 2017 respectively. Overall, predation rate per transect varied from 1 to 71%, with a median predation risk of 7%.

6.4.1 Experiment 1 – colour, iridescence and predation risk

6.4.1.i Iridescence

Experiment 1 was designed to test the effect of different aspects of prey coloration, including hue, luminance, and iridescence, on predation rates. There was a slight trend for iridescent prey to be attacked more than the matte ones, but this was not significant according to survival analysis ($OR_{I\ vs.\ N}$=1.270, coxme, treatment, df=1, X^2=0.182, p=0.700; likelihood ratio test, G=2.332, df=1, p=0.127; see Figure 6.7).

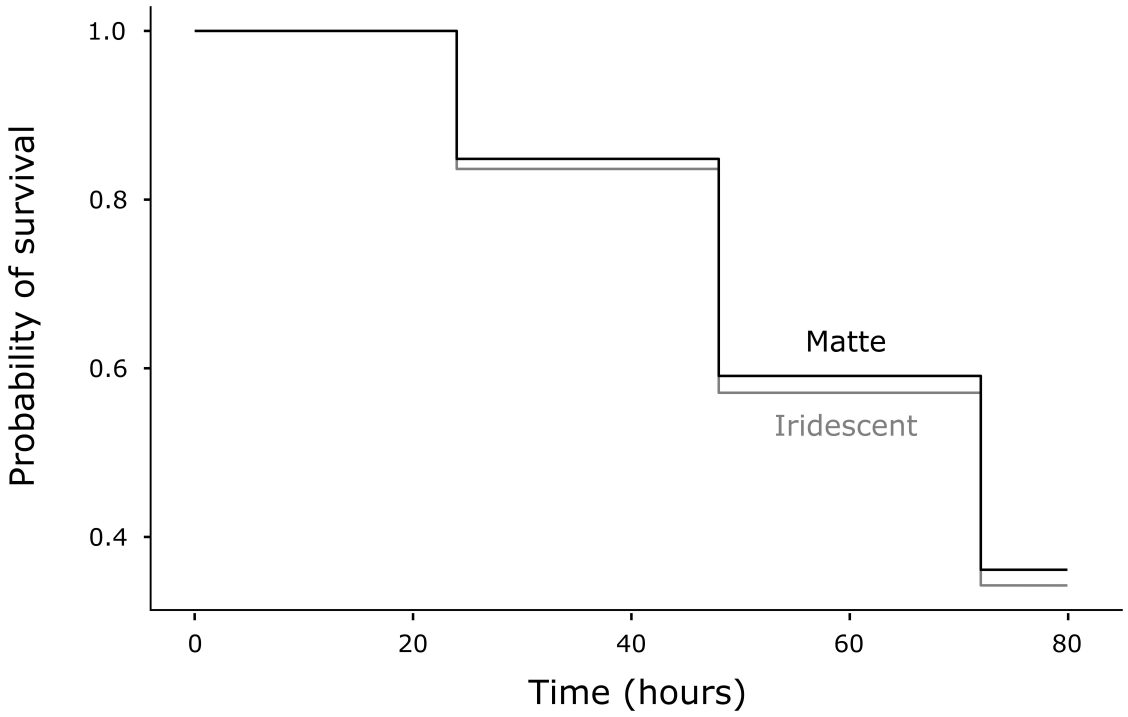


Figure 6.7: Survival of matte and iridescent artificial prey in Experiment 1. The difference in predation risk between these groups was not significant.

6.4.1.ii Natural prey coloration

The closeness of spot colour in the artificial prey to their natural burnet moth models had no impact on predation rates. Luminance and colour contrasts relative to the median red colour of the wild *Z. filipendulae* dataset had no effect on prey survival (coxme; luminance contrast to median red, df=1, X^2=0.0227, p=0.880; chromatic contrast to median red, df=1, X^2=2.883, p=0.0895). Similarly, whether spot colour was considered to be within the natural range of *Z. filipendulae* coloration (within 3 JNDs of the median, 5th percentile or 95th percentile of the dataset) or outside the range (>3 JNDs above all these values) had no effect (coxme, range; df=1, X^2=0.849, p=0.357).

6.4.1.iii Hue, luminance and internal contrast

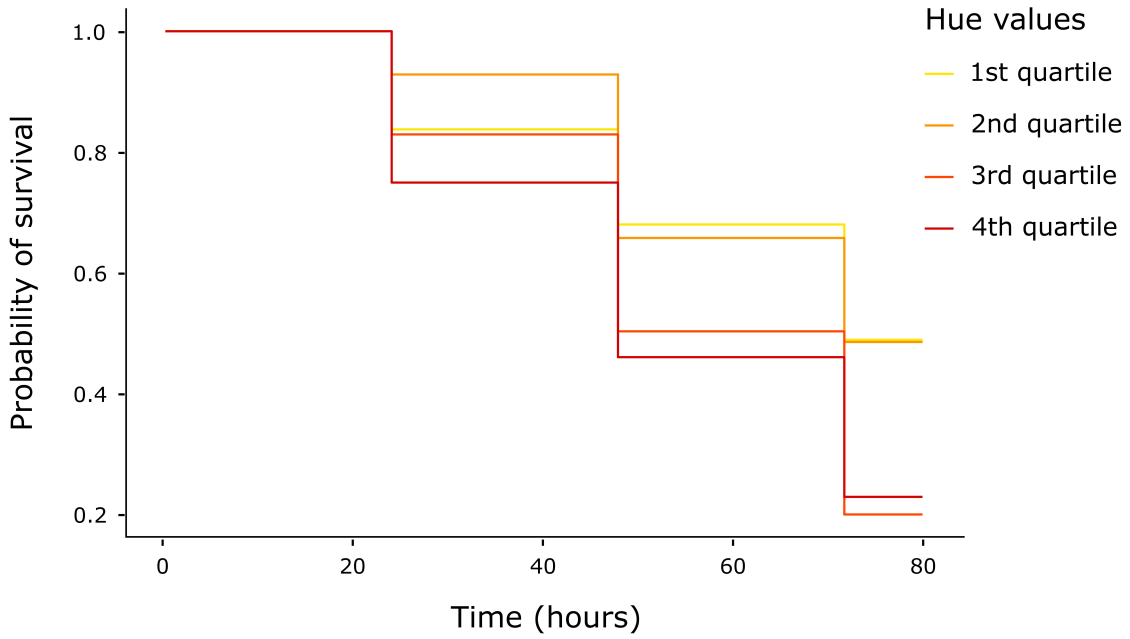
Spot colour did have a discernible effect on survival of the artificial prey. While marking luminance had no effect on predation risk, prey whose spots had higher hue values, representing redder colours, incurred greater attack rates (Figure 6.8a; coxme, hue, df=1, X^2=4.620, p=0.0316). Variation in spot hue also affects the chromatic and luminance contrasts between the spots and black

background colour of the artificial prey, which may be relevant to detection and attack by predators. Testing the effects of internal contrasts and iridescence on predation yielded a significant effect of chromatic contrast alone, with other factors dropping out of the model (coxme; chromatic contrast, df=1, X^2=7.52, p=0.00610): prey with more contrasting patterns (higher JNDs between spots and background) had reduced survival (Figure 6.8b). The high JND values (>13) mean that all artificial prey had strongly contrasting markings, with spots easily discernible from the background wing colour on the basis of colour, yet greater chromatic contrast still appeared to further increase predation risk. Model comparison using AIC suggests that hue and internal chromatic contrast explain the experimental results equally well ($AIC_{coxme(hue)}$=1403.84 , $AIC_{coxme(chromatic\ contrast)}$= 1400.823).

6.4.1.iv Conspicuousness to natural backgrounds

Based on the subset of prey items photographed, all artificial prey were highly conspicuous to avian vision: chromatic contrast between prey colours and their natural environment was significantly greater than the threshold for discrimination, at JND=3 (one-tailed t-tests; T=66.641, df=99, p<0.001 and T= 52.649, df=99, p<0.001 for spot and background colours respectively), as was luminance contrast (one-tailed t-tests; T=18.947, df=99, p<0.001 and T= 9.490, df=99, p<0.001 for spot and background colours respectively). There was no difference between the chromatic conspicuousness of iridescent and matte prey colours ($F_{1, 98}$=0.0631, p=0.802 and $F_{1, 98}$=3.241, p=0.0749 for spot and background colours respectively). However, iridescent prey were more contrasting to the natural backgrounds than matte prey, in terms of luminance ($F_{1, 98}$=6.056, p=0.0156 and $F_{1, 98}$=53.21, p<0.001 for spot and background colours respectively, Figure 6.9).

a.



b.

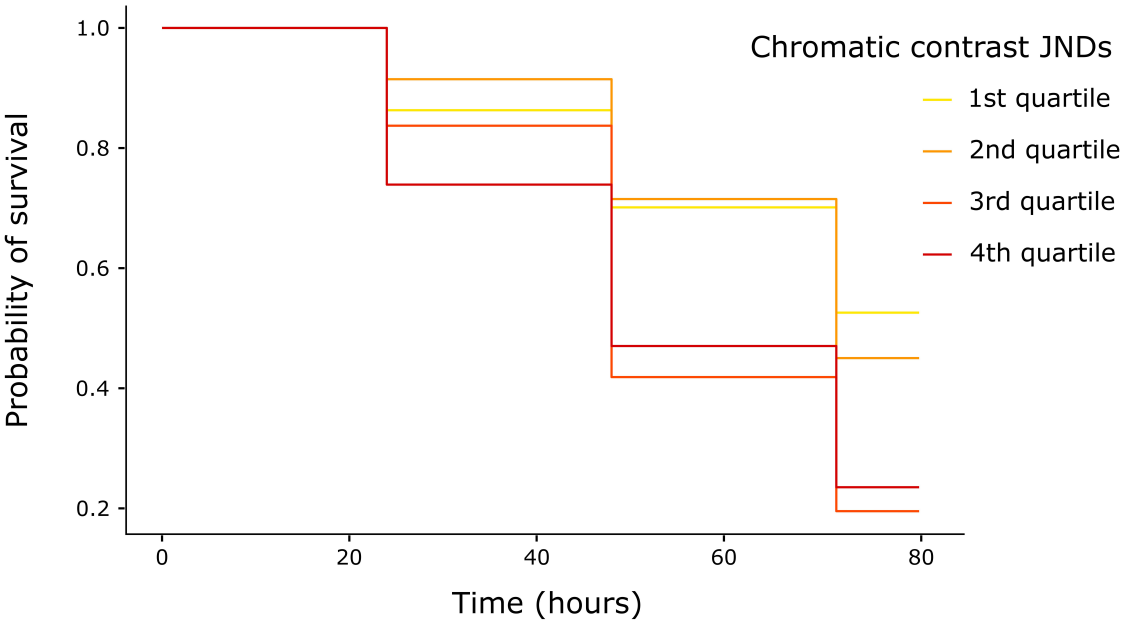


Figure 6.8: Prey with higher spot hue values (a) and higher chromatic contrasts (b) had lower survival. For visualisation, spot hue and chromatic contrast values were split into quartiles. Higher hue values represent redder colours (equation 6.1).

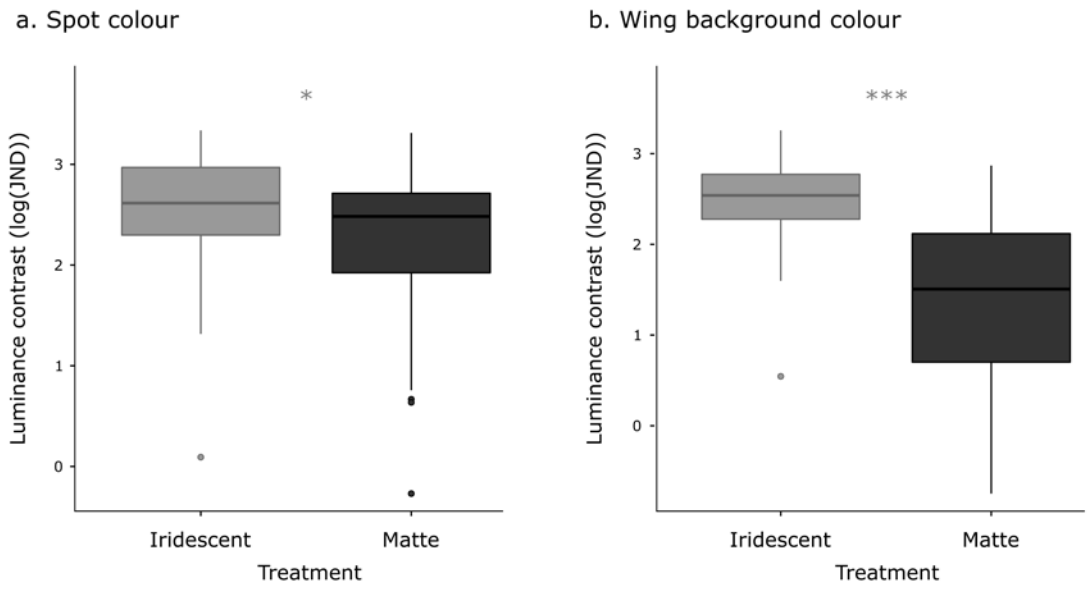


Figure 6.9: Luminance contrast between artificial prey spot colour (a) and wing background colour (b) and their natural backgrounds in the field. Boxplots show the median and interquartile range. Significance levels: ***:p<0.001, **:p<0.01, *:p<0.05.

6.4.2 Experiment 2 – spot size and predation risk

Experiment 2 was designed to test the effect of the presence and size of spots on predation risk for artificial moth-like prey, and was run multiple times to investigate the possibility of differences in predation between years and seasons. Predation risk for the artificial prey was much higher overall in 2017 than in 2016 (coxme; season, df=2, X^2=59.176, p<0.001, with Tukey's post-hoc tests: $p_{\text{Spring 2016 – Spring 2017}}$<0.001, $p_{\text{Spring 2016 – Summer 2017}}$<0.001, $p_{\text{Spring 2017 – Summer 2017}}$= 0.329). However, there was no interaction between treatment and season (coxme; season:treatment, df=6, X^2=9.615, p=0.142), suggesting that preferences for particular spot patterns did not significantly differ between seasons. There was also no effect of treatment overall (coxme; treatment, df=3, X^2=1.930, p=0.587; Figure 6.10), indicating no preference for prey with or without spots. This was confirmed by tests on each field season separately (Table 6.3). Overall, spotted prey were attacked slightly less than their uniform equivalents, and the redder prey were attacked slightly more than the darker ones, but neither trend was significant ($OR_{\text{LS and SS vs.ULS and USS}}$=0.932, G=1.016, df=1, p=0.314; $OR_{\text{LS and ULS vs.SS and USS}}$=1.085, G=1.383, df=1, p=0.240 respectively).

Table 6.3: Effect of treatment (LS, SS, ULS and USS) on predation rates in each season for Experiment 2, as analysed by coxme models and likelihood ratio tests. LS= Large spots, SS= Small Spots, ULS= Uniform equivalent to large spot treatment, USS= Uniform equivalent to small spot treatment.

Season	coxme			Likelihood ratio test		
	df	X^2	p	df	G	p
All seasons	3	1.930	0.587	3	4.123	0.249
Spring 2016	3	5.446	0.142	3	2.920	0.404
Spring 2017	3	3.243	0.356	3	3.184	0.364
Summer 2017	3	0.908	0.824	3	0.853	0.837

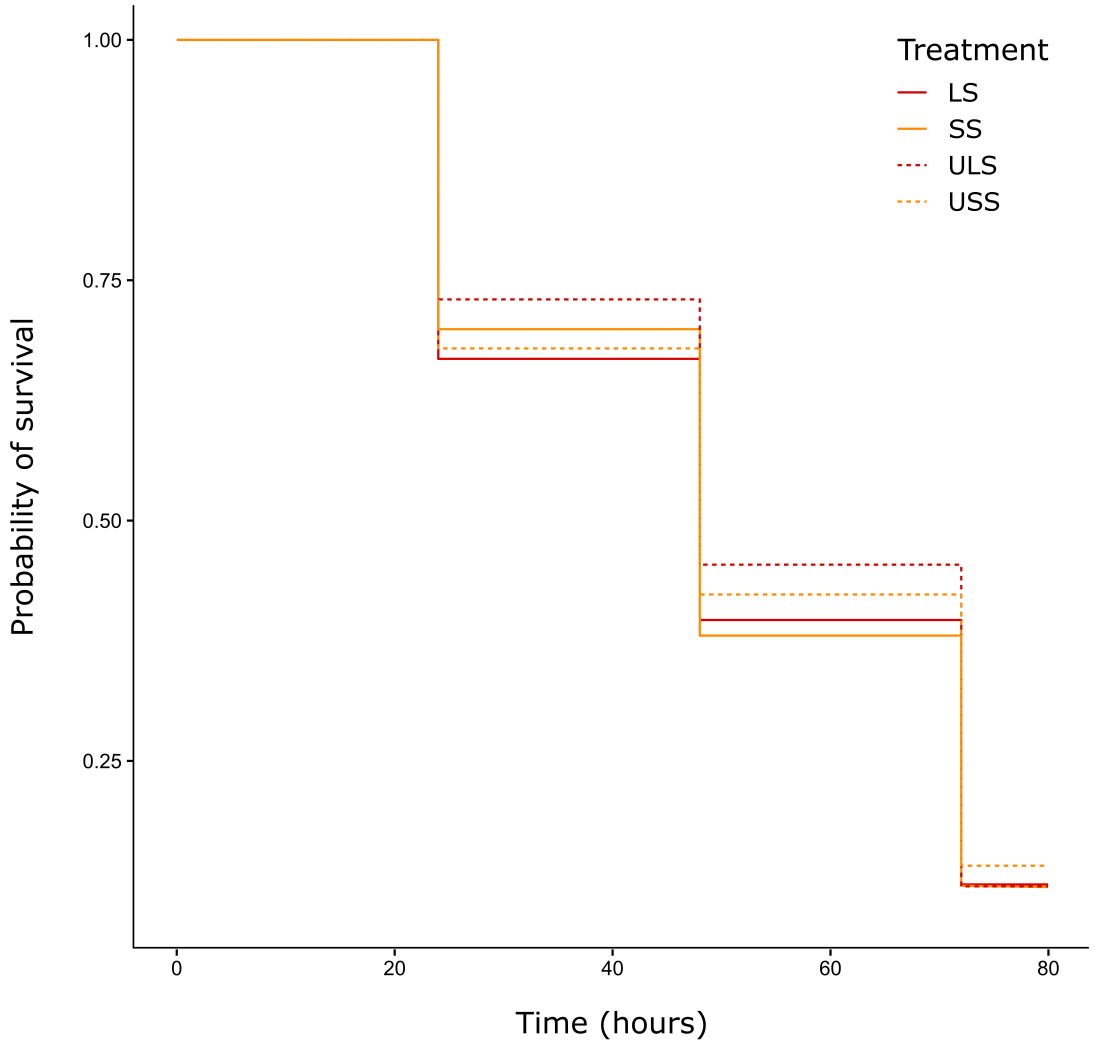


Figure 6.10: Survival of artificial prey in Experiment 2, across all three seasons together. There are no significant differences between groups. Dotted lines indicate uniform prey. SS=small spots, USS=uniform small spots, LS=large spots, ULS=uniform large spots.

6.5 Discussion

Of all the coloration and pattern metrics tested across Experiments 1 and 2, only chromatic components of the forewing markings had a significant impact on predation rates in the field. Using continuous variation in colour to design the artificial prey stimuli proved an effective technique to test the role of multiple colour variables in enhancing prey survival. Artificial prey with spots both redder in hue and more highly contrasting to the background colour of the wing had reduced survival. In Experiment 2, predation rates varied between field seasons but were not affected by the presence or size of red spots, echoing results of predation experiments on spot size and contrast in ladybird models (Arenas, 2015).

6.5.1 Surprising effects of colour, but not pattern or iridescence

Collectively, these results are contrary to expectations for warning signals. The presence of spots, and an increased spot size, would typically be considered to constitute stronger warning signals, yet these characteristics did not have a protective effect on artificial prey in the field. Similarly, more contrasting and thus more conspicuous colours, as well as redder hues, are generally thought of as boosting the efficacy component of warning signals, making them more effective at generating avoidance (Stevens and Ruxton, 2012). In terms of strategic value, in an honest signalling paradigm, redder and more conspicuous individuals would be expected to be more strongly defended and thus more readily avoided. Among ladybirds (Coccinellidae) for example, redder species were found to be more toxic than orange, yellow and brown ones, and red ladybird models were correspondingly avoided more than artificial prey of other colours (Arenas, Walter and Stevens, 2015). The results of the present experiments seem to support previous work suggesting that colour may be more important than pattern (Finkbeiner, Briscoe and Reed, 2014), as colour does have an impact on predation, whereas spot presence and size do not. However, and contrary to predictions, the more conspicuous moths, in terms of internal contrast, were more likely to be predated; it is important to note that all prey in Experiment 1 were highly conspicuous against their natural backgrounds. While it is not possible to disentangle the relative importance of increasing internal chromatic contrast or redder hues in driving the higher

predation rates, in both cases the results contradict expectations based on warning signal theory. Detectability of the artificial prey thus appears to be the most important factor in determining the likelihood of attack by avian predators here, rather than any specific avoidance of aposematic patterns.

In addition to these surprising results, this study found no evidence that iridescence was an effective aposematic signal; though the trend was non-significant, if anything iridescent prey were attacked more than non-iridescent ones. This supports previous work on the pipevine swallowtail butterfly, *Battus philenor* (Pegram, Han and Rutowski, 2015), which similarly found no effect of iridescence on prey survival in the field. In the Procridinae, or forester moths, a sub-family of the Zygaenidae which share the chemical defences of burnet moths (though at a lower concentration; Mika Zagrobelny, pers. comm.) and are characterised by bright green, turquoise or brown iridescent wings, iridescence is thought to have a dual function: crypsis at rest in grasses and aposematism when feeding on exposed flowers (McNamara *et al*., 2011). In the present experiment, iridescence conferred no protection on artificial prey, whether by aposematism or improved crypsis, raising the question of the true function of iridescence in zygaenid moths. However, the effect of iridescence in these predation trials may be masked by the high detectability of the contrasting black and red markings; iridescence might thus still play a role in anti-predator defence in the Procridinae, which lack these markings. Compounding this issue, iridescent prey in this experiment displayed greater luminance contrasts to the surrounding environment than the matte prey, again increasing their visibility to predators. Alternatively, iridescence may be more relevant to the visual components of mate choice rather than to anti-predator defence, as has been suggested for a range of iridescent invertebrates (Doucet and Meadows, 2009; Fabricant *et al*., 2013), including *B. philenor* (Rajyaguru *et al*., 2013) and other Lepidoptera (Rutowski *et al*., 2005; Papke, Kemp and Rutowski, 2007; Kemp, 2008; Rutowski, Nahm and Macedonia, 2010; Rajyaguru *et al*., 2013).

6.5.2 Considerations when interpreting results, limitations and strengths of this study, and future perspectives

An inherent weakness of artificial predation experiments such as those presented here is their inability to separate out the different phases of predation,

in particular initial detection of prey and acceptance of the item as a suitable food source after discovery. The results of Experiment 1 collectively suggest that more conspicuous prey items were more likely to be attacked, but cannot reveal whether many more of these items were located and subsequently rejected by predators or not. Moreover, the identical performance of spotted prey and their uniform colour equivalents could suggest that predators were not able to resolve the spots, or simply ignored them. If the decision to attack the prey is taken from a sufficient distance away, distance-dependent effects could make the colours of the spotted prey blend together, giving them a more cryptic appearance, as has been suggested for striped caterpillars (Barnett and Cuthill, 2014; Barnett *et al*., 2017). However, this seems unlikely, as the moth and marking sizes are based on real moth measurements, and all predation events recorded on camera showed birds foraging at ground level, with plenty of opportunity to observe the stimuli before pecking them, rather than swooping in from above.

Relatively few studies attempt to discover exactly which species are responsible for the attacks they record. In most cases, only general observations of the most commonly found avian species on site are used to provide a basic indication of potential predators, although more in-depth assessments of the predator community are sometimes carried out, such as transect counts (Valkonen *et al*., 2012; Nokelainen *et al*., 2014) or using data from ringing and mist-netting (Mappes *et al*., 2014). These more detailed observations have informed conclusions as to the impact of specific predator groups on predation risk (Valkonen *et al*., 2012; Nokelainen *et al*., 2014), and have been used to estimate encounter rates between specific predator species and prey morphs between microhabitats (Willmott *et al*., 2017). Deploying camera traps is a less labour-intensive method for acquiring accurate data on the actual predators participating in the experiment, although only a small subset of predations can be captured. Camera-trap footage, along with evidence from tracks and other clues, can help determine the visual systems through which prey appearance is processed in the wild, enabling researchers to analyse visual signals, whether aposematism or camouflage, in the most relevant ways (e.g. Troscianko *et al*., 2016). Moreover, camera traps can uncover the role of unsuspected predators and provide information on the methods of predation, increasing the accuracy of

the end dataset. In these experiments, observing predation of moths by foxes in 2017 allowed me to recognise predations for which foxes were most likely to be responsible, and which otherwise would have been erroneously attributed to birds. Observations of small birds, such as stonechats (*Saxicola rubicola*), pecking at the wings of the artificial prey without leaving any marks, highlighted the potential limitations of recording predations on the plasticine baits. Conversely, footage of a mistle thrush (*Turdus viscivorus*) entirely consuming the plasticine body enabled baits with missing plasticine bodies to be more confidently included as predations in the dataset.

A further strength of the experiments reported here lies in repeating the predation trials over multiple seasons. While there was no change in predator preference for moths with or without spots across field seasons, there was a significant increase in average predation risk for all items between 2016 and 2017. Environmental conditions are likely to be responsible for this change, although this is difficult to ascertain with only two years of data. Following an extremely dry winter, there were no water sources on site in 2017, where there had previously been several lakes and a stream running through the dunes; these visibly harsher conditions may have affected insect life on site and made foraging more difficult for insectivorous birds. As suggested by laboratory experiments, birds experiencing these harsher conditions may have been more willing to attack potentially defended prey (Barnett, Bateson and Rowe, 2007; Chatelain, Halpin and Rowe, 2013). In summer 2017, the presence of a new generation of young and naïve predators (as recorded by camera-trap footage) may have additionally contributed to the overall increase in predation rates, while making no distinction between spotted and plain prey. Mappes *et al*. (2014) demonstrated that the survival of artificial prey mimicking wood tiger larvae (*Arctia plantaginis*) varied across seasons in northern Finland: aposematic models were more at risk than cryptic models in July, coinciding with a high number of naïve fledgling birds. Correspondingly, aposematic lepidopteran larvae were less common at that time of year, when aposematism was a less-rewarding strategy. Differences in average predator naivety over time may also contribute to the maintenance of ontogenetic colour polymorphism in the striated shieldbug, *Graphosoma lineatum*, in which bugs emerging in early spring have conspicuous red and black markings, while later-

emerging individuals have a paler, more cryptic form (Johansen *et al*., 2010; Tullberg *et al*., 2008). By contrast, the emergence of *Z. filipendulae* in coastal habitats in Cornwall in early summer coincides with the first foraging experiences of many fledgling birds, when predation risk for aposematic prey is expected to be at its highest. Predation risk for spotted prey, more closely resembling real burnet moths, was initially expected to decrease in the summer repeat of Experiment 2, when temperatures were warmer, more alternative invertebrate prey should be available and predators had had recent opportunities to experience the distasteful prey. However, the presence of naïve predators may have counteracted these effects, such that overall predation rates of spotted prey did not decrease in June-July. It would be interesting to repeat the experiment once more, later in summer, when all potential predators would have presumably experienced unprofitable aposematic prey.

Nevertheless, the results of my experiments seem to suggest that colour alone may not be a sufficiently protective trait for wild burnet moths. Burnet moths advertise their toxicity, not only through visual conspicuousness, but also with several other types of signals, in particular gustatory and olfactory. When attacked, larvae of *Z.filipendulae* produce droplets of defensive fluids from cuticular cavities, and adults secrete both fluids from their mouthparts and haemolymph from their legs (Zagrobelny et al. 2008). Larval defensive droplets contain compounds deterring predation by other invertebrates (Pentzold et al. 2016), as well as cyanogenic glucosides, whose bitter taste may lead to taste-rejection by avian predators. If an attack continues and the droplets come into contact with moth haemolymph or the predator's digestive enzymes, the breakdown of the cyanogenic glucosides will release toxic cyanide. Moreover, in adults, pyrazines, "warning odours" commonly found in defended insects including *Z. filipendulae* and *Z. lonicerae* (Rothschild et al. 1984; Tremewan 2006) combine with hydrogen cyanide (HCN) to produce a foul odour (Zagrobelny, Bak and Møller, 2008), vividly described by Miriam Rothschild as "close to physical pain", and blamed for triggering asthma attacks (Rothschild, 1961). Pyrazines in particular are known to play a key role in the multimodal signals of defended insects (Guilford *et al*., 1987). The combination of pyrazine odours and warning signals, such as red and yellow coloration, can cause birds to avoid even palatable stimuli (Rowe and Guilford, 1996), and pyrazines have

further been shown to contribute to avoidance of novel and conspicuous stimuli, as well as promote faster and more accurate learning of associations between signals and defences (reviewed in Rowe and Guilford, 1999). The relative effects of vision, smell and taste in triggering and maintaining avoidance of defended prey by predators are currently being investigated in the wood tiger moth (*Arctia plantaginis*; Emily Burdfield-Steel and Bibiana Rojas, pers. comm.), and similar work in the Zygaenidae is the next logical step towards a better understanding of the anti-predator strategy of these species. Experiments with stimuli mimicking burnet moths paired to distasteful baits, or associated with pyrazine odours, may come closer to a realistic test of avian responses to burnet moths. Moreover, these present experiments were strictly concerned with avian predation, but burnet moths are also vulnerable to many invertebrate predators such as ants and spiders, against which they deploy a suite of chemical defences in defensive droplets (Penzold *et al*., 2016). The combination of chemical and visual signals may be critical for survival in the wild, providing protection against a broader range of predators.

Even without resorting to distasteful baits, this study highlights several ways in which artificial predation experiments can be improved to provide more detailed and reliable information about how visual signals affect predation risk in the wild. A better understanding of the most likely predators facilitates more accurate data collection, and can also help improve the design of artificial stimuli, by identifying the most relevant predator visual systems. Using more continuous variation in coloration, rather than discrete treatments, improves the statistical power of the analysis, which can be valuable in these typically very large experiments, in which thousands of artificial baits are deployed to ensure significant results despite low predation rates (e.g. over 12000 in a study of adder markings, Wüster *et al*., 2004). Finally, while it is not uncommon for predation trials to run over multiple field seasons, very few other studies have explicitly tested for an effect of seasonality on predation risk (but see Mappes *et al*. [2014] and Arenas [2015]). Predator naïvety, but also other relevant factors such as environmental conditions, predator condition and the community of alternative prey available, will all vary over time. Experiments attempting to unpick the importance of these variables, rather than testing only the role of

signal features, will help build a more comprehensive picture of predation risk for aposematic prey in the wild.

Chapter 7

Discussion:
Aposematism in burnet moths – new insights and opportunities

A pair of six-spot burnet moths, *Z. filipendulae*.
Photograph: E. S. Briolat

7.1 Abstract

Despite a long history of research since the theory was first proposed in the 19th century, warning coloration remains a fertile field of investigation. In this thesis, I studied the warning signals of Lepidoptera to explore how specific features of aposematic patterns may relate to quantitative variation in prey toxicity and may ultimately contribute to prey survival in the wild. I was able to take full advantage of recently-developed tools for analysing visual signals from their likely receivers, and sophisticated techniques to quantify defensive chemicals, to provide sensitive and ecologically-relevant insights. Having established that the six-spot burnet moth, *Zygaena filipendulae*, is more variable in appearance to avian predators than it seems to humans, and that reliable measurements of coloration could be obtained, I focused primarily on this species and its close relatives in the Zygaenidae. My investigations into the patterns of British moths using museum specimens suggested that most expectations for the form of aposematic wing patterns, based on typical warning signals across taxa and the predictions of efficacy theory, were met, at least to some extent. Defended species generally tended to possess more saturated, redder and more contrasting colours, making them more conspicuous against natural backgrounds. Concentrating on the six-spot burnet moth, very few signal features seemed to correlate with levels of cyanogenic defences, whether between or within populations, suggesting a lack of quantitative relationships between coloration and defences, and in some cases hinting at dishonesty in signalling. I found similar trends across species in the Zygaenidae, but these results also highlighted the importance of environmental variation and sex-specific patterns in determining the relationship between colour and unprofitability. Finally, my predation experiments confirmed that variation in coloration does affect predator behaviour, albeit in sometimes unexpected ways. Collectively, these studies point to several general conclusions, principally the primary role of chromatic features, and especially internal chromatic contrast, in aposematic signals, and the complexity of the selective landscape affecting the relationship between coloration, toxicity and predator behaviour. These first forays into the warning signals of the Zygaenidae from the perspective of avian predators also highlight the significant potential of this family of moths as a study system in which to explore questions relating to aposematism.

7.2 General conclusions concerning the form of warning signals

7.2.1 Quantitative signal honesty is not ubiquitous in aposematic species

Both within and between populations of the six-spot burnet moth, *Zygaena filipendulae*, and across species of Zygaenidae, I found no clear evidence of quantitative signal honesty. There were very few significant correlations between measures of coloration and toxin levels, suggesting that in general, there was no quantitative relationship between signals and defences in these species. In terms of coloration, the trends that did emerge pointed to dishonesty in signalling, as more saturated, redder and more contrasting markings were associated with lower levels of cyanogenic glucosides, for example within the Holywell Bay population of *Z. filipendulae* and across females of the nine Zygaenidae species I studied in 2015. These results are in conflict with those of many studies of quantitative honesty in warningly-coloured species, including recent work on nudibranchs and ladybirds (Cortesi and Cheney, 2010; Arenas, Walter and Stevens, 2015; see Chapter 1). Although many theoretical models do find that quantitative honesty in aposematic species is possible, there are also many factors which may prevent the evolution of quantitative honesty as a stable strategy (Summers *et al.*, 2015). First and foremost amongst these are disjunctions in the relative costs of signals and defences. Theoretical models suggest that, if the costs of one of these elements increases, while the other does not, investment in the least costly component will be prioritised, leading to negative correlations between quantitative measures of signals and defences (Speed and Ruxton, 2007). Even if coloration and defensive chemicals compete for shared resources (as hypothesised in the resource-allocation theory; Blount *et al.*, 2009), aposematic species are expected to invest more in defences when resources are plentiful. High toxicity will be an effective deterrent on its own, while conspicuousness carries inevitable costs of visibility to resistant, naïve or highly-motivated predators. Strategic trade-offs between coloration and defensive chemicals may explain other negative correlations between signals and toxins, found in particular in very toxic poison frogs (Wang, 2011; Crothers *et al.*, 2016). Many other circumstances promoting variation in signal form, such as trade-offs with other functions of visual signals and differences in predator communities (Mappes, Marples and Endler, 2005), could be responsible for a lack of signal honesty across populations and species. In burnet moths, the high

aversiveness of their defences and the fragility of their coloration, fading over time in natural populations, are likely to be important factors.

Nevertheless, there were positive correlations between the luminance, or perceived lightness, of red markings and cyanogenic glucosides levels, both within the six-spot burnet, *Zygaena filipendulae*, and across species (Chapters 4 and 5). Intuitively, lighter markings seem unlikely to constitute more conspicuous signals. In addition, increased marking luminance did not seem to result in increased luminance contrast between the markings and background colour of the wings, or increased conspicuousness against natural backgrounds. However, positive correlations between luminance contrast and the concentration of defensive compounds were found across species in 2016 (Chapter 5). Achromatic information is generally considered less important than chromatic cues in determining avoidance learning, at least in birds, but may still have a role to play (Stevens and Ruxton, 2012). Luminance contrast can be relevant to avoidance learning in invertebrate predators, such as mantids (Prudic, Skemp and Papaj, 2007), suggesting that visual communication with invertebrate enemies should not be discounted. Moreover, contrasting melanic patterns are widespread in aposematic species, and experiments testing their value for avoidance learning have yielded somewhat conflicting conclusions. Overall, studies of the reaction of birds to patterned prey have suggested that contrasting markings are less important than overall colour (Exnerová *et al.*, 2006; Aronsson and Gamberale-Stille, 2008, 2009), but may nevertheless further speed up learning (Aronsson and Gamberale-Stille, 2012b), or provide further information on prey quality (Aronsson and Gamberale-Stille, 2012a). Predation experiments with artificial prey models in the field have similarly indicated that pattern and luminance cues are less relevant than colour (Hegna *et al.*, 2011; Flores *et al.*, 2015), although patterns can have an effect on survival in some cases (Tan, Reid and Elgar, 2016). In addition, there is some evidence that luminance contrast could be important, even for avian predators: experiments testing the response of great tits (*Parus major*) to mealworms painted to resemble different morphs of vapourer moth larvae (*Orygia antiqua*) found that high luminance contrast between the painted markings and the mealworm promoted initial avoidance of the prey (Sandre, Stevens and

Mappes, 2010). As a result, the possibility of quantitative signal honesty based on luminance must be considered.

7.2.2. The relative importance of various signal and defence properties is difficult to assess

These difficulties in interpreting my results highlight a key problem in the study of quantitative honesty in aposematic animals: the lack of knowledge about which aspects of coloration, and to a lesser extent defences, are truly relevant to predators. Broad differences between the sensory modalities used by the principal predators of defended prey when foraging, or in the sensitivity of their visual systems, are known to be important in determining the evolution of warning signals (Willink *et al.*, 2014; Fabricant and Herberstein, 2015). Yet which specific features of visual signals are most important in promoting predator avoidance is still relatively unclear, despite considerable work in this area (Stevens and Ruxton, 2012). As discussed above, there is still some debate over the relative importance of chromatic and achromatic information, and of colour versus pattern. While it does not constitute a test of avian responses to signal features, my comparative analysis of British moths suggests that a diverse palette of colours, and strong internal chromatic contrast, are important characteristics of warning signals (Chapter 3). In addition, the predation experiments I carried out suggested that variation in colour had an impact on prey survival, while wing iridescence and the presence or size of spots did not (Chapter 6). My results thus support the idea that chromatic information is most important, although the role of marking luminance would benefit from further study.

Without clear predictions concerning which signal properties would be most relevant to potential predators, I tested for trends in a wide range of signal features, and my results are correspondingly not straightforward to interpret. Many existing studies of honesty in aposematic signals have focused on specific visual components of signals (such as the area or luminance of a specific colour patch, see Chapter 1), but this may lead to misleadingly simple conclusions. In most cases, signal strength is measured as conspicuousness against natural backgrounds, a key feature of warning coloration and an inherently costly trait (Sherratt, 2002). Yet some experiments have suggested

that colour contrasts are more difficult to learn than simple associations between colours and defences (Gamberale-Stille and Guilford, 2003), so conspicuousness may not be the only signal property to which predators attend. When multiple signal traits and types of defences have been considered in studies of quantitative honesty, the results are much more complex, with few trends emerging and occasionally inconsistent patterns (Bezzerides *et al.*, 2007; Blount *et al.*, 2012; Maan and Cummings, 2012; Winters *et al.*, 2014; Arenas, Walter and Stevens, 2015; Crothers *et al.*, 2016). For example, in the seven-spot ladybird, *Coccinella septempunctata*, the relationship between coloration and defences varies between sexes and dietary treatments, and according to which alkaloid defence (coccinelline or precoccinelline) is quantified (Blount *et al.*, 2012). These complicated trends may provide a more accurate representation of the signalling landscape that predators must navigate.

Ideally, studies of quantitative honesty should measure coloration, defences and how these traits affect predator behaviour (see Arenas, Walter and Stevens, 2015). However, my own artificial predation experiments did not support the idea of quantitative honesty (Chapter 6). More conspicuous patterns, with more highly-contrasting and redder markings, were in fact more likely to be attacked, suggesting that greater detectability was more relevant than avoidance of aposematic signals in this study. My work is difficult to compare to that of Arenas, Walter and Stevens (2015), as I considered only visual properties inherent in the wing patterns themselves, rather than conspicuousness against natural backgrounds, in my cross-species analysis. The wing patterns of burnet moths differ from the elytra of ladybirds, as long wavelength colours generally occupy most of the ladybird elytra and are in direct contact with natural backgrounds, whereas in burnet moths the long wavelength markings are embedded in dark background scales. As such, conspicuousness of these colours to natural backgrounds may be less relevant in this family. Moreover, measurements in *Zygaena filipendulae* suggested that all specimens were highly conspicuous (Chapter 4), and this is expected to be the case for all Zygaeninae. The species that might be considered less visible against natural backgrounds, such as the brown *Aglaope infausta* (Chalcosiinae) and *Rhagades pruni* (Procridinae), in fact contained the highest levels of cyanogenic glucosides (Chapter 5). However, as they are also the

smallest Zygaenidae I analysed, they possessed the least cyanogenic glucosides in total. This then raises the question of whether the total amount of defensive compounds or their concentration is most relevant to predators (see Chapter 5). The levels of defensive chemicals are generally thought to be more important, which would make sense for species which, like burnet moths, produce defensive secretions (Rothschild, 1985). Nevertheless, more detailed information on the anti-predator strategies of the more discreet and smaller Procridinae, as well as observations of avian responses to them, would help determine whether these insects are likely to be tasted and rejected on the basis of toxin levels, or would be consumed whole.

Much like coloration, measuring defences in a meaningful way, relevant to potential predators, is not a trivial task. The best method would be to test the responses of natural predators of the aposematic species in question to individual prey, varying in their level of defence. Yet this is rarely feasible due to practical considerations and ethical restrictions when working with vertebrate predators. Many experiments offering distasteful prey to model species such as great tits (*Parus major*) or starlings (*Sturnus vulgaris*) have nevertheless been carried out, whether with real invertebrates (e.g. Wiklund and Jarvi, 1982) or mealworms injected with bitter-tasting compounds (e.g. Barnett, Bateson and Rowe, 2014; Rowland, Fulford and Ruxton, 2017). Experiments with natural prey provide information about which species are most likely to be avoided in nature (Sargent, 1995), while those with manipulated baits allow controlled tests of the effects of toxin levels, bitter taste, and how these interact with other prey features (reviewed in Skelhorn, Halpin and Rowe, 2016). As a drawback, interpreting the behavioural responses of predators can be difficult and hard to compare across studies. Quantifying defensive compounds with analytical techniques such as liquid chromatography – mass spectrometry (LC-MS) has the advantage of producing quantitative data, which is easily comparable across species with the same type of defences. However, this may not capture the true impact of prey defences on predator physiology, as the mix of compounds in defensive fluids may interact to enhance the unpleasant experience. Equally, some predators may have evolved a level of resistance to prey toxins, as seen in some populations of garter snakes, *Thamnophis sirtalis*, resistant to tetrodotoxin (Geffeney, 2002). An intermediate strategy is to conduct bioassays,

often based on injecting extracts from prey into mice (Marsh and Rothschild, 1974; Summers and Clough, 2001) and now increasingly using invertebrates such as *Daphnia* water fleas (Arenas, Walter and Stevens, 2015) and *Formica* ants (Lindstedt *et al.*, 2017). These provide a measure of the biological effects of the prey's defences, albeit not to the most relevant predator. Verifying results with multiple techniques is probably the best solution to these difficulties (see Maan and Cummings, 2012), and I would be keen to validate my work on burnet moth defences by carrying out complimentary bioassays.

7.2.3 Complex selective pressures shape the relationship between coloration and defences

Aside from the limitations of our understanding of how predators respond to different signal and defence properties, one of the main sources of complexity brought to the fore by my research is variation in selective pressures between the sexes. Different costs and benefits of signalling to predators may arise due to disparities in traits such as body size (e.g. ladybirds; Blount *et al.*, 2012) or activity patterns (e.g. more active males in burnet moths). In the comparative analysis of museum specimens, two of the species measured are sexually dimorphic and have contrasting flight times (the muslin moth, *Diaphora mendica* and the clouded buff, *Diacrisia sannio* [Erebidae]; Chapter 3). Moreover, aposematic signals may interact with sexual signalling and mate choice, and this is likely to have different effects on males and females. In some species, separate body parts or visual features may be used to mediate intra- and interspecific signalling, such as dorsal and ventral surfaces in poison frogs and swallowtail butterflies (Siddiqi *et al.*, 2004; Rutowski, Nahm and Macedonia, 2010, but see Maan and Cummings, 2008). Others adopt sexual signals that cannot be seen by other potential receivers, creating private channels of communication. For example, the chromatic features of male damselflies can be perceived by prospective female partners, but not by their dipteran prey (Outomuro *et al.*, 2017), while correlated evolution of UV-sensitive photoreceptors and UV-reflective yellow pigments allows *Heliconius* species to discriminate between co-mimics which appear indistinguishable to birds (Bybee *et al.*, 2012). However, this is not always possible, and trade-offs between sexual and anti-predator signalling do occur. In the wood tiger moth, *Arctia plantaginis*, yellow males are more effectively avoided by avian predators, but

white morphs have greater reproductive success (Nokelainen *et al.*, 2012). Conversely, sexual selection and predator preferences could both favour the same signal form, as suggested by experiments in *Heliconius erato* (Finkbeiner, Briscoe and Reed, 2014). Sex-specific trends are rarely, if ever, considered in studies of quantitative honesty in aposematic signalling. This omission may be concealing interesting trends, so future work should consider the possibility of different relationships in males and females (Maan and Cummings, 2009).

Further complications arise due to changes in the selective landscape over time, and depending on ecological conditions. Measurable differences in defence levels and signal salience between field seasons forced me to consider specimens collected in 2015 and 2016 separately, which I had not anticipated (Chapter 5). Similarly, the stark differences in environmental conditions, and in particular water levels and its downstream effects on invertebrate populations, are likely to have affected the results of my artificial predation experiments in 2016 and 2017 (Chapter 6). I collected specimens for my analyses of the relationship between coloration and defences from many separate locations, to improve my chances of finding sufficiently large sample sizes, especially across multiple species. However, considering the subtle effects of different predator and prey communities, as well as of contrasting environmental conditions, it seems important to firstly gain a better understanding of how individuals invest in signals and defences within the same location. These individuals would be facing the same ecological conditions, making the results easier to interpret. Measuring colours and defences at a single location at any given point in time, effectively a snapshot of what a predator might encounter, would test whether prey coloration does actually provide useful quantitative information to a foraging predator.

Conversely, tracking changes in coloration and defences in the same location across time would enable more detailed investigations of how prey might invest in signals and defences, in response to changing environmental conditions and available resources, or other seasonal patterns. The need for thermoregulation is likely to change in space, along gradients of altitude and latitude, and in time, with the seasons, or at an even shorter scale with the time of day, affecting the relative costs and benefits of aposematic signals (see work on thermoregulation

and signalling in the wood tiger moth, *A. plantaginis*; Lindstedt, Lindström and Mappes, 2009; Hegna *et al.*, 2013). Predator communities are also inherently variable, both seasonally and on a daily cycle (e.g. the prevalence of bats and birds hunting tiger moths; Ratcliffe and Nydam, 2008). Even within the same class of predator, experience and motivation will vary, and may follow general trends across time. The striated shieldbug, *Graphosoma lineatum*, is typically red with black stripes, but adults emerging late in the season in Sweden adopt a paler form, with a cream background colour, thus switching to a more cryptic rather than aposematic strategy (Tullberg *et al.*, 2008). Changes in the predator community, and especially an abundance of naïve fledglings, who have not learnt the association between red colours and unprofitability, may be responsible for this change in phenotype, tipping the balance of costs and benefits of signalling towards camouflage rather than conspicuousness (Johansen *et al.*, 2010). Similar considerations may have shaped broader trends in the abundance of aposematic Lepidoptera (Mappes *et al.*, 2014). Multiple species of burnet moths often co-occur in continental Europe, so these species may represent a promising system in which to investigate how whole communities of aposematic prey respond to variation in ecological conditions.

7.3 Future perspectives for research on aposematism in the Zygaenidae

7.3.1 Challenges and opportunities for studies of aposematism in burnet moths

As I discovered over the course of my research project, working with burnet moths does pose some challenges, primarily relating to collection and rearing. Males provide females with nuptial gifts of cyanogenic glucosides during mating, and females invest their defensive resources in their eggs, leading to substantial changes in cyanogenic glucoside levels pre- and post-reproduction. Males lose approximately 30% of their body weight during mating (Zagrobelny *et al.*, 2013) and females invest up to 20% of their total cyanogenic glucoside resources obtained as larvae into their eggs (Zagrobelny *et al.*, 2007a). Obtaining meaningful measurements of cyanogenic glucoside levels therefore requires virgin individuals, such that wild specimens have to be collected at the larval or pupal stage. The six-spot burnet, *Z. filipendulae*, is easy to find in Cornwall (UK) as the larvae feed openly on bird's foot trefoil, *Lotus corniculatus*, and spin their cocoons high up on grass stems. However, there is considerable

variation in the behaviour of Zygaenidae larvae as well as in the appearance and position of their cocoons, and many cocoons are concealed low in vegetation or under rocks (Hofmann and Tremewan, 2017). Some larvae, such as *Aglaope infausta* and both *Z. filipendulae* and *Z. occitanica* feeding on tufts of *Dorycnium pentaphyllum*, can be collected easily with beating trays from their host plants. Others, such as *Z. sarpedon* and *Z. erythrus*, have to be prised from the central whorls of low-growing and spiky *Eryngium* plants. Many Procridinae, or foresters, are leaf-miners, their larvae feeding inside the leaves of their host plants (Drouet, 2016), making them very difficult to locate. In addition, while all the Zygaeninae other than *Z. brizae* lay their eggs in batches (Hofmann and Kia-Hofmann, 2011), most forester moths lay their eggs singly (Tarmann, 2005), reducing their density in the field. I had hoped to include many more foresters in my cross-species analysis, as their defences have been examined in comparatively little detail. Unfortunately, I only succeeded in finding fewer than 10 specimens of *Jordanita* spp. (with the help of Eric Drouet), only 1 of which survived to adulthood. Rearing burnet moths from eggs or even small larvae is also non-trivial, due to their obligatory larval diapause in winter (Tremewan, 1985), summer aestivation in some species (e.g. *Z. fausta*), and the propensity for some species to return into diapause multiple times (eg. *Z. cynarae* and *Z. transalpina*, pers. obvs.). Even with single-diapause strains, successfully rearing less than 5% of eggs to adulthood would not be unusual (Tremewan, 2006). Low sample sizes are one of the main limitations of the studies presented in this thesis, especially in the dietary experiment, testing the effect of acyanogenic diet on resource allocation to signals and defences in *Z. filipendulae* (see Chapter 4). This remains a valuable opportunity to test resource allocation in an aposematic species, so would be worth repeating with larger sample sizes.

However, these difficulties can be overcome with experience and if more resources and personnel are dedicated to rearing the specimens. Conversely, burnet moths also present considerable advantages. There is a dedicated community of both professional researchers and amateur entomologists interested in burnet moths and foresters. These experts represent a phenomenal source of knowledge of their natural history, can provide assistance with field locations and rearing techniques, and even host a biennial

conference devoted to these species. Moreover, there have been extensive investigations into the phylogenetic relationships between species in the Zygaenidae, providing a sound basis for comparative analyses across species (e.g. Niehuis, Naumann and Misof, 2006a, 2006b; Niehuis *et al.*, 2007). Most importantly, the chemical defences of burnet moths have been elucidated with a rare level of detail among aposematic species, down to the genetic and metabolic pathways involved in *de novo* synthesis and sequestration of cyanogenic glucosides (Jensen *et al.*, 2011; Fürstenberg-Hägg *et al.*, 2014b). Since all Zygaenidae tested so far possess the same defensive compounds, linamarin and lotaustralin (Davis and Nahrstedt, 1982), and these can be quantified using LC-MS techniques, defence levels can be compared both within and between species.

Further investigations of warning coloration in the Zygaenidae could develop the work I carried out in this thesis in a number of ways. As I relied on specimens from fairly easily-accessible collection sites in the UK, France and Denmark, I was restricted to certain species, which do not display the most variable wing patterns in the family. Selecting from a broader range of species, even within the Western Palearctic, would be informative, enabling a test of signal honesty across species with more divergent wing patterns. Within species, there are some striking examples of polymorphisms and polytypisms which also warrant further research. For example, the pale phenotype of *Z. carniolica kappadokiae*, which has white background scales, has been linked to thermoregulation (Buntebarth, 2004) and crypsis against the volcanic soil of its habitat in Cappadocia (Tremewan, 2006), but its cyanogenic glucoside content and effectiveness in stimulating avoidance by predators have not been tested. Similarly, many species have melanistic coastal populations with reduced red markings (a phenomenon known as “littoral melanism”; Tremewan, 2006; Tarmann and Tremewan, 2013), but how these populations vary in their toxicity and behaviour is unknown. Finally, I would also be interested in applying more rigorous measurements of colour, from the perspective of avian predators, and techniques for quantifying cyanogenic glucosides, to investigate the putative Müllerian mimicry between *Zygaena ephialtes* and the nine-spotted, *Amata phegea* (Turner, 1971; Sbordoni *et al.*, 1979). The red-spotted Zygaeninae have also traditionally been considered to mimic each other, potentially as part of a

wider mimicry ring with other red insects, including the cinnabar moth, *Tyria jacobaeae*, and red beetles (Tremewan, 2006). In continental Europe, the distributions of different *Zygaena* species greatly overlap, so individual predators may well encounter multiple species, making mimicry a viable strategy. Whether zygaenid species are truly mutualistic Müllerian mimics, such that the presence of multiple co-mimics decreases predation risk for all species concerned (Müller, 1879), remains untested. However, if the differences in toxin levels between species are biologically relevant to predators, the more weakly-defended species may be acting as parasitic quasi-Batesian mimics, benefitting from their resemblance to more highly-defended species (Speed, 1999; Rowland *et al.*, 2010). Quantifying cyanogenic glucosides and coloration in several populations of sympatric species would help resolve this question.

Zygaena species are well-suited to the study of quantitative signal honesty, as their ability to sequester and *de novo* synthesise cyanogenic glucosides allows for a test of the effect of resource limitation on their investment in signals and defences (Blount *et al.*, 2009, 2012). Yet while the energetic costs of *de novo* synthesis and sequestration of cyanogenic glucosides are fairly well-understood (Zagrobelny *et al.*, 2007a; Fürstenberg-Hägg *et al.*, 2014b), much less is known about the production of colourful wing patterns in the Zygaenidae. Melanin is responsible for the dark background colour, and pteridines (including erythropterin and another compuound similar to drosopterin; Tremewan, 2006) for the white, yellow and red markings, although carotenoids are also involved in the yellow colour of adult haemolymph (Feltwell and Rothschild, 1974). As pteridines are nitrogen-rich (Chittka, 2013), they may compete with other nitrogen-based compounds, including cyanogenic glucosides, if resources are limited. This could apply to other species, such as the stinkbug *Tectocoris diopthalmus*, whose red patches are also produced by erythropterin (Fabricant *et al.*, 2013). An understanding of the costs of pteridine production, and how investment in pigments may trade off with other functions, would help complete the picture of the costs and benefits relevant to quantitative signal honesty. A growing number of studies are investigating the basis of colour signals in aposematic species, focusing primarily on carotenoid pigments (e.g. Lindstedt *et al.*, 2010; Blount *et al.*, 2012; Fabricant *et al.*, 2013), although there has also been work on pteridines, particularly in sulphur butterflies, *Colias* spp. (Rutowski

et al., 2007). In those species, pteridines are implicated in long wavelength colours as well as iridescence and ultraviolet reflectance (Rutowski *et al.*, 2005, 2007). The red scales of burnet moths present high levels of ultraviolet reflectance (pers. obvs.), a phenomenon previously observed in a few species of butterflies and moths (e.g. the common rose, *Pachliopta aristolochiae*, and the rosy underwing, *Catocala electa*; Eguchi and Meyer-Rochow, 1983). This is another aspect of the visual features of burnet moths that would warrant further investigation, both of the mechanistic basis of red and ultraviolet coloration, and of the relative importance of these two components in aposematic and intraspecific communication.

7.3.2 Interactions between aposematic and sexual signalling

The tight link between anti-predator defences and sexual selection in burnet moths is both a complicating factor and an especially interesting aspect of signalling in these species. Similarly to tiger moths (Erebidae), in which males provide nuptial gifts of alkaloids (Weller, Jacobson and Conner, 1999; Conner and Weller, 2004) the defensive chemicals of burnet moths are an integral part of their life cycle and play a major role in sexual signalling and mate choice. Males provide females with a substantial nuptial gift of cyanogenic glucosides in their spermatophore (Zagrobelny *et al.*, 2007b; Zagrobelny *et al.*, 2013), and females accept and reject males on the basis of their levels of these compounds. Experiments on female choosiness in *Z. filipendulae* showed that rejected males have a body mass 25% smaller and cyanogenic glucoside levels 60% lower than those which are accepted (Zagrobelny *et al.*, 2013), and that this bias can be overcome if the males are injected or painted with extra linamarin (Zagrobelny *et al.*, 2015). Both sexes also release hydrogen cyanide (HCN) as part of a cocktail of volatiles, and levels of emission are especially high in virgin females, suggesting that HCN may play a role in mate attraction (Zagrobelny *et al.*, 2015).

While cyanide-based compounds clearly have multiple functions in the Zygaenidae, the role of warning coloration in other types of signalling is less clear. Visual characteristics are known to be important for mate choice in butterflies, with pheromones operating in the later stages of courtship, while moths typically rely on long-distance pheromones emitted by females to attract

males (Vane-Wright and Boppré, 1993). Yet one family of day-flying moths, the Castniidae ("butterfly-moths", or "sun-moths"), have evolved to utilise visual cues: females appear to have lost their abdominal pheromone-producing glands, and thus the capacity for producing long-distance pheromones, while males adopt the perching and patrolling strategies seen in butterfly species (Sarto i Monteys *et al.*, 2016). The day-flying Zygaenidae appear to have adopted an intermediate strategy, in which pheromones are predominantly important, but visual cues may also come into play (Sarto i Monteys *et al.*, 2016). Several *Zygaena* species are thought to use optical cues to orient themselves towards their potential mates at close range (Zagatti and Renou, 1984; Koshio, 2003; Friedrich and Friedrich-Polo, 2005). In addition, *Zygaena trifolii* males switch between two alternative searching strategies, following pheromone plumes to find females in the morning, then locating them using visual information in the afternoon when females are not emitting pheromones (Naumann, Tarmann and Tremewan, 1999; Hofmann and Kia-Hofmann, 2010). These moths also use a combination of chemical and visual information when choosing flowers to nectar on, and are attracted to the blue colour of field scabious, *Knautia arvensis* (Naumann *et al.*, 1991; Ockenfels and Schmidt, 1992), lending further support to the idea that visual stimuli are relevant to their behaviour.

Given that my research also suggests that there are differences in coloration between the sexes, it would be interesting to gain a better understanding of the visual features burnet moths themselves may attend to. I did not find evidence of quantitative honesty in signalling in either sex in *Z. filipendulae*, suggesting that colour would not provide detailed information about the cyanogenic glucoside levels of prospective partners. However, coloration may be a useful signal of the freshness of a potential mate, which could act as a crude proxy for quality, as older males are likely to have given away substantial nuptial gifts in previous matings, and females lay most eggs in their first batch (see Chapter 4). As yet, there have been very few attempts to investigate the role of visual cues in intraspecific communication in the Zygaenidae. Early experiments using artificial baits and preserved specimens suggested that males may have a preference for more saturated colours, although chemical cues from the specimens may have confounded the results (Zagatti and Renou, 1984). Males

also failed to distinguish between the wing patterns of closely-related species. These experiments, and more recent work on the vine bud moth, *Theresimima ampellophaga* (Toshova, Subchev and Toth, 2007) suggest only a very crude perception of colour patterns, but they did not attempt to account for the moths' visual system. This is a difficult task, as the specific characteristics of vision in the Zygaenidae are not yet known, and the diversity of lepidopteran visual systems makes it difficult to draw inferences from other species, especially with relatively little data available for moths (Briscoe and Chittka, 2001; Stavenga and Arikawa, 2006). Nevertheless, it would be valuable to at least consider reflectance in the UV wavelengths, as most Lepidoptera possess UV-sensitive photoreceptors (Briscoe and Chittka, 2001). Finally, the potential for female choice based on visual signals is completely untested. Unfortunately, female preference for more toxic males makes designing experiments testing their response to visual cues particularly difficult. Any underlying variation in male toxin content will confound the results of choice tests, while injecting the males with glycosides to eliminate this variation will result in very high quality males whose high levels of glycosides might override the effect of any other available information. Although doubtlessly challenging, experimental work assessing the role of intraspecific visual signalling in burnet moths would be extremely valuable in clarifying the potential trade-offs between aposematic coloration and other functions.

7.3.3 Multimodal signalling in burnet moths

Burnet moths have a complex and intricate anti-predator strategy, or multimodal defensive display (as defined by Rowe and Halpin, 2013), going beyond a simple association of colour signals and toxic cyanogenic glucosides. This is exemplified by the defensive fluids of *Zygaena* larvae, secreted from cuticular cavities when the larvae are disturbed. The bitter taste of cyanogenic glucosides may be aversive to birds, which could taste and reject them, as has been demonstrated by experiments exposing starlings to bitter compounds (Skelhorn and Rowe, 2009, 2010). If predators continue to attack, and they swallow the defensive droplets or wound the caterpillar such that the droplets come into contact with haemolymph, toxic cyanide will be released. At the same time, the viscous fluids inhibit predation by ants, forming precipitates that block their mandibles (Pentzold *et al.*, 2016). Equally, adults possess multiple layers

of signals and defences, including emission of HCN (Zagrobelny *et al.*, 2015), pyrazine odours (Moore, Brown and Rothschild, 1990), conspicuous wing patterns and brightly-coloured haemolymph, defensive secretions (Jones, Parsons and Rothschild, 1962), and cyanogenic glucosides distributed throughout adult tissues (Zagrobelny *et al.*, 2014b).

There are many hypotheses for the benefits of multimodal signalling, based either on the strategic message they carry, or concerns of signal efficacy (Guilford and Dawkins, 1991; Rowe and Halpin, 2013). In terms of strategic components, each modality may convey a separate 'message' regarding quality, or they may act synergistically to provide more accurate information. Adopting multiple types of signals also increases signal efficacy in a number of ways (reviewed in Rowe and Halpin, 2013), primarily facilitating predator learning and enabling communication with multiple predator types, which may differ in their preferred sensory modality (e.g. tiger moths [Erebidae] using warning coloration to signal to avian predators, and acoustic warning signals to deter bats; Ratcliffe and Nydam, 2008). Signalling to several taxa is likely to be important in burnet moths, which are vulnerable to both invertebrate and vertebrate predators at all life stages. While research on aposematism has overwhelmingly focused on avian predators, the importance of other predator guilds, and especially invertebrates is increasingly being recognised (e.g. (Prudic, Skemp and Papaj, 2007; Willink *et al.*, 2014; Fabricant and Herberstein, 2015). Similarly to the defensive secretions of burnet moth larvae, adult wood tiger moths protect themselves from both avian and invertebrate enemies: their abdominal defensive fluids are aversive to ants, but not birds, while their thoracic fluids deter avian predators, primarily due to the presence of a pyrazine odour (Rojas *et al*., 2017). It is likely that adult burnet moths are similarly aversive to different types of predators. Burnet moths are also targeted by many species of parasitoids, especially at the larval stage (e.g. Žikić *et al.*, 2013). Defensive compounds such as alkaloids in the tiger moth *Utetheisa ornatrix* (Bezzerides *et al.*, 2004), have been shown to protect invertebrates against parasitism, so the potential for burnet moth defences aimed at parasitoids should also be considered.

Nevertheless, the multiple components of burnet moth signals and defences may also work together to improve their protection from avian predators. Multimodal signalling could contribute to some of the more unexpected results from my studies. For example, my predation experiments found no evidence that birds avoided prey with red spots, even when noxious burnet moths were present in the field. This could indicate that the visual signals of burnet moths need to be reinforced with other cues, such as odours (pyrazines and/or hydrogen cyanide) to be effective, especially if predators are highly-motivated to feed due to poor environmental conditions. Experiments with avian predators, unpicking the respective roles of colour, taste and smell in inducing avoidance of burnet moths, would shed some light on this possibility. Similar work has already been carried out in the seven-spot ladybird (*Coccinella semptempunctata*), by presenting domestic quail (*Coturnix coturnix japonicus*) with palatable beetles endowed with one or several of ladybird signals (Marples, van Veelen and Brakefield, 1994). Colour was the most effective feature in generating avoidance when presented alone, but the combination of taste and colour led to increased rejection. Moreover, no single cue or pairwise combination of cues elicited as strong an avoidance response as the ladybirds themselves, suggesting that these insects benefit from combining all these elements. More recently, the interactions between alkaloid defences, conspicuous wing patterns and pyrazine odours have been suggested to play a role in predator deterrence by aposematic wood tiger moths. Different colour morphs may even employ alternative strategies, relying more heavily on either initial avoidance of visual signals and unpleasant odours, such as pyrazines, or last-ditch defences by taste-rejection (Emily Burdfield-Steel and Bibiana Rojas, pers. comm.). So far, experiments presenting burnet moths to birds have shown that they are generally rejected as prey, and can survive attacks, suggesting that taste-rejection is likely to occur in the wild, but these studies have not specifically set out to separate the effects of different signal components (Heikertinger, 1939; Wiklund and Järvi, 1982; Rammert, 1992). Multimodal signalling has received an increasing amount of attention in the last two decades, and is still a key avenue of research in the field of animal communication and aposematism in particular (Cuthill *et al.*, 2017). The especially complex strategies of burnet moths may yield more insights into the interactions between different elements of anti-predator defences.

7.4 Concluding remarks

Burnet moths provide an attractive study system in which to investigate many complex facets of aposematic signalling. These include the relationship between defence levels and variable signals, both within and between species, the relative importance of multiple predator types in shaping defensive strategies, and the interactions between inter- and intraspecific signalling. In this thesis, I have only scratched the surface of many questions that could be addressed in the Zygaenidae, yet my results nevertheless make valuable contributions. In particular, my work has highlighted the complexity of the selective pressures affecting the relationship between aposematic signal features and defences. Selective forces may change through time due to dynamic predator communities and variable environmental conditions, and may vary between sexes and different populations or species. While this complexity has been recognised (e.g. Mappes, Marples and Endler, 2005; Mappes *et al.*, 2014; Skelhorn, Halpin and Rowe, 2016), it is worth emphasising, especially in the context of the debate over quantitative honesty in aposematic species. If multiple signal properties and types of defences are considered, as well as sex-specific trends and other complicating factors, results are likely to be more nuanced than a simple honest or dishonest dichotomy may allow for. Moving forward, several of my experiments, such as dietary manipulations in *Zygaena* species, comparative analyses of coloration and defence levels across the Zygaenidae, and meticulously-designed artificial predation experiments in multiple seasons, are worth pursuing and expanding to confirm and further explore the conclusions of my PhD. I sincerely hope that my results will inspire future work on the warning colours and other signalling strategies of this charismatic family of moths.

Appendices

(for Chapters 2 - 5)

Appendix 2.1: Coordinates of sites on which *Z. filipendulae* larvae and pupae were collected.

Site name	Country	Latitude	Longitude	Altitude (m)	N	Collectors	Year
Taastrup	Denmark	55.6346	12.2625	30	25	Mika Zagrobelny	2015
Cabasse	France	43.4201	6.2355	250	4	Emmanuelle Briolat	2015
La Chapelle-en-Valgaudémar	France	44.8169	6.1953	2100	1	Eric Drouet	2016
Lardier-et-Valenca	France	44.2512	5.5689	830	5	Eric Drouet	2015
Le Fournas	France	44.0753	5.9722	480	1	Emmanuelle Briolat, Eric Drouet	2015
Les Piles, La Saulce	France	44.4432	6.0288	600	1	Eric Drouet	2016
Mortiès	France	43.7700	3.8223	200	1	Alain Migeon	2016
Mouans-Sartoux	France	43.6204	6.9725	150	2	Emmanuelle Briolat, Alain Bourgon, Pierre Desriaux	2015
St Bauzille	France	43.3542	3.3233	140	2	Alain Migeon	2016
St-Cézaire-sur-Siagne	France	43.3848	6.4928	450	1	Emmanuelle Briolat, Alain Bourgon, Pierre Desriaux	2016
St Félix de Tournegat	France	43.1312	1.7483	310	2	David Demergès	2015
Vacquières	France	43.8445	3.9436	110	2	Alain Migeon	2016
Veynes	France	44.3239	5.4924	900	4	Eric Drouet	2015
Holywell Bay	United Kingdom	50.3910	-5.1430	20	23	Emmanuelle Briolat	2015
Lamorna Cove	United Kingdom	50.0610	-5.5544	30	25	Emmanuelle Briolat	2015
Pendeen Watch	United Kingdom	50.1636	-5.6705	60	9	Emmanuelle Briolat	2015
Porthnanven	United Kingdom	50.1157	-5.6996	20	5	Emmanuelle Briolat	2015
Upton Towans	United Kingdom	50.2100	-5.3972	40	2	Emmanuelle Briolat	2015

Appendix 3.1: Classification of species used in the comparative analysis of museum specimens, based on palatability to predators and activity patterns. Activity patterns based on Newland, Still and Swash (2013).

A) Palatable species		
Species	**Evidence of profitability or chemical defences**	**Activity**
Acronicta psi	One record of wings found at a bat feeding site (Poulton, 1929); no negative effect when pupal tissues are injected into mice (Marsh and Rothschild, 1974); closely related North American species of dagger moths range from very highly to very acceptable to birds in feeding trials (Sargent, 1995)	Nocturnal
Agrotis ipsilon	Very highly acceptable to birds in feeding trials (Sargent, 1995); found in the diet of starlings in the USA (Lindsey, 1939) and great tit nestlings in Spain (Barba, López and Gil-Delgado, 1996)	Nocturnal
Alcis repandata	Two records of wings found at bat feeding sites (Poulton, 1929); larvae found in the stomach of jays (Campbell, 1936); one record in the diet of a scops owl chick (Bavoux *et al*., 1993); adults were studied as an example of industrial melanism for camouflage, suggesting that they were predated by birds (Ford, 1940)	Nocturnal
Amphipyra pyramidea	Found in collections of wings at bat feeding sites (Poulton, 1929) and in the diet of scops owl chicks (Bavoux *et al*. 1993), jays, magpies (Owen, 1956) and great tits (Royama, 1970); highly acceptable to birds in feeding trials (Sargent, 1995)	Nocturnal
Amphipyra tragopoginis	Commonly found in collections of wings at bat feeding spots, suggesting palatability at least to bats (Poulton, 1929)	Nocturnal
Anaplectoides prasina	Highly acceptable to birds in feeding trials (Sargent, 1995)	Nocturnal
Biston betularia	One record of wings found at a bat feeding site, adults readily eaten by common noctule bats (Poulton, 1929); no negative effects when pupal extracts are injected into mice (Marsh and Rothschild, 1974); *Biston betularia cognataria* was highly acceptable to birds in feeding trials (Sargent, 1995); adults predated by 9 species of British birds (Cook *et al*., 2012)	Nocturnal
Catocala nupta	Two records of wings found at bat feeding sites (Poulton, 1929); *Catocala* species are readily eaten by birds and other predators (Jones, 1932; Brower, 1985); North American species ranged from very highly to very acceptable to birds in feeding trials (Sargent, 1995)	Nocturnal, but can be flushed easily in the daytime
Conistra vaccinii	Wings found in a cave frequented by bats (Poulton, 1929); adults found in food samples from blackbird nestlings (Torok, 1981)	Nocturnal
Drepana falcataria	No negative effects when pupal extracts are injected into mice (Marsh and Rothschild, 1974)	Nocturnal
Hoplodrina blanda	A closely-related species, *H. superstes*, was recorded in the diet of a scops owl (Bavoux *et al*., 1993)	Nocturnal

Species	Evidence of profitability or chemical defences	Activity
Hypomecis roboraria	One record in the diet of a scops owl chick (Bavoux *et al*., 1993); adults used to study the role of body orientation on detectability, suggesting that this species is attractive to predators (Kang *et al*., 2012)	Nocturnal
Laothoe populi	Despite the presence of histamine in adult tissues (Bisset *et al*., 1960), there is evidence of palatability from multiple sources: one specimen seen to be eaten by a common noctule bat (Poulton, 1929); no effect of pupal tissues when injected into mice (Marsh and Rothschild, 1974); polymorphic *L. populi* caterpillars used in a predation experiment, likely eaten by corvids or *Turdus* spp. (Edmunds and Grayson, 1991); the related species, *Laothoe juglandis,* was very to moderately acceptable to birds in feeding trials (Sargent, 1995); overall, *Laothoe spp.* are considered undefended, based on published literature on caterpillars (Tullberg and Hunter, 1996)	Nocturnal, but may be found in the daytime on exposed resting places
Noctua pronuba	Collections of wings at feeding spots suggest they are a popular prey for bats (Poulton, 1929); adults were observed to be eaten by birds, including sparrows (Veley, 1902) and found in the diet of great tits (Betts, 1955; Barba, López and Gil-Delgado, 1996), scops owl chicks (Bavoux *et al*., 1993) and golden plover (Campbell, 1936)	Nocturnal, but can be easily disturbed during the day
Orthosia cerasi (Oc)	The closely-related species *O. cruda, O. populi* and *O. stabilis* were found in the diet of great tits (Betts, 1955; Royama, 1970); adults and larvae of several *Orthosia* species were found in the diet of jays, magpies (Owen, 1956) and blackbirds (Torok, 1981)	Nocturnal
Peridea anceps	The closely related *Peridea ferruginea* is highly acceptable to birds in feeding trials (Sargent, 1995)	Nocturnal
Smerinthus ocellata (So)	Despite the presence of histamine in adult tissues (Bisset *et al*., 1960), adults are attacked by passerines (Blest, 1957; Vallin, Jakobsson and Wiklund, 2007); two *Smerinthus spp.* are considered undefended, based on published literature on caterpillars (Tullberg and Hunter, 1996)	Nocturnal, but may be found in the daytime on exposed resting places
Sphinx pinastri	No negative effect when pupal extracts are injected into mice (Marsh and Rothschild, 1974); considered to be undefended, based on published literature on caterpillars (Tullberg and Hunter, 1996)	Nocturnal
Xestia c-nigrum (Xcn)	Two records of wings found at bat feeding sites (Poulton, 1929); closely related North American *Xestia spp*. are highly acceptable to birds in feeding trials (Sargent, 1995)	Nocturnal

B) Diurnal defended species

Species	Evidence of profitability or chemical defences	Activity
Adscita geryon	Defensive “quinolene-like” smell in *Adscita spp*. (Rothschild, 1961); HCN released when adult specimens are crushed (Jones, Parsons and Rothschild, 1962)	Flies in sunshine
Adscita statices	Defensive “quinolene-like” smell in *Adscita spp*. (Rothschild, 1961); presence of cyanogenic glycosides in adult tissues (Davis and Nahrstedt, 1982)	Fly in sunshine, males can be seen in the evening
Arctia plantaginis	Unpalatable to a range of vertebrate and invertebrate predators (Lindstedt, Lindström and Mappes, 2008)	Considered day-flying; males fly fast in the sunshine

Species	Evidence of profitability or chemical defences	Activity
Atolmis rubricollis	Lichen polyphenolics found in some adult specimens, but palatability untested (Hesbacher *et al*., 1995).	Seen in hot, sunny weather
Callimorpha dominula	Defensive “quinolene-like” smell, rejected by house mice (Rothschild, 1961); female extracts toxic when injected into mice (Marsh and Rothschild, 1974); pyrrolizidine alkaloids found in larvae (Von Nickish-Rosenegk and Wink, 1993; Weller, Jacobson and Conner, 1999)	Both sexes can be seen at rest during the day, and males can be very active towards the end of the day
Diacrisia sannio male	Larvae capable of sequestering pyrrolizidine alkaloids (Von Nickish-Rosenegk and Wink 1993; Weller, Jacobson and Conner, 1999)	Often flies during the day, as well as at night
Diaphora mendica female	Pyrrolizidine alkaloids present in larvae and pupae (Rothschild *et al.,* 1979; Weller, Jacobson and Conner, 1999)	Fly in bright sunny weather
Euplagia quadripunctaria	Defensive “quinolene-like” smell (Rothschild, 1961); pyrrolizidine alkaloids found in adults for pheromone use, defensive role untested (Schneider *et al*., 1998; Weller, Jacobson and Conner, 1999)	Considered day-flying, as adults will sometimes fly by day or feed and rest on exposed plants and surfaces
Phragmatobia fuliginosa	Despite two records of their presence in jay food samples (Owen 1956), there is evidence suggesting toxicity: a defensive “quinolene-like” smell (Rothschild 1961); eggs, adults and possibly larvae containing pyrrolizidine alkaloids and a histamine or acetylcholine-like compound (Von Nickish-Rosenegk and Wink 1993; Weller, Jacobson and Conner, 1999)	Considered day-flying; first generation adults fly both during the day and at night, while second generation moths are more reluctant to fly in the daytime
Setina irrorella	Lichen polyphenolics present in some adult specimens, though palatability is untested (Hesbacher *et al*., 1995; Weller, Jacobson and Conner, 1999; Scott *et al*., 2014)	Considered a day-flying moth, active in the morning and late afternoon
Tyria jacobaeae	This species is accepted by some animals, including some birds, a meerkat and a capuchin monkey (Pocock, 1911), but is rejected by many others, such as bats (Poulton, 1929), flycatchers (Pocock, 1911), Himalayan crested tits and terrapins (Rothschild, 1961); it emits a defensive “quinolene-like” smell (Rothschild, 1961); presence of histamine in adult tissues (Bisset *et al*., 1960); pupal extracts are toxic when injected into mice (Marsh and Rothschild, 1974); specimens contain pyrrolizidine alkaloids at larval, pupal and adult stages (Aplin, Benn and Rothschild, 1968; Ehmke *et al*., 1990; Von Nickish-Rosenegk and Wink, 1993; Weller, Jacobson and Conner, 1999)	Fly on sunny days
Zygaena exulans	Defensive “quinolene-like” smell in *Zygaena spp.* (Rothschild, 1961); presence of cyanogenic glycosides in adult tissues (Davis and Nahrstedt, 1982)	Day-flying

Species	Evidence of profitability or chemical defences	Activity
Zygaena filipendulae	Signs of disgust when fed to birds (Pocock, 1911); defensive "quinolene-like" smell in *Zygaena spp*. (Rothschild, 1961); *Zygaena* extracts lethal when injected into mice (Rocci, 1916); all life cycle stages release HCN when crushed (Jones, Parsons and Rothschild, 1962); presence of acetylcholine (Morley and Schachter, 1963) and cyanogenic glycosides in all life stages (Davis and Nahrstedt, 1982)	Day-flying
Zygaena lonicerae	Presence of histamine and acetylcholine (Morley and Schachter, 1963) in adult tissues (Bisset *et al*., 1960); larval and adult tissues cause distress when injected into mice (Marsh and Rothschild, 1974); all life cycle stages release HCN when crushed (Jones, Parsons and Rothschild, 1962); presence of cyanogenic glycosides in adult tissues (Davis and Nahrstedt, 1982)	Day-flying
Zygaena loti	Presence of cyanogenic glycosides in adult tissues (Davis and Nahrstedt, 1982)	Day-flying
Zygaena purpuralis	Presence of cyanogenic glycosides in adult tissues (Davis and Nahrstedt, 1982)	Day-flying
Zygaena trifolii	All life cycle stages release HCN when crushed (Jones, Parsons and Rothschild, 1962); female and egg extracts toxic when injected into mice (Marsh and Rothschild, 1974); presence of cyanogenic glycosides in adult tissues (Davis and Nahrstedt, 1982)	Day-flying
Zygaena viciae	Presence of cyanogenic glycosides in adult tissues (Davis and Nahrstedt, 1982)	Day-flying

C) Nocturnal defended species

Species	Evidence of profitability or chemical defences	Activity
Abraxas grossulariata	Rejected by several bird species (Pocock, 1911) as well as a common noctule bat in feeding trials, and induced signs of disgust in a pipistrelle bat (Poulton, 1929); rejected by house mice (Rothschild, 1961); pupal extracts toxic when injected into mice (Marsh and Rothschild, 1974); specimens contain sarmentosin, a bitter-tasting cyanogenic glucoside (Nishida, 2002)	Occasionally seen flying during the daytime, but not usually classed as a day-flying moth
Arctia caja	Defensive "quinolene-like" smell (Rothschild, 1961); tissue extracts toxic when injected into mice (Marsh & Rothschild 1974); specimens contain toxic substances (Rothschild *et al*., 1970) including a choline ester in adult tissues (Bisset *et al*., 1960), acetylcholine (Morley and Schachter, 1963; Weller, Jacobson and Conner, 1999), pyrrolizidine alkaloids (Rothschild *et al*., 1970) and cyanogenic glucosides (Weller, Jacobson and Conner, 1999); larvae considered to have repellent defences, based on published literature on caterpillars (Tullberg and Hunter, 1996)	Nocturnal, not considered a day-flyer
Arctia villica	Defensive "quinolene-like" smell (Rothschild, 1961); acetylcholine in male tissues (Morley and Schachter, 1963; Weller, Jacobson and Conner, 1999)	Nocturnal, not considered day-flying
Diacrisia sannio female	Larvae capable of sequestering pyrrolizidine alkaloids (Von Nickish-Rosenegk and Wink, 1993; Weller, Jacobson and Conner, 1999)	Mainly flies at night, though can sometimes be found at rest during the day
Diaphora mendica male	Pyrrolizidine alkaloids larvae and pupae (Rothschild *et al*., 1979; Weller, Jacobson and Conner, 1999)	Generally flies only at night

Species	Evidence of profitability or chemical defences	Activity
Eilema complana	Lichen polyphenolics present in some adult specimens, but palatability is unknown (Hesbacher *et al*., 1995; Weller, Jacobson and Conner, 1999; Scott *et al*., 2014)	Nocturnal
Eilema depressa	Lichen polyphenolics present in some adults (Hesbacher *et al*., 1995; Weller, Jacobson and Conner, 1999)	Nocturnal
Eilema lurideola	Lichen compounds present in adults, possibly in larvae (Weller, Jacobson and Conner, 1999)	Nocturnal
Eilema sororcula	Lichen polyphenolics present in some adults (Hesbacher *et al*., 1995; Weller, Jacobson and Conner, 1999)	Nocturnal
Eulithis mellinata	Distasteful to birds and bats (Rothschild, 1985); the closely-related species *E. prunata* considered to have repellent defences, based on published literature on caterpillars (Tullberg and Hunter, 1996)	Nocturnal
Euproctis chrysorrhea	Irritating hairs, with esterases, proteases and other noxious compounds, causing dermatitis in humans, present mainly on the larvae but also protecting other life stages such as adults and eggs (Edmunds, 1974; Deml and Dettner, 1997; de Jong and Bleumink, 1977)	Nocturnal
Hydriomena furcata	There is evidence that this species can be eaten: it is found in the diet of great tits (Royama, 1970) and on one occasion in a food sample from a jay (Owen, 1956). However, it appears to be distasteful to birds and bats (Rothschild, 1985), and larvae of 4/8 *Hydriomena spp*. examined are considered to have repellent defences, based on published literature on caterpillars (Tullberg and Hunter, 1996)	Nocturnal
Miltochrista miniata	Lichen polyphenolics present in some adult specimens, although palatability is unknown, and there is no negative effect of extracts when injected into mice (Marsh and Rothschild, 1974; Hesbacher *et al*., 1995; Weller, Jacobson and Conner, 1999; Scott *et al*., 2014)	Nocturnal
Pelosia muscerda	Lichen polyphenolics present, though palatability is unknown (Hesbacher *et al*., 1995; Scott *et al*., 2014)	Nocturnal
Phalera bucephala	The larvae are considered to have both physical (hairs) and chemical defences (Tullberg and Hunter, 1996); adults can be eaten but there is evidence of distastefulness: partially eaten bodies at bat feeding sites, rejection by lizards (Poulton, 1929), great reluctance of birds to feed on them (Marsh and Rothschild, 1974). They also emit defensive secretions (Pocock, 1911) and adult and pupal tissues are toxic when injected into mice (Marsh and Rothschild, 1974).	Nocturnal

Appendix 3.2: Provenance of the specimens photographed in the comparative analysis of museum specimens.

Family	Subfamily	Species		Location of specimens
Erebidae	Arctiinae	Garden tiger	*Arctia caja* (Linnaeus, 1758)	Baikenridge and Griffiths collections, Bristol museum
		Wood tiger	*Arctia plantaginis* (Linnaeus, 1758)	Baikenridge collection, Bristol museum
		Cream-spot tiger	*Arctia villica* (Oberthür, 1911)	Baikenridge and Griffiths collections, Bristol museum
		Scarlet tiger	*Callimorpha dominula* (Linnaeus, 1758)	Baikenridge and Griffiths collections, Bristol museum
		Clouded buff	*Diacrisia sannio* (Linnaeus, 1758)	Baikenridge, Coney and Griffiths collections, Bristol museum
		Muslin moth	*Diaphora mendica* (Clerck, 1759)	Baikenridge and Coney collections, Bristol museum
		Jersey tiger	*Euplagia quadripunctaria* (Poda, 1761)	Coney, Griffiths and Hadley collections, Bristol museum; Natural History Museum, London
		Ruby tiger	*Phragmatobia fuliginosa* (Linnaeus, 1758)	Baikenridge collection, Bristol museum
		White ermine	*Spilosoma lubricipeda* (Linnaeus, 1758)	Griffiths collection, Bristol museum
		Buff ermine	*Spilosoma lutea* (Hufnagel, 1766)	Baikenridge collection, Bristol museum
		Cinnabar	*Tyria jacobaeae* (Linnaeus, 1758)	Baikenridge and Griffiths collections, Bristol museum
	Lithosiinae	Red-necked footman	*Atolmis rubricollis* (Linnaeus, 1758)	Baikenridge and Coney collections, Bristol museum
		Scarce footman	*Eilema complana* (Linnaeus, 1758)	Baikenridge and Coney collections, Bristol museum; Bignell collection, Plymouth museum
		Buff footman	*Eilema depressa* (Esper, 1787)	Baikenridge and Coney collections, Bristol museum
		Common footman	*Eilema lurideola* (Zincken, 1817)	Baikenridge collection, Bristol museum
		Orange footman	*Eilema sororcula* (Hufnagel, 1766)	Baikenridge and Coney collections, Bristol museum
		Rosy footman	*Miltochrista miniata* (Forster, 1771)	Baikenridge and Coney collections, Bristol museum
		Dotted footman	*Pelosia muscerda* (Hufnagel, 1766)	Baikenridge, Bartlett and Coney collections, Bristol museum
		Dew moth	*Setina irrorella* (Linnaeus, 1758)	Baikenridge collection, Bristol museum
	Lymantriinae	Brown-tail	*Euproctis chrysorrhoea* (Linnaeus, 1758)	Drawer Ma26065, Natural History Museum, London

Family	Subfamily	Species		Location of specimens
Drepanidae	Drepaninae	Pebble hook-tip	*Drepana falcataria* (Linnaeus, 1758)	Bignell and Moore collections, Plymouth museum
Geometridae	Ennominae	The Magpie	*Abraxas grossulariata* (Linnaeus, 1758)	Drawer Ma26353, Natural History Museum, London
		Mottled Beauty	*Alcis repandata (Linnaeus, 1758)*	Drawer Ma26456, Natural History Museum, London
		Peppered moth	*Biston betularia (Linnaeus, 1758)*	Drawer Ma26423, Natural History Museum, London
		Great oak beauty	*Hypomecis roboraria* (Denis & Schiffermüller, 1775)	Moore collection, Plymouth museum; Drawer Ma24657, Natural History Museum, London
	Larentiinae	The Spinach	*Eulithis mellinata* (Fabricius, 1787)	Drawer Ma26227, Natural History Museum, London
		July Highflyer	*Hydriomena furcata* (Thunberg, 1784)	Drawer Ma26252, Natural History Museum, London
Noctuidae	Acronictinae	Grey dagger	*Acronicta psi* (Linnaeus, 1758)	Bignell collection, Plymouth museum
	Amphipyrinae	Copper underwing	*Amphipyra pyramidea* (Linnaeus, 1758)	Bignell collection, Plymouth museum
		Mouse moth	*Amphipyra tragopoginis (Clerck, 1759)*	Drawer Ma25774, Natural History Museum, London
	Catocalinae	Red underwing	*Catocala nupta* (Linnaeus, 1767)	Bignell collection, Plymouth museum
	Hadeninae	Common quaker	*Orthosia cerasi* (Fabricius, 1775)	Drawer Ma25575, Natural History Museum, London
	Noctuinae	Dark sword-grass	*Agrotis ipsilon* (Hufnagel, 1766)	Moore collection, Plymouth museum
		Green arches	*Anaplectoides prasina*. (Denis & Schiffermüller, 1775)	Drawer Ma25452, Natural History Museum, London
		Large yellow underwing	*Noctua pronuba* (Linnaeus, 1758)	Bignell collection, Plymouth museum
		Setaceous Hebrew character	*Xestia c-nigrum* (Linnaeus, 1758)	Bignell collection, Plymouth museum
	Xyleninae	The Chestnut	*Conistra vaccinii (Linnaeus, 1761)*	Drawer Ma25723, Natural History Museum, London
		The Rustic	*Hoplodrina blanda* (Denis & Schiffermüller, 1775)	Drawer Ma27364, Natural History Museum, London
Notodontidae	Heterocampinae	Great prominent	*Peridea anceps* (Goeze, 1781)	Bignell and Moore collections, Plymouth museum; Drawer Ma26135, Natural History Museum, London

Family	Subfamily	Species		Location of specimens
	Phalerinae	Buff-tip	*Phalera bucephala* (Linnaeus, 1758)	Drawer Ma26132, Natural History Museum, London
Sphingidae	Smerinthinae	Eyed hawkmoth	*Smerinthus ocellata* (Linnaeus, 1758)	Bignell collection, Plymouth museum
		Poplar hawkmoth	*Laothoe populi* (Linnaeus, 1758)	Drawer Ma26088, Natural History Museum, London
		Pine hawkmoth	*Sphinx pinastri* (Linnaeus, 1758)	Bignell collection, Plymouth museum; Drawer Ma26079, Natural History Museum, London
Zygaenidae	Procridinae	Cistus forester	*Adscita geryon* (Hübner, 1813)	Coney collection, Bristol museum
		Forester	*Adscita statices* (Linnaeus, 1758)	Coney collection, Bristol museum
	Zygaeninae	Scotch burnet	*Zygaena exulans* (Hohenwarth, 1792)	Coney collection, Bristol museum
		Six-spot burnet	*Zygaena filipendulae* (Linnaeus, 1758)	Baikenridge collection, Bristol museum
		Narrow-bordered five-spot burnet	*Zygaena lonicerae* (Scheven, 1777)	Baikenridge, Coney and Davis collections, Bristol museum
		Slender scotch burnet	*Zygaena loti* (Denis & Schiffermüller, 1775)	Drawer Ma28387, Natural History Museum, London
		Transparent burnet	*Zygaena purpuralis* (Brünnich, 1763)	Baikenridge collection, Bristol museum
		Five-spot burnet	*Zygaena trifolii* (Esper, 1783)	Baikenridge collection, Bristol museum
		New Forest burnet	*Zygaena viciae* (Denis & Schiffermüller, 1775)	Coney collection, Bristol museum

Appendix 3.3: Validation of museum study with freshly-eclosed specimens of four species.

Methods

Provenance of specimens and photography

Comparisons between museum and fresh specimens were based on five individuals of the Scarlet tiger (*Callimorpha dominula*) and Cinnabar (*Tyria jacobaeae*), as well as five randomly-selected individuals of the Six-spot Burnet (*Zygaena filipendulae*) and Five-spot Burnet (*Zygaena trifolii*) used in subsequent work (see Chapters 4 and 5). Cinnabars and scarlet tigers were obtained as pupae, then frozen upon eclosion; Cinnabars were collected by Andrew Spicer in Bedford, UK and Scarlet tigers were obtained commercially from Worldwide Butterflies. Five and six-spot burnets were collected in Cornwall, UK (Bostraze Bog and Holywell Bay, respectively, see chapter 5 for details). The wings were photographed and analysed as described above. In the photographs of fresh specimens, two PTFE reflectance standards (reflecting 5 and 95% of all wavelengths) were used rather than a single one. Comparisons between museum and fresh specimens are based on the UVS (blue tit) visual system alone. Colour patches on the moth wings were selected to match those on the museum specimens of the same species.

Statistical analyses

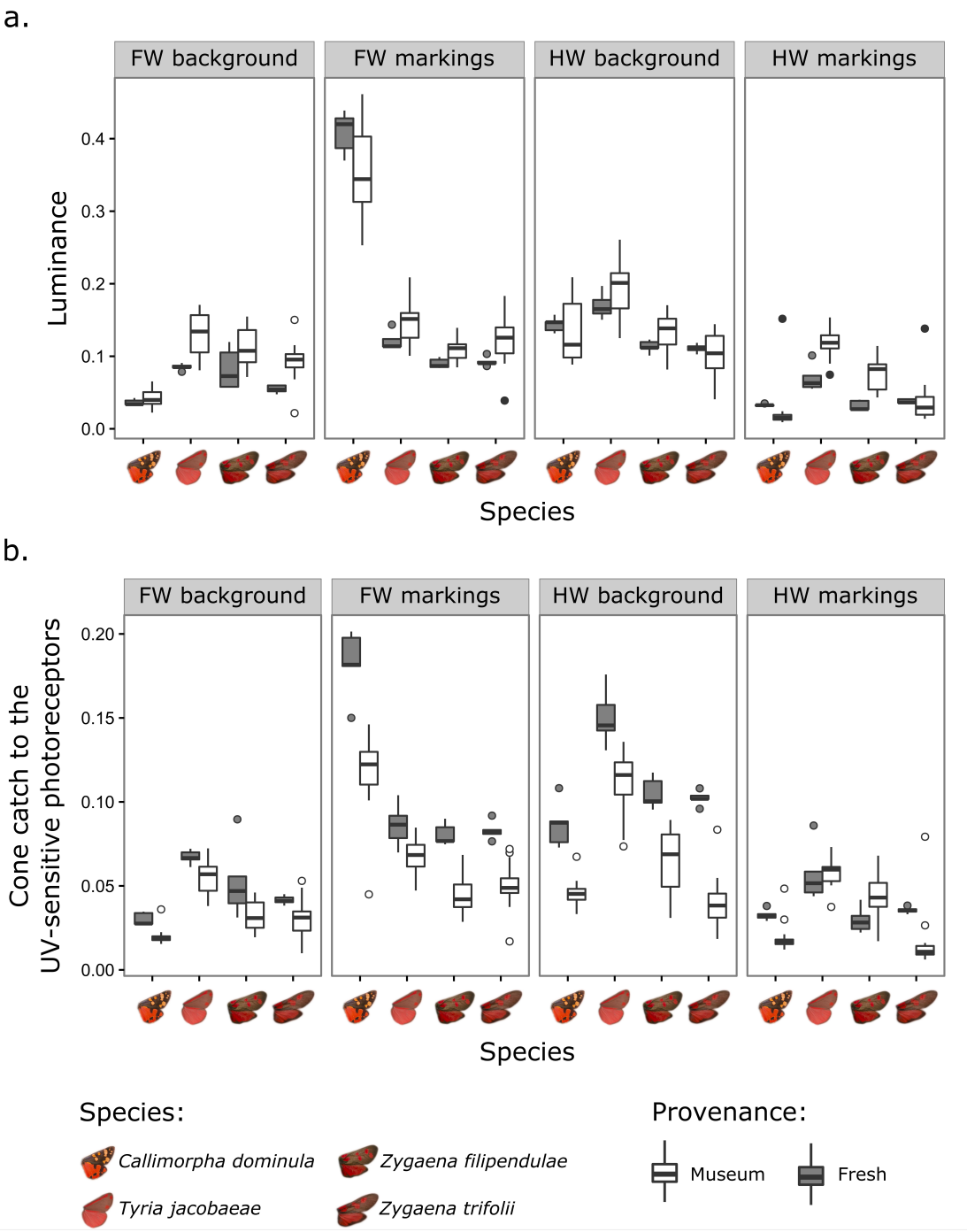
The colours of fresh and museum specimens of four species of moths were analysed using linear models, for each wing area separately, with provenance (museum or fresh) and species as fixed effects. The dependent variables tested were luminance and cone catch values for each photoreceptor type. The effect of the interaction between provenance and species was tested with a likelihood ratio test, to test whether the relative differences between the colours of each species were consistent between fresh and museum specimens. Colour metrics were log-transformed to fit the assumptions of linear models.

Results

The relative differences between species in terms of luminance and cone catch values for the ultraviolet-, short wavelength-, medium wavelength- and long wavelength-sensitive photoreceptors are generally not affected by the provenance of specimens (museum collections or freshly-frozen). With the exception of cone catch values for the medium-wavelength-sensitive photoreceptors, there is no effect of provenance on the relative differences between species' colours in the forewings (Supplementary table 3.3). However, the relative differences between the hindwing colours of these four test species do appear to be less stable between museum and fresh specimens (Supplementary table 3.3), suggesting that some caution may be required when interpreting the results of analyses based on the hindwing colours of museum specimens. Supplementary figure 3.3 shows two metrics particularly expected to be affected by the storage of museum specimens, luminance and cone catch values for the UV-sensitive photoreceptors, for all wing areas.

Supplementary table 3.3: Significance of the provenance:species interaction in analysis of museum and fresh specimens, for the UVS (blue tit) visual system. Significant interactions, highlighted in italics indicate a difference in the relative values of each species depending on their provenance, from museum collections or freshly-frozen.

Colour metric	Wing area	F	df	p
Luminance	Forewing background	1.056	3,72	0.373
	Forewing markings	2.563	3,72	0.0614
	Hindwing background	1.263	3,72	0.294
	Hindwing markings	8.640	3,72	*<0.001*
UV	Forewing background	0.532	3,72	0.662
	Forewing markings	1.614	3,72	0.194
	Hindwing background	4.796	3,72	*0.00421*
	Hindwing markings	11.18	3,72	*<0.001*
SW	Forewing background	0.919	3,72	0.436
	Forewing markings	0.835	3,72	0.479
	Hindwing background	1.863	3,72	0.144
	Hindwing markings	9.877	3,72	*<0.001*
MW	Forewing background	0.900	3,72	0.446
	Forewing markings	4.563	3,72	*0.00554*
	Hindwing background	0.577	3,72	0.632
	Hindwing markings	10.05	3,72	*<0.001*
LW	Forewing background	1.192	3,72	0.319
	Forewing markings	2.21	3,72	0.0943
	Hindwing background	2.629	3,72	0.0567
	Hindwing markings	7.422	3,72	*<0.001*



Supplementary figure 3.3: Luminance (a) and cone catch values for the UV-sensitive photoreceptors (b) for fresh and museum specimens of four species. FW = forewing, HW = hindwing. Museum specimens appear generally lighter and with lower UV cone catch values. However, the relative differences between species appear consistent whether the specimens measured are fresh or from a museum collection, with the exception of the hindwing marking areas (see Supplementary table 3.3). Boxplots show the median and interquartile range.

Discussion

In general, the relative differences between species are fairly consistent between fresh and collection specimens, However, there were some differences in the cone catch values to each photoreceptor type when the hindwing markings were considered, affecting the relative values of the different species when fresh or preserved in museums. This may suggest that more caution would be needed when interpreting results concerning hindwing marking colours, but is likely to be a result of the hindwing patches selected in these particular species. In all four species, the hindwing markings were very dark, and for three of these (*Z. filipendulae*, *Z. trifolii* and *Tyria jacobaeae*), were selected from very small border areas. Moreover, individuals will also experience fading with age in nature, so will not all have colours as vivid as freshly-eclosed specimens. As such, comparisons between moth wings based on this museum sample can be interpreted with relative confidence.

Appendix 3.4: Details of regions selected for colour analysis in the comparative analysis of museum specimens.

Species	Forewing background	Forewing marking	Hindwing background	Hindwing markings
Abraxas grossulariata	5 circular areas of yellow colour	5 circular patches from black markings	5 circular areas of white background colour	5 circular patches from black markings
Acronicta psi	5 circular areas of grey background colour, avoiding distinctive markings	4 largest black markings	5 circular patches of wing, avoiding wing veins	NA
Adscita geryon	uniform green forewing area	dark forewing border	uniform green hindwing area	dark hindwing border
Adscita statices	uniform green forewing area	dark forewing border	uniform green hindwing area	dark hindwing border
Agrotis ipsilon	5 circular areas of brown background colour, avoiding distinctive markings and wing veins	single dark brown triangular marking	5 circular patches of wing, avoiding wing veins	NA
Alcis repandata	5 circular areas of brown background colour, avoiding distinctive markings	5 small dark markings	5 circular areas of brown background colour, avoiding distinctive markings	5 small dots from dark edge line
Amphipyra pyramidea	5 circular areas of brown background colour, avoiding distinctive markings	5 small white patches	large orange hindwing area	brown area of hindwing
Amphipyra tragopoginis	5 circular areas of brown background colour	3 dark markings	uniform large wing area	2 patches in hindwing border
Anaplectoides prasina	5 circular areas of green background	5 small areas from dark markings	uniform large wing area	2 patches in hindwing border
Arctia caja	5 circular areas of cream background colour	5 dark brown patches	5 circular areas of orange background colour	5 dark blue patches, excluding the black rim
Arctia plantaginis	5 circular areas of cream background colour	5 black patches	5 circular areas of yellow background colour	5 black patches
Arctia villica	5 circular areas of black background colour	5 cream patches	5 circular areas of orange background colour	5 black patches
Atolmis rubricollis	uniform brown forewing	NA	uniform brown hindwing	NA
Biston betularia - melanic	5 circular areas of dark background colour	5 small white markings	5 circular areas of dark background colour	5 small white markings

Species	Forewing background	Forewing marking	Hindwing background	Hindwing markings
Biston betularia – pale morph	5 circular areas of pale background colour	5 small dark markings	5 circular areas of pale background colour	5 small dark markings
Callimorpha dominula	5 circular areas of black background colour	5 cream patches	5 circular areas of orange background colour	5 black patches
Catocala nupta	5 circular areas of grey background colour	3 dark patches	2 areas of orange areas	2 areas of brown areas
Conistra vaccinii	5 circular areas of brown background colour, avoiding dark areas	single dark marking	large grey wing area	2 patches in pale hindwing border
Diacrisia sannio female	5 circular areas of russet background colour	single large dark brown marking	5 circular areas of russet background colour	5 dark brown patches
Diacrisia sannio male	5 circular areas of yellow background colour	single large reddish marking	5 circular areas of cream background colour	5 brown patches
Diaphora mendica female	5 circular areas of white background colour	up to 5 black markings	5 circular areas of white background colour	up to 5 black markings
Diaphora mendica male	5 circular areas of brown background colour	up to 5 black markings	5 circular areas of brown background colour	up to 5 black markings
Drepana falcataria	5 circular areas of cream background colour	single large black marking	5 circular areas of cream background colour	5 small patches from dark lines
Eilema complana	large greyish area in mainly uniform wing	yellow stripe on upper edge of wing	uniform cream hindwing	NA
Eilema depressa	large greyish area in mainly uniform wing	yellow stripe on upper edge of wing	uniform grey hindwing area	yellow hindwing border
Eilema lurideola	large greyish area in mainly uniform wing	yellow stripe on upper edge of wing	uniform cream hindwing	NA
Eilema sororcula	uniform orange forewing	NA	uniform orange hindwing	NA
Eulithis mellinata	5 circular areas of pale yellow background colour	5 small patches from dark lines	large uniform wing area	2 small patches from yellow line at wing edge
Euplagia quadripunctaria	5 circular areas of cream background colour	5 black patches	5 circular areas of orange background colour	up to 5 black patches
Euproctis chrysorrhoea	large white wing area	NA	large white wing area	NA

Species	Forewing background	Forewing marking	Hindwing background	Hindwing markings
Hoplodrina blanda	5 circular areas of brown background colour, avoiding dark markings	single dark marking	large uniform wing area	2 patches in hindwing border
Hydriomena furcata	5 circular areas of pale background colour	5 dark markings	large uniform wing area	2 patches from darker line at wing edge
Hypomecis roboraria	5 circular areas of grey background colour, avoiding distinctive markings	5 largest dark brown markings	5 circular areas of grey background colour	5 dark markings
Laothoe populi	5 circular areas of cream background colour	5 circular areas from darker markings	5 circular areas of cream background colour	single russet marking
Miltochrista miniata	5 circular areas of dark pink/orange background colour, at upper edge of wing	1 patch from each of 4 dark vertical waves and lines of markings	uniform cream hindwing	NA
Noctua pronuba	5 circular areas of brown background colour, avoiding distinctive markings	single large black marking	large yellow area	dark stripe
Orthosia cerasi	5 circular areas of pale brown background colour, avoiding markings	2 central patches from dark markings	large wing area	2 patches from hindwing border
Pelosia muscerda	5 circular areas of brown background colour	5 small black markings	uniform brown hindwing	NA
Peridea anceps	5 circular areas of grey background colour, avoiding distinctive markings	5 small brown patches	large white area	single dark patch
Phalera bucephala	5 circular areas of brown background	single yellow marking	5 circles of pale background colour	single darker grey marking
Phragmatobia fuliginosa	5 circular areas of brown background colour	2 dark brown spots	large red area	thick black hindwing border
Setina irrorella	5 circular areas of yellowish background colour	5 small black markings	large cream area in mainly uniform hindwing	up to 5 small black markings
Smerinthus ocellata	5 circular areas of grey background colour, avoiding distinctive markings	5 dark brown patches	red area	blue eyespot, without black ring

Species	Forewing background	Forewing marking	Hindwing background	Hindwing background
Sphinx pinastri	5 circular areas of grey background colour	3 darkest markings	large brown wing area	5 patches of white hindwing fringe
Spilosoma lubricipeda	5 circular areas of white background colour	5 largest black markings	5 circular areas of white background colour	up to 5 black markings
Spilosoma lutea	5 circular areas of cream background colour	5 largest black markings	5 circular areas of cream background colour	up to 5 black markings
Tyria jacobaeae	5 circular areas of black background colour	4 red patches	uniform large red area	black hindwing border
Xestia c-nigrum	5 circular areas of brown background colour, avoiding distinctive markings	4 dark brown patches	large cream wing area	darker wing sector
Zygaena exulans	5 circular areas of black background colour, avoiding wing veins	5 red patches	5 circular patches of red background colour, avoiding wing veins	5 circular patches from black hindwing border, avoiding wing veins
Zygaena filipendulae	5 circular areas of black background colour	5 red patches	large red area	2 patches in hindwing border
Zygaena lonicerae	5 circular areas of black background colour	5 red patches	large red area	2 patches in hindwing border
Zygaena loti	5 circular areas of black background colour	5 red patches	large red area	2 patches in hindwing border
Zygaena purpuralis	5 circular areas of black background colour, avoiding wing veins	3 red patches	large red area	2 patches in hindwing border
Zygaena trifolii	5 circular areas of black background colour	5 red patches	large red area	2 patches in hindwing border
Zygaena viciae	5 circular areas of black background colour, avoiding wing veins	5 red patches	large red area	2 patches in hindwing border

Appendix 3.5: Equations for Hue2, based on museum specimens of Lepidoptera.

$$Hue2_{UVS}(FW\ background) = Hue2_{VS}(FW\ background) = \frac{UV+LW}{SW+MW} \quad \text{(S3.5.1)}$$

$$Hue2_{UVS}(FW\ markings) = \frac{MW+LW}{UV+SW} \quad \text{(S3.5.2)}$$

$$Hue2_{VS}(FW\ markings) = \frac{SW+MW+LW/3}{UV} \quad \text{(S3.5.3)}$$

$$Hue2_{UVS}(HW\ background) = Hue2_{VS}(HW\ background) = \frac{MW+LW}{UV+SW} \quad \text{(S3.5.4)}$$

$$Hue2_{UVS}(HW\ markings) = \frac{UV+LW}{SW+MW} \quad \text{(S3.5.5)}$$

$$Hue2_{VS}(HW\ markings) = \frac{SW+MW+LW/3}{UV} \quad \text{(S3.5.6)}$$

UV, SW, MW, LW = standardised cone catch values for the UV-, SW-, MW- and LW- sensitive photoreceptors respectively. UVS = ultraviolet-sensitive (blue tit) visual system, VS = violet-sensitive (peafowl) visual system. FW = Forewings, HW = Hindwings.

Appendix 3.6: Host plants photographed for each species of moth in the comparative analysis of museum specimens.

Moth species	Chosen hostplants
Abraxas grossulariata	Gooseberry, *Ribes uva-crispa*
Acronicta psi	Alder, *Alnus glutinosa*; Birch, *Betula pendula*; Oak, *Quercus robur*; Willow, *Salix sp.*
Adscita geryon	Rockrose, *Helianthemum nummularium*
Adscita statices	Sorrel, *Rumex acetosa*
Agrotis ipsilon	Hawthorn, *Crataegus sp.*; Red clover, *Trifolium pratense*; White clover, *Trifolium repens*
Alcis repandata	Birch, *Betula pendula*; Gooseberry, *Ribes uva-crispa*; Heather, *Calluna vulgaris*; Meadowsweet, *Filipendula ulmaria*; Willow, *Salix sp.*
Amphipyra pyramidea	Honeysuckle, *Lonicera sp.*; Oak, *Quercus robur*
Amphipyra tragopoginis	Willow, *Salix sp.*
Anaplectoides prasina	Honeysuckle, *Lonicera sp.*
Arctia caja	Apple, *Malus sp.*; Birch, *Betula pendula*; Dandelion, *Taraxacum officinale*; Gooseberry, *Ribes uva-crispa*; Meadowsweet, *Filipendula ulmaria*; Ribwort plantain, *Plantago lanceolata*; Red clover, *Trifolium pratense*; Sorrel, *Rumex acetosa*; White clover, *Trifolium repens*
Arctia plantaginis	Ribwort plantain, *Plantago lanceolata*; Sorrel, *Rumex acetosa*
Arctia villica	Forget-me-not, *Myosotis sp.*; Gorse, *Ulex europaeus*; Ribwort plantain, *Plantago lanceolata*; Sorrel, *Rumex acetosa*
Atolmis rubricollis	Lichens*
Biston betularia	Alder, *Alnus glutinosa*; Birch, *Betula pendula*
Callimorpha dominula	Comfrey, *Symphytum officinale*; Oak, *Quercus robur*
Catocala nupta	Willow, *Salix sp.*
Conistra vaccinii	Blackthorn, *Prunus spinosa*; Oak, *Quercus robur*; Willow, *Salix sp.*
Diacrisia sannio	Heather, *Calluna vulgaris*
Diaphora mendica	Ribwort plantain, *Plantago lanceolata*
Drepana falcataria	Alder, *Alnus glutinosa*
Eilema complana	Lichens*
Eilema depressa	Lichens*
Eilema lurideola	Lichens*
Eilema sororcula	Lichens*
Eulithis mellinata	Gooseberry, *Ribes uva-crispa*
Euplagia quadripunctaria	Hemp agrimony, *Eupatorium cannabinum*; Nettle, *Urtica dioica*; Ribwort plantain, *Plantago lanceolata*
Euproctis chrysorrhoea	Apple, *Malus sp.*; Blackthorn, *Prunus spinosa*; Hawthorn, *Crataegus sp.*
Hoplodrina blanda	Ribwort plantain, *Plantago lanceolata*; Sorrel, *Rumex acetosa*
Hydriomena furcata	Heather, *Calluna vulgaris*
Hypomecis roboraria	Oak, *Quercus robur*
Laothoe populi	Willow, *Salix sp.*
Miltochrista miniata	Dog Lichen, *Peltigera canina* (only 1 specimen)
Noctua pronuba	Dandelion, *Taraxacum officinale*
Orthosia cerasi	Oak, *Quercus robur*; Willow, *Salix sp.*
Pelosia muscerda	Lichens*
Peridea anceps	Oak, *Quercus robur*
Phalera bucephala	Oak, *Quercus robur*
Phragmatobia fuliginosa	Dandelion, *Taraxacum officinale*
Setina irrorella	Lichens*
Smerinthus ocellata	Apple, *Malus sp.*; Willow, *Salix sp.*
Sphinx pinastri	Scots pine, *Pinus sylvestris*

Moth species	Chosen hostplants
Spilosoma lubricipeda	Oak, *Quercus robur*; Ribwort plantain, *Plantago lanceolata*; Red clover, *Trifolium pratense*; Sorrel, *Rumex acetosa*; White clover, *Trifolium repens*
Spilosoma lutea	Oak, *Quercus robur*; Ribwort plantain, *Plantago lanceolata*
Tyria jacobaeae	Ragwort, *Senecio jacobaea*
Xestia c-nigrum	Nettle, *Urtica dioica*
Zygaena exulans	Heather, *Calluna vulgaris*
Zygaena filipendulae	Bird's foot trefoil, *Lotus corniculatus*; Greater bird's foot trefoil, *Lotus pedunculatus*
Zygaena lonicerae	Bird's foot trefoil, *Lotus corniculatus*; Greater bird's foot trefoil, *Lotus pedunculatus*; Meadow Vetchling, *Lathyrus pratensis*; Red clover, *Trifolium pratense*; White clover, *Trifolium repens*
Zygaena loti	Bird's foot trefoil, *Lotus corniculatus*
Zygaena purpuralis	Mother-of-thyme, *Thymus praecox*
Zygaena trifolii	Bird's foot trefoil, *Lotus corniculatus*; Greater bird's foot trefoil, *Lotus pedunculatus*
Zygaena viciae	Bird's foot trefoil, *Lotus corniculatus*; Meadow vetchling, *Lathyrus pratensis*

Appendix 3.7: Sequences used for phylogenetic reconstruction in the comparative analysis of museum specimens.

Species	Sequence accession numbers
Abraxas grossulariata	CGUKB462-09;CGUKB619-09;CGUKC095-09;CGUKC176-09;CGUKD557-09;FBLMX151-11;GBGL10423-12;GBLAA428-14;GBMIN33722-13;GWOAL003-08;GWOSI561-10;LEFIC261-10;LEFIC262-10;LEFIJ163-10;LEFIJ164-10;LEFIJ166-10;LENOA1047-11
Acronicta psi	FBLMT860-09;FBLMU113-09;FBLMV189-09;FBLMV190-09;GBLAB685-13;GBLAC543-13;GBLAC568-13;GBLAC683-13;GBLAF312-14;GWORL337-09;GWOSZ261-11;LEATA113-13;LEATA194-13;LEATA303-13;LEATD057-13;LEFIA461-10;LEFIA472-10;LEFIC344-10;LEFIC610-10;LEFIF661-10;LEFIL176-10;LENOA436-11;PHLAA464-09;PHLAE350-11;PHLAF338-11;CGUKA031-09;CGUKA123-09;CGUKA290-09;CGUKA432-09;CGUKA496-09;CGUKA732-09;CGUKA782-09;CGUKB130-09;CGUKB699-09;CGUKC107-09;CGUKD385-09;DGH070-15;GBGL18051-15;GBLAA906-14;GBLAA907-14;LEATH689-14;NLLEA437-12
Adscita geryon	FBLMX101-11;FBLMX103-11;LENOA1386-11;LENOA1387-11;LENOA1388-11;LENOA1389-11;LENOA1390-11;LENOA1391-11;PHLAH704-12;PHLSA437-11;ZYGMO086-09;ZYGMO087-09;ZYGMO088-09;ZYGMO089-09;ZYGMO090-09;ZYGMO091-09;ZYGMO249-10;ZYGMO250-10;ZYGMO251-10;ZYGMO252-10;ZYGMO253-10;ZYGMO254-10;ZYGMO255-10;ZYGMO256-10;ZYGMO257-10;ABOLA484-14;ABOLB247-15;LEATD139-13;LEATH469-14;LEATH572-14;ZYGMO1000-14;ZYGMO258-10;ZYGMO259-10;ZYGMO260-10;ZYGMO261-10;ZYGMO262-10;ZYGMO263-10;ZYGMO264-10;ZYGMO265-10;ZYGMO266-10;ZYGMO267-10;ZYGMO268-10;ZYGMO269-10;ZYGMO300-10;ZYGMO448-10;ZYGMO449-10;ZYGMO450-10;ZYGMO451-10;ZYGMO994-14;ZYGMO999-14;LEATJ240-15
Adscita statices	FBLMV677-09;FBLMX102-11;FBLMX104-11;GWORA2519-09;LEATA406-13;LEATH573-14;LEATJ271-15;LEFIB041-10;LEFID566-10;LEFID567-10;LEFIJ739-10;LEFIL320-10;LENOA1435-11;LENOA1436-11;LENOA1437-11;LON825-11;ZYGMO241-10;ZYGMO242-10;ZYGMO243-10;ZYGMO244-10;ZYGMO245-10;ZYGMO246-10;ZYGMO443-10;ZYGMO444-10;ZYGMO446-10;LEFIA967-10
Agrotis ipsilon	ANICL135-10;ANICL136-10;ANICL137-10;ANICL138-10;BBLEC543-09;BBLOD1661-11;BBLOD900-11;BBLOD955-11;BBLOD967-11;BBLOD968-11;BBLPC033-09;BBLPC508-09;BBLSY416-09;BCMI277-11;BCMI359-11;CMAZA226-09;CMAZA900-12;CMAZA909-12;FBLMU935-09;FBLMX167-11;GMLC1298-12;GMLC1300-12;GMLC597-11;GMLC968-12;GWORL296-09;GWORZ500-10;GWOSZ254-11;GWOTF340-12;JGLL060-10;JGLL061-10;JGLL062-10;LALPA175-10;LEFIC502-10;LEFIJ587-10;LEFIK425-10;LENOA064-11;LGSMG881-10;LILLA632-11;LILLA833-11;LOQTI451-11;LPOKA934-09;LPOKB1002-09;LPOKB323-09;LPOKB329-09;LPOKC617-09;MAIMB145-09;MAIMB147-09;MAMOT1454-12;MAMOT1455-12;MAMOT1729-12;MAMOT1460-12;MAMOT1461-12;MAMOT1464-12;MAMOT1465-12;MAMOT1470-12;MAMOT1498-12;MAMOT1559-12;MAMOT1560-12;MAMOT1561-12;MAMOT1562-12;MAMOT1563-12;MAMOT1565-12;MAMOT2231-12;MAMOT2239-12;MAMOT2241-12;MAMOT2243-12;MAMOT2245-12;MAMOT2252-12;MAMOT2284-12;MAMOT2365-12;MAMOT2425-12;MAMOT451-10;MAMOT452-10;MAMOT455-10;MAMOT472-10;GBLAB337-13;GBLAB613-13;LEATA234-13;LEATA314-13;LEATB707-13;LEATC655-13;LEATD370-13;MAMOT3674-13;MAMOT3726-13;NSWLP134-13;NSWLP223-13;PHLAF435-11;PHLAV014-12;PHLCC475-11;PHLCC476-11;PHLCC483-11;PHLCH800-11;PHLPM058-11;RWWC227-11;RWWC926-12;SMTPB17386-13;SMTPD1181-13;WALPA3546-13;WALPA3717-13;WALPA4514-13;BLTIB545-08;BLTIB830-08;CGUKA044-09;CGUKB107-09;GBLAA931-14;GBLAC320-13;GBLAC347-13;GBLAC868-13;GBLAD183-14;LOCBF4664-15;MBIOJ021-13;PMANL4500-15;PMANL4501-15;RWWC1460-14;SMTPD2879-13;SMTPD5182-13;SMTPF1886-14;SMTPF4899-14;SMTPI5898-14;SMTPJ3152-14;SMTPJ5448-14;WALPA3537-13;WALPA3544-13;WALPB3858-14;WALPB3859-14;CGUKB765-09;CGUKB906-09;CGUKC203-09;IMLQ715-08;LNC572-06;LNC573-06;LNCNW114-06;LOCB742-06;LOCB744-06;LOCB822-06;LOCBB235-06;LOCBB236-06;LOCBB237-06;LOCBB238-06;LOCBC158-06;LOCBC169-06;LOCBE106-06;LOCT058-05;LOCT104-05;LOQB201-05;LOTSE059-08;LPOKA042-08;LPOKA191-08;LPOKA521-09;LPOKA525-09;LPOKA629-09;LPOKA670-09;LPOKA676-09;LPSO022-08;LPSOD1045-09;LTOL929-08;MHCOL007-07;MHCOL107-07;MHCOL113-07;MHCOL114-07;MHCOL156-07;MHCOL167-07;MHCOL284-07;MHCOL360-07;MHCOL366-07;MHCOL368-07;MHLEP015-07;MHLEP078-07;MHLEP085-07;MHLEP093-07;RDLQF818-06;RDNMD382-06;TTMNB420-06;TTMNB421-06;XAJ813-06;LBCS028-07;LBCS184-07;LBCS250-07;LGSM492-04;LGSMC653-05;LOTB235-05;LOTB310-05;LOTB312-05;MNBB179-

Species	Sequence accession numbers
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Alcis repandata	GBLAB193-13;GWORA2612-09;GWORL316-09;GWORL353-09;GWOSH464-10;GWOSK918-11;GWOSN701-11;GWOSN707-11;GWOSN708-11;GWOSN724-11;GWOSN737-11;GWOSN926-11;GWOSN927-11;GWOSZ185-11;LEATC217-13;LEATC270-13;LEFIA452-10;LEFIA453-10;LEFIB843-10;LENOA1127-11;LON840-11;PHLAC369-10;PHLAF282-11;PHLAG885-12;PHLAV330-12;CGUKA501-09;CGUKB054-09;CGUKB507-09;CGUKD225-09;CGUKD329-09;GBLAA1395-15;GBLAA2044-15;GBLAC072-13;GBLAC073-13;GBLAC265-13;GBLAC265-13;GBLAC893-13;GBLAD312-14;GBLAD441-14;GBLAD441-14;GBLAD746-14;GBLAD747-14;GBLAD852-14;GBLAF453-14;GWOR3768-09;GWORB783-07;GWORC752-07;GWORD1632-08;GWORD1633-08;GWORD798-08;GWORE2220-09;LEATD189-13;GBGL7176-10;GBLAA2383-15;GBLAA2384-15;GBLGC102-12;GBLGC113-12;GBLGC114-12;GBLGC115-12;GWOR3779-09;GWOR3780-09;GWOR3781-09;GWOR4056-09;GWOR4077-09;GWOR4080-09;GWOR4095-09;GWORB782-07;GWORC070-07;GWORE2219-09
Amphipyra pyramidea	ABOLA875-15;FBLMV103-09;FBLMV304-09;FBLMW128-10;GBLAA193-14;GBLAC205-13;GBLAC322-13;GBLAC350-13;GBLAC597-13;GBLAC598-13;GBLAD188-14;GBLAD507-14;GWORA2534-09;GWOTL001-13;LEATC178-13;LEATD100-13;LEATI067-15;LEFIC744-10;LEFIG397-10;LEFII105-10;LENOA416-11;LENOA417-11;PHLAA253-09;PHLAC575-10;CGUKA166-09;CGUKA175-09;CGUKA762-09;CGUKA828-09;CGUKA858-09;CGUKA976-09;CGUKA977-09;CGUKA978-09;CGUKA979-09;CGUKA980-09;CGUKB954-09;CGUKC115-09;CGUKD429-09;GBGL14135-14;GBMIN22916-13;GWORK485-09;GWORK486-09;GWOTS257-17;LON602-08;NLLEA377-12
Amphipyra tragopoginis	GBLAB720-13;GWORL395-09;GWORR896-10;GWORZ527-10;GWORZ528-10;GWORZ528-10;GWOSA244-10;IBLAO442-12;LALPA1279-11;LALPA600-10;LALPA759-10;LALPA760-10;LALPA770-10;LALPA802-10;LBCH1503-10;LBCH7951-10;LEFIA616-10;LEFIA617-10;LEFIC716-10;LENOA420-11;LENOA421-11;LNCC1175-11;LNCC1177-11;LNCC1178-11;PHLAH731-12;PHLSA584-11;ABOLA729-14;CGUKA984-09;CGUKA985-09;CGUKB283-09;CGUKC104-09;CGUKC186-09;CGUKC452-09;CGUKD468-09;CNCLB2631-14;GBLAA1134-15;GBLAA1309-15;GBLAA1338-15;GBLAB308-13;GBLAF534-14;LALPA1345-12;LBCW059-08;LEATB734-13;LEATD122-13;LEATD405-13;LEATD555-13;LNAUU943-15;LOWCB916-05;NGAAB055-14;NGAAB056-14;RRSSC7290-15;CGUKA509-09;GWOSN915-11;LBCG630-09;LOWCB917-05;LOWCB918-05;LOWCB919-05;LPVIB231-08;LPVIB508-08;NORIN072-13;PHMO276-03;PHMO343-03;RDLQG219-06;TTNFS067-09;XAD156-04
Anaplectoides prasina	BBLEC272-09;BBLEC422-09;BBLEC790-09;BBLPB661-10;BBLPB662-10;BBLPC026-09;BBLPC099-09;BBLPC113-09;BBLPC598-09;BBLPC620-09;GWORA2541-09;GWORL340-09;GWORO839-09;GWOSZ266-11;LALPA720-10;LALPA764-10;LALPA801-10;LALPA803-10;LALPA804-10;LBCH013-10;LBCH110-10;LBCH1426-10;LBCH1563-10;LBCH224-10;LBCH2242-10;LBCH3143-10;LBCH3144-10;LBCH3328-10;LBCH3333-10;LBCH345-10;LBCH3465-10;LBCH3766-10;LBCH4111-10;LBCH4415-10;LBCH4416-10;LBCH4417-10;LBCH4418-10;LBCH4419-10;LBCH4420-10;LBCH4421-10;LBCH4422-10;LBCH4671-10;LBCH4672-10;LBCH4673-10;LBCH4674-10;LBCH4675-10;LBCH4676-10;LBCH4677-10;LBCH4678-10;LBCH490-10;ALLEP398-13;CNWBG3125-13;LBCH6524-10;LBCH803-10;LBCH933-10;LBCH934-10;LEATB574-13;LEATC192-13;LEATD236-13;LEFIC855-10;LEFIF641-10;LENOA202-11;LENOA203-11;LEPPK007-13;LEPPK023-13;LEPPK027-13;LEPPK046-13;LEPPK047-13;LEPPK075-13;LGSMG845-10;LGSMG915-10;LGSMG916-10;LGSMG917-10;PHLAC367-10;PHLAV317-12;CGUKA344-09;CGUKB110-09;CGUKB256-09;CGUKD319-09;CNFNT127-14;CNPEP065-14;CNRVE039-15;CONTA1185-14;GBLAA207-14;GBLAD169-14;GMOCL278-15;LBCB613-05;LBCB614-05;LBCC014-05;LBCC326-05;PREXP235-14;RWWC1473-14;SSFDB2985-14;SSFDB3012-14;SSFDB3013-14;SSFDB3450-14;SSGLB2136-15;SSJAC2076-13;UAMIC3045-15;UAMIC3116-15;LBCC751-05;LBCC752-05;LBCC829-05;LBCD092-05;LBCD093-05;LBCD094-05;LBCD330-05;LBCG007-08;LBCG2315-09;LBCW054-08;LHLEP402-06;LHLEP403-06;LHLEP404-06;LOWC847-05;LOWC848-05;LOWC849-05;LOWC850-05;LOWC851-05;LOWC852-05;LOWC853-05;LOWC854-05;LOWC855-05;LOWCD583-06;LOWCD584-06;LOWCD585-06;LBCB888-05;LBCG818-09;LBCS367-07;LOWCD586-06;LOWCD587-06;LOWCD588-06;LOWCD589-06;LOWCD590-06;LOWCD591-06;LOWCD592-06;LPABC023-09;LPABC278-09;LPABC339-09;LPGVA599-08;LPMNB419-

Species	Sequence accession numbers
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Arctia caja	ALLEP233-13;FBLMU561-09;FBLMZ510-12;FGMLB659-13;GBLAA359-14;GBLAD206-14;GWORA2556-09;GWORZ115-10;GWOTL161-13;IBLAO551-12;IBLAO552-12;LEATB711-13;LEEUA664-11;LEFIA864-10;LEFIC785-10;LEFID959-10;LNCC229-10;ODOPE713-11;PHLSA661-11;RWWA728-09;RWWA776-09;RWWA832-09;RWWB952-10;RWWC467-11;SSWLB2311-13;CGUKB237-09;CGUKC151-09;CGUKC432-09;CNTGF001-15;GBLAF517-14;LEATG181-14;LEATG183-14;LEATH576-14;LEFIA1001-10;RDLQB571-05;RDLQB836-05;RDMAB386-05;RDMAB387-05;RDMAB388-05;RDNMB385-05;RDNMB386-05;RDNMB387-05;RDNMB388-05;RDNME157-07;TMNBD404-07;XAG404-05;XAG579-05;XAG615-05;XAG617-05;XAK250-06;GBGL14204-14;LON562-08;NLLEA802-12;NLLEA807-12;NLLEA809-12
Arctia plantaginis	ABKWR046-07;ABOLB364-15;ABOLB365-15;BBLPA385-10;BBLPA386-10;BBLPA387-10;GWORA2565-09;GWORA2566-09;GWOSD396-10;IBLAO856-12;IBLAO857-12;LEATH574-14;LEATH575-14;LEFIB998-10;LEFIE849-10;LEFIE870-10;LOWCE081-06;LOWCE082-06;LOWCE083-06;LPSK104-08;LPSK105-08;ODOPE710-11;PHLAA416-09;PHLAB1208-10;PHLAW048-13;GBGL12952-14;GBGL13707-14 ;GBGL13708-14;GBGL13709-14;GBGL13711-14;GBGL13712-14;GBGL13713-14;GBGL13734-14;GBGL13735-14;GBGL13742-14;GBGL13743-14;GBGL13744-14;GBGL13745-14;GWORK565-09;LON485-08;LPSK106-08;RDMAB736-06;RDNME158-07;RDNME163-07;RDNME164-07;RDNME165-07
Arctia villica	CGUKD205-09;CGUKD277-09;FBLMZ558-12;GWORZ116-10;GWOSI563-10;LEATC649-13;PHLAC475-10;VNMB683-08;VNMB684-08;VNMB685-08;VNMB686-08;VNMB687-08;VNMB688-08
Atolmis rubricollis	GBLAA1236-15;GBLAA888-14;GBLAC579-13;GBLAD933-14;GBLAF412-14;GBLGC275-12;GMGRF5574-13;GWOR4226-09;GWORA2562-09;GWORK365-09;GWORK366-09;GWOSZ223-11;GWOTL141-13;GWOTL142-13;GWOTL143-13;GWOTL185-13;GWOTL186-13;LEATD201-13;LEATD275-13;LEATD324-13;LEFIA095-10;LEFIA096-10;LEFIC620-10;PHLAE180-11;PHLAV032-12
Biston betularia	BBLEC403-09;BBLEC406-09;BBLPB300-10;BBLPB301-10;BBLPB302-10;BBLPB303-10;BBLPB304-10;BBLPC968-09;BBLPE237-09;CNGIF035-12;CNPAF970-13;FBLMV459-09;FBLMZ502-12;GMNCG021-12;GWORE2209-09;GWOSI024-10;GWOSZ160-11;LALPA1150-11;LALPA1157-11;LBCH056-10;LBCH162-10;LBCH277-10;LBCH3009-10;LBCH553-10;LBCH708-10;CNPAN001-13;GBLAB091-13;GBLAB092-13;GWOTL1115-13;LBCH864-10;LEFIA157-10;LEFIA158-10;LEFIA204-10;LEFIB836-10;LENOA1076-11;LENOA1077-11;MAMOT180-10;MAMOT2149-12;MAMOT224-10;MAMOT2608-12;MAMOT2611-12;MAMOT2887-12;MAMOT2888-12;MAMOT2890-12;PHLAC582-10;PHLAF283-11;PHLAV022-12;RWWC188-11;RWWC259-11;RWWC354-11;CNRVG054-15;EII711-15;GBLAA2038-15;GBLAA2039-15;GBLAC064-13;GBLAC065-13;GBLAC1059-13;GBLAD106-14;GBLAD749-14;GBLAF426-14;GWOTL926-13;LEATC582-13;LEATC583-13;LEATC653-13;LEPPK010-13;LNAUS5386-13;RWWA054-09;RWWA056-09;SSEID010-13;SSEID011-13;SSJAF2156-13;SSPAC13697-13;SSPAC13698-13;SSPAC2099-13;SSWLF1368-13;CGUKA099-09;CGUKA500-09;CGUKB141-09;CGUKB223-09;CGUKB505-09;CGUKB749-09;CGUKB933-09;CGUKC214-09;CGUKD260-09;EII650-15;GWOAL042-08;GWOAL045-08;GWORB038-07;GWORB039-07;GWORB040-07;GWORB042-07;GWORB043-07;GWORB046-07;GWORB047-07;GWORB048-07;GWORD398-07;GWORD399-07;GWORD400-07;GWORD401-07;GWORD402-07;GWORD403-07;GWORD404-07;GWORD405-07;LBCA245-05;LBCA266-05;LBCA278-05;LBCA464-05;LBCA465-05;LBCA747-05;LBCA750-05;LBCA751-05;LBCA752-05;LBCA754-05;LBCB048-05;LBCB156-05;LBCB157-05;LBCB158-05;LBCB159-05;LBCB160-05;LBCB161-05;LBCB162-05;LBCB163-05;LBCB164-05;LBCB165-05;LBCB166-05;LBCB170-05;LBCB176-05;LBCB357-05;LBCB605-05;LBCB607-05;LBCB608-05;LBCB609-05;LBCB611-05;LBCB612-05;LBCB751-05;LBCB816-05;LBCB824-05;LBCB881-05;LBCB884-05;LBCC327-05;LBCC383-05;LBCC386-05;LBCC388-05;LBCC389-05;LBCC390-05;LBCC391-05;LBCC392-05;LBCC395-05;LBCC734-05;LBCC735-05;LBCC736-05;LBCC737-05;LBCC738-05;LBCC739-05;LBCC740-05;LBCC741-05;LBCC905-05;LBCC906-05;LBCC908-05;LBCC911-

Species	Sequence accession numbers
	05;LBCC913-05;LBCD150-05;LBCD261-05;LNCNW042-06;LOWC750-05;LOWC751-05;LOWC752-05;LOWCC932-05;LOWCC934-05;LPAB311-08;LPMN244-08;LPMN664-08;LPSOB309-08;LPSOB375-08;LPSOB787-08;LBCB153-05;LBCB155-05;LBCB606-05;LOWC753-05;LPSOC100-08;LPSOC206-08;LPSOC280-08;LPSOD316-09;LPSOD317-09;LPSOD360-09;LPSOD522-09;LPSOD701-09;LPSOD723-09;LPSOD729-09;TMNBC737-07;TMNBC738-07;TMNBC739-07;TMNBC740-07;TMNBC741-07;XAJ347-06;XAJ483-06;XAJ486-06;XAJ542-06;XAJ581-06;XAJ585-06;CGUKA805-09;LBCB154-05;LBCB610-05;LBCB882-05;LBCB885-05;LBCC379-05;LBCC381-05;LBCC382-05;LBCC384-05;LBCC385-05;LBCC387-05;LBCC907-05;LCH347-04;LCH596-04;LEFIA1024-10;LGSM196-04;LGSM372-04;LGSMC223-05;LGSMC224-05 ;LGSMC499-05;LGSMC500-05;LGSMC501-05;LOT379-04;LOT380-04;LPSOD312-09;LOT381-04;LOT382-04;LOT383-04;LOT537-04;LOWC756-05;LOWC757-05;LOWC759-05;MNBB020-05;MNBB198-05;MNBB252-05;MNBB336-05;PHMNB047-03;PHMNB053-03;PHMNB184-04;PHMNB219-04;PHMNB564-04;PHMNB594-04;PHMNB721-05;PMG023-03;RRINV3952-15;RRINV3957-15;TMG18-03;TMG19-03;XAB079-04;XAB238-04;XAC234-04;XAC260-04;XAC267-04;XAC399-04;XAC431-04;XAC432-04;XAC434-04;XAC435-04;XAC475-04;XAC479-04;XAC483-04;XAC725-04;XAC810-04;XAC826-04;XAD428-04;XAE243-04;XAE393-04;XAE395-04;XAE446-04;XAE469-04;XAE523-04;XAF574-05;XAF642-05;XAG912-05;XAH365-05;GBGL17626-15;GBGL17627-15;GBGL17628-15;GBGL3835-07;GWNC628-07;GWNC640-07;GWNC642-07;GWNC643-07;GWNC661-07;GWNN024-07;GWNR123-07;GWNR124-07;GWNR125-07;GWNR127-07;GWNR128-07;GWNS090-07;GWNS092-07;GWNW032-07;GWORB037-07;GWORB786-07;GWORB787-07;GWORE1433-08;GWORG120-08;LBCG023-08;LBCG2919-09
Callimorpha dominula	CGUKA700-09;FBLMV287-09;GBGL10155-12;GBLAA2795-15;GBLAA355-14;GBLAF270-14;GWOR3966-09;GWORA2560-09;GWORE2018-09;GWORL402-09;GWOTL162-13;GWOTL163-13;GWOTL164-13;GWOTL172-13;LEATB704-13;LEATD577-13;LEATG182-14;LEATG256-14;LEFIJ828-10;LEFIJ829-10;PHLAA682-09
Catocala nupta	CGUKA188-09;CGUKB632-09;CGUKD442-09;GBLAA288-14;GBLAB331-13;GBLAC204-13;GBLAC351-13;GBLGC343-12;GWORK533-09;GWOTI370-12;GWOTI371-12;GWOTL428-13;IBLAO1088-14;IBLAO753-12;LEATD503-13;LEATH596-14;LEATH651-14;LEATH809-14;LEFIC508-10;LEFIC710-10;LEFIG987-10;LENOA704-11;LON650-09;PHLAW058-13;GBLAA2353-15
Conistra vaccinia	FBLMS305-09;FBLMV400-09;GMGRA1367-13;GWOSI801-10;GWOTI313-12;GWOTK435-12;GWOTL170-13;GWOTL251-13;GWOTL252-13;LEATA010-13;LEATA026-13;LEATA043-13;LEFIB104-10;LEFIC658-10;LENOA410-11;LENOA411-11;LENOA412-11;LENOA413-11;LENOA414-11;LENOA415-11;LON698-09;LON705-09;NOCJH356-10;NOCJH359-10;NOCJH398-11;CGUKA215-09;CGUKA903-09;CGUKA906-09;CGUKA907-09;CGUKB275-09;CGUKB668-09;GBLAA252-14;GBLAA947-14;GBLAA948-14;GBLAA949-14;GBLAB741-13;GBLAB742-13;GBLAC1139-13;GBLAC234-13;GBLAC235-13;GBLAC314-13;GBLAC797-13;GBLAD815-14;LEATH525-14;LEFIA498-10;LEFIA499-10;NOCJH360-10;NOCJH361-10;PHLAC624-10;PHLSA739-11;GBLAA2346-15;GWOR3948-09;NLLEA064-12;NLLEA065-12;NLLEA066-12;NLLEA1133-12
Diacrisia sannio	FBLMT905-09;FBLMU453-09;FBLMU559-09;GBLAA1126-15;GBLAA882-14;GBLAA883-14;GWORL400-09;GWORO853-09;GWORU318-10;GWORZ114-10;GWOSZ221-11;GWOTL160-13;IBLAO1033-14;IBLAO538-12;LEATB377-13;LEATB615-13;LEATC262-13;LEATG184-14;LEFIA118-10;LEFIA119-10;LEFIC882-10;LON822-11;PHLAA233-09;PHLAC445-10;PHLAG871-12;CGUKB146-09;CGUKD341-09;GBLAA2339-15;GBLGC271-12;GWOR3967-09;GWOR4222-09;LON028-08;RDMAB745-06
Diaphora mendica	CGUKA007-09;CGUKA260-09;CGUKA417-09;CGUKA555-09;CGUKA645-09;CGUKA778-09;CGUKB803-09;CGUKB893-09;CGUKD232-09;CGUKD233-09;FBLMU487-09;GBLGC187-12;GWOR3853-09;GWORZ112-10;GWOSZ104-11;GWOTD392-12;IBLAO542-12;IBLAO790-12;LASTS118-14;LEATH580-14;LEFIA110-10;LEFIA111-10;LEFIC589-10;LGEN058-14
Drepana falcataria	CGUKA128-09;CGUKA315-09;CGUKA644-09;CGUKC227-09;FBLMT359-09;FBLMU922-09;GBLAA1664-15;GBLAA401-14;GBLAB051-13;GBLAB052-13;GBLAC040-13;GBLAC1000-13;GBLAC584-13;GBLAD126-14;GBLAF261-14;GBLAF797-14;GWOSZ218-11;LEATA224-13;LEATA225-13;LEATG546-14;LEFIA223-10;LEFIA224-10;LEFIE168-10;LENOA765-11;PHLAC649-10;CGUKC108-09;CGUKD420-09;GBGL0271-06;GBGL4823-08;GBLGC144-12;GBLGC145-12;GWOR3810-09;GWOR3811-09;GWORE1420-08;LON042-08;NLLEA109-12;NLLEA264-12

Species	Sequence accession numbers
Eilema complana	FBLMU530-09;FBLMV679-09;GBLAA1681-15;GBLAA1682-15;GBLAB630-13;GBLAB631-13;GBLAD158-14;GBLAD180-14;GBLAF403-14;GWORZ105-10;GWOTF329-12;GWOTL151-13;GWOTL152-13;LEATB429-13;LEATB430-13;LEFIA101-10;LEFIA102-1;LEFIC752-10;LENOA1158-11;LENOA1159-11;PHLAA239-09;PHLAC532-10;PHLAC560-10;PHLAF318-11;PHLSA658-11;CGUKA267-09;CGUKA481-09;CGUKA512-09;CGUKB621-09;CGUKC062-09;CGUKC149-09;CGUKC158-09;CGUKD330-09;GBGL15092-14;GBLAA1752-15;GBLGC183-12;GBLGC184-12;GBLGC223-12;GWOR3849-09;GWOR3850-09;GWOR4174-09;LON542-08;NLLEA846-12;NLLEA847-12;NLLEA848-12
Eilema depressa	FBLMU531-09;FBLMU532-09;FBLMU533-09;FBLMW365-10;GBLAA393-14;GBLAB629-13;GBLAC580-13;GBLAD181-14;GBLAD244-14;GWORU327-10;GWOTL147-13;GWOTL148-13;GWOTL190-13;IBLAO917-12;IBLAO918-12;LEATB414-13;LEATB467-13;LEATD568-13;LEFIA108-10;LEFIA109-10;LEFIC766-10;LEFIC767-10;PHLAC462-10;PHLAC561-10;PHLAH735-12;CGUKB358-09;CGUKD226-09;CGUKD419-09;EPNG8667-14;EPNG8668-14;EPNG8669-14;GBLAA1683-15;GBLAA2287-15;GBLAA2348-15;GWOR4176-09;GWOR4181-09;LON555-08
Eilema lurideola	CGUKA355-09;CGUKB1004-09;FBLMU536-09;FBLMU537-09;FBLMV288-09;GBLAD243-14;GWORZ106-10;GWOSZ216-11;GWOTL149-13;GWOTL150-13;IBLAO522-12;IBLAO523-12;LEATB415-13;LEATB466-13;LEATD571-13;LEEUA665-11;LEFIA099-10;LEFIA100-10;LEFIJ574-10;LENOA1160-11;LENOA1161-11;ODOPE704-11;PHLAC463-10;PHLAC507-10;PHLAV379-12;CGUKA462-09;CGUKA816-09;CGUKB561-09;CGUKB974-09;CGUKC075-09;CGUKC317-09;CGUKC413-09;CGUKD224-09;CGUKD382-09;GWOR4149-09;LON526-08
Eilema sororcula	GBLAA885-14;GBLAA886-14;GBLAB632-13;GBLAB633-13;GBLAC1053-13;GBLAF265-14;GBLAF793-14;GBLAF794-14;GWORA2561-09;GWORZ102-10;GWOTL156-13;GWOTL157-13;IBLAO830-12;IBLAO889-12;LEATA176-13;LEFIA690-10;LEFIC346-10;LEFID960-10;LENOA1169-11;LENOA1170-11;PHLAA453-09;PHLAC506-10;PHLAC524-10;PHLAG932-12;PHLAV043-12;CGUKA056-09;CGUKA259-09;CGUKA590-09;CGUKB044-09;CGUKB775-09;CGUKC251-09;CGUKD250-09;CGUKD493-09;EII407-15;EII491-15;GBLGC276-12;GWOR4218-09;GWOR4227-09;NLLEA129-12;NLLEA309-12;NLLEA324-12
Euplagia quadripunctaria	CGUKA161-09;FBLMU553-09;GBGL15167-14;GBLAA1439-15;GBLAA887-14;GBLAD186-14;GBLAD354-14;GMGMB413-14;GMGMB421-14;GMGMB430-14;GWOR3964-09;GWOTL165-13;GWOTL166-13;GWOTL171-13;IBLAO1104-14;IBLAO1105-14;LEATB709-13;LEATB710-13;LEATB831-13;LEATD630-13;LEFIL416-10;PHLAH687-12
Eulithis mellinata	CGUKA474-09;CGUKA608-09;CGUKB096-09;GBLAC1083-13;GBLAF389-14;GWOSN648-11;LEFIA424-10;LEFIA425-10;LEFIB846-10
Euproctis chrysorrhoea	BTM001-10;BTM011-10;BTM014-10;BTM023-10;BTM026-10;BTM027-10;BTM032-10;BTM039-10;BTM040-10;BTM048-10;BTM049-10;BTM050-10;BTM051-10;BTM052-10;BTM053-10;BTM054-10;BTM055-10;BTM056-10;BTM057-10;BTM058-10;BTM076-10;BTM077-10;BTM078-10;BTM079-10;BTM080-10;BTM081-10;BTM082-10;BTM083-10;BTM084-10;BTM085-10;BTM092-10;CGUKA293-09;CGUKC451-09;FBLMU524-09;GBLAB617-13;GBLAD136-14;GBLAF429-14;GBLGC217-12;GBLGC264-12;GWOR4168-09;GWOR4215-09;GWORU379-10;GWORZ097-10;GWOSP624-11;IBLAO564-12;IBLAO565-12;LEATH447-14;LEATH448-14;NLLEA383-12;PHLSA637-11;NLLEA782-12;NLLEA797-12
Hoplodrina blanda	FBLMV302-09;FBLMW367-10;FBLMZ126-12;FBLMZ495-12;GWORO674-09;GWORO981-09;GWORZ540-10;GWOTL012-13;GWOTL013-13;GWOTL425-13;LEATB731-13;LEATB732-13;LEFIC866-10;LEFIJ740-10;LEFIK470-10;LEFIK471-10;LENOA620-11;LENOA621-11;NOCJH337-10;NOCJH338-10;NOCJH340-10;ODOPE274-11;PHLAE289-11;PHLAE446-11;PHLAH691-12;CGUKA795-09;CGUKA809-09;CGUKA915-09;CGUKA920-09;CGUKB025-09;CGUKC404-09;CGUKD408-09;GBLAA117-14;GBLAA124-14;GBLAA1518-15;GBLAA1582-15;GBLAA300-14;GBLAC211-13;GBLAC455-13;GBLAC505-13;GBLAC588-13;GBLAD825-14;GBLAF554-14;GBLGC300-12;GWOR3946-09;LEATC144-13;LEATC145-13;LEATG119-14;NLLEA754-12;NOLEP040-14
Hydriomena furcata	BBLPA869-10;BBLPB053-10;BBLPB161-10;BBLPB163-10;BBLPB175-10;BBLPB179-10;BBLPB204-10;BBLPB210-10;BBLPB211-10;CHIP127-12;CHIP614-12;CHIP615-12;CHIP616-12;CHIP617-12;CNEIE1774-12;CNEIE1775-12;CNEIE1805-12;CNEIE1807-12;CNEIE1808-12;CNEIE1811-12;CNEIE1813-12;CNEIE1815-12;CNEIE1816-12;CNEIE1817-12;LBCH4375-10;CNEIE1818-12;CNEIE1821-12;CNEIE1822-12;CNEIE1824-12;CNEIF2109-12;CNEIF2111-12;CNEIF2118-12;CNEIF2119-12;ENLCW009-09;ENLCW015-09;ENLCW059-09;GWORM125-09;GWORO951-09;GWORO959-09;GWORO964-09;GWORO968-09;GWORO974-09;GWOSN644-11;GWOTH326-12;GWOTI862-12;LALPA1218-11;LALPA432-10;LALPA503-10;LALPA651-10;LALPA960-11;LALPA969-11;LBCH1359-10;LBCH1588-10;LBCH1626-10;LBCH1955-10;LBCH1992-10;LBCH2022-

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Hypomecis roboraria	EII738-15;FBLMZ161-12;GBLAC074-13;GBLAC246-13;GBLAF208-14;GBMIN33829-13;GWOR4079-09;GWORG119-08;GWORG132-08;GWOSM028-11;GWOSM029-11;GWOSM032-11;GWOTI427-12;GWOTL902-13;LEATB501-13;LEATD246-13;LEFIA408-10;LEFIC867-10;LEFIE735-10;LENOA1120-11;LENOA1121-11;ODOPE198-11;PHLAV346-12
Laothoe populi	CGUKA052-09;CGUKA307-09;CGUKA434-09;CGUKA579-09;CGUKA650-09;CGUKA720-09;FBLMU939-09;GBLAA1666-15;GBLAA358-14;GBLAD210-14;GBLAF494-14;GBLAF772-14;GWORA2547-09;GWOSZ103-11;LEATA232-13;LEATB328-13;LEATB702-13;LEATC590-13;LEATD346-13;LEFIA064-10;LEFIB838-10;LEFIF632-10;LENOA1255-11;PHLAV299-12;SPUEB242-07;CGUKB231-09;CGUKB762-09;CGUKD265-09;GBGL19549-15;GBLGC283-12;GWOR4234-09;LEFIA1198-10;LON017-08;NLLEA511-12;NLLEA512-12;NLLEA594-12;NLLEA595-12;SOWC609-06;SPBIS138-09;SPTOL013-07;SPUEB244-07;SPUEB245-07
Miltochrista miniata	CGUKC170-09;FBLMV289-09;FBLMZ115-12;GBLAA394-14;GBLAB626-13;GBLAC582-13;GBLAD117-14;GBLAF266-14;GWORZ098-10;GWOTL138-13;GWOTL139-13;GWOTL140-13;GWOTL183-13;GWOTL184-13;IBLAO511-12;IBLAO512-12;LEATC225-13;LEATD597-13;LEATG178-14;LEFIA104-10;LEFIA105-10;LEFIF382-10;LENOA1162-11;PHLAC559-10;PHLAV293-12;CGUKD217-09;CGUKD391-09;CGUKD552-09;GBGL15717-14;GBGL15718-14;GBGL15719-14;GBGL15720-14;GBGL15721-14;GBGL15722-14;GBGL15723-14;GBGL15724-14;GBGL15725-14;GBGL15726-14;GBGL15727-14;GBGL15728-14;GBLGC210-12;GWOR4161-09;GWORK364-09

Species	Sequence accession numbers
Noctua pronuba	AWCLB329-10;AWCLB330-10;AWCLB378-10;AWCLB379-10;AWCLB380-10;BBLCU086-09;BBLEC049-09;BBLEC132-09;BBLEC416-09;BBLEC418-09;BBLEC419-09;BBLEC420-09;BBLEC423-09;BBLEC441-09;BBLEC453-09;BBLEC455-09;BBLEC456-09;BBLEC485-09;BBLEC564-09;BBLEC682-09;BBLPC035-09;BBLPC100-09;BBLPC101-09;BBLPC392-09;BBLPC395-09;BBLPC396-09;BBLPC402-09;BBLPC438-09;BBLWU077-09;BCMI035-11;BCMI423-11;BCMI426-11;CNBPF461-13;CNPPB1403-12;CNWLD1009-12;FBLMZ509-12;GMLC1208-12;GMLC523-11;GMLC561-11;GMLC609-11;GMLC619-11;GMLC754-12;GWORL292-09;GWORO843-09;GWORZ204-10;GWOSP677-11;GWOSZ109-11;GWOTG523-12;GWOTL103-13;IBLAO286-12;IBLAO669-12;LALPA121-10;LALPA122-10;LALPA706-10;LBCH7117-10;LBCH7326-10;LBCH7424-10;LEFIA580-10;LEFIA581-10;LEFIC780-10;LENOA111-11;LENOA112-11;LENOA113-11;LENOA114-11;LGSMG920-10;LGSMG921-10;LGSMG922-10;PAJUL2040-12;PAJUL2042-12;PHJUN3992-11;PHLAV335-12;PHLPM055-11;RWWA163-09;RWWA194-09;RWWA328-09;CNROO662-13;CNWLM2436-13;GBLAB596-13;GBLAC330-13;GBLAC403-13;GBLAD247-14;GBLAF274-14;GMGMD022-14;GMGMD029-14;HPPPD1636-13;LEATB703-13;LEATC134-13;LEATC646-13;LEATD235-13;MBIOD020-13;MBIOJ919-13;RWWA807-09;RWWA897-09;RWWB216-09;RWWB218-09;RWWC156-10;RWWC266-11;RWWC850-12;TRLEP056-13;TRLEP058-13;BLTIB1000-08;BLTIB311-08;BLTIB704-08;BLTIB783-08;CGUKA040-09;CGUKA251-09;CGUKA349-09;CGUKA435-09;CNGUB107-14;GBLAA1775-15;GBLAA2024-15;GMGMK175-14;GMGMM051-14;NOLEP032-14;PREXP140-14;RRGCO206-15;RRGCO207-15;RRGCO650-15;RRGCO651-15;RRGCO676-15;RRMFI4043-15;SMTPJ001-14;SMTPM5888-15;SMTPP3776-15;SSGIA2115-15;CGUKA568-09;CGUKA712-09;CGUKA794-09;CGUKB073-09;CGUKB241-09;CGUKB722-09;CGUKB950-09;DUNLP176-08;GMLC046-09;GMLC072-09;GMLC109-09;JBAZ031-09;LBCG3745-09;LBCG3747-09;LBCG3748-09;LBCG3749-09;LBCW044-08;LBCW045-08;LCHP771-07;LCHP814-07;LCHQ320-08;LCHQ658-08;LHLEP023-06;LHLEP024-06;LHLEP061-06;LBCS001-07;LNCNW012-06;LNCNW056-06;LOCBB214-06;LOCBE116-06;LOCT045-05;LOCT085-05;LOCT314-05;LPMNB466-09;LPSO631-08;LPSO731-08;LPSO732-08;LPSO933-08;LPSOB079-08;LPSOB705-08;LPSOB798-08;LPSOB946-08;RDLQF474-06;TTMNB430-06;TTNFS176-09;UDLEP153-09;XAJ684-06;XAJ812-06;XAJ948-06;XAK448-06;LBCS025-07;LBCS026-07;LBCS027-07;LBCS104-07;LBCS105-07;LBCS106-07;LBCS107-07;LBCS108-07;LBCS181-07;LBCS182-07;LBCS183-07;LBCS243-07;LBCS244-07;LBCS245-07;LBCS246-07;LBCS247-07;LBCS362-07;LBCS363-07;LBCS364-07;LBCS365-07;LBCS366-07;LBCS438-07;LBCS439-07;LBCS637-07;LBCS638-07;LBCS639-07;MNBB131-05;MNBB132-05;MNBB380-05;MNBB381-05;MNBB475-05;MNBB575-05;MNBB576-05;MNBB643-05;PHMNB084-03;PHMNB182-04;PHMNB229-04;PHMO004-03;PHMO100-03;PMG138-03;PPGB342-12;RRINV3896-15;RRINV3897-15;XAC570-04;XAD098-04;XAD173-04;XAF573-05;XAG911-05;XAH097-05;XAH336-05;GBGL12286-13;GBGL19777-15;GWOR4045-09;LEUAE1113-12;LON580-08;MPSC047-11;NLLEA207-12;NLLEA510-12;NLLEA786-12;NORIN064-13;SMTPR3823-16;UAMIC648-13;UAMIC649-13;XAH450-05;ZMBN353-16
Orthosia cerasi	EII434-15;GBLAA995-14;GBLAA996-14;GBLAA997-14;GBLAC1010-13;GBLAC1040-13;GBLAC684-13;GBLAC774-13;GBLAF272-14;GWORB3937-14;GWORZ488-10;GWOSI753-10;GWOSI754-10;GWOTL275-13;LEATA004-13;LEATA035-13;LEFIC656-10;LEFIC657-10;LEFIF508-10;LENOA278-11;LENOA279-11;LENOA280-11;PHLAC603-10;PHLAE374-11;PHLAV052-12;CGUKA411-09;CGUKA635-09;CGUKA956-09;CGUKB262-09;CGUKB811-09;CGUKB853-09;EII397-15;EII505-15;EII543-15;EII603-15;EII677-15;EII717-15;EII723-15;EII753-15;GBLAA2347-15;GWOR4027-09;NLLEA055-12;NLLEA056-12;NLLEA057-12;NLLEA166-12
Pelosia muscerda	CGUKD521-09;FBLMZ159-12;GBLAB628-13;GBLAF402-14;GWORA2564-09;GWORO775-09;GWORO776-09;GWORZ099-10;LASTS388-14;LEFIA094-10;LEFIC249-10;LEFIF742-10
Peridea anceps	CGUKD269-09;CGUKD836-09;FBLMV429-09;GBLAC1063-13;GBLAF247-14;GWORA2568-09;GWORZ132-10;GWOTF718-12;GWOTF719-12;GWOTF720-12;LEATA108-13;LEFID121-10;LEFIJ1004-11;LEFIL168-10;NLLEA305-12;NLLEA310-12;PHLAC600-10;PHLAV006-12
Phalera bucephala	CGUKA336-09;CGUKA362-09;CGUKA587-09;CGUKA822-09;CGUKB037-09;CGUKB525-09;GBLAA1124-15;GBLAA1607-15;GBLAC262-13;GBLAD104-14;GBLAD769-14;GBLAF269-14;GWORZ141-10;GWOSZ225-11;LEATB379-13;LEATB450-13;LEATC648-13;LEATD220-13;LEFIA068-10;LEFIE529-10;LENOA1273-11;ODOPE192-11;PHLAC479-10;PHLAF286-11;PHLAV007-12;CGUKA426-09;CGUKB734-09;CGUKB881-09;CGUKC294-09;GBLGC191-12;GBLGC201-12;GBLGC285-12;GWOR4142-09;GWOR4152-09;GWOR4236-09;LEFIA1291-10;LEFIA1292-10;NLLEA487-12

Species	Sequence accession numbers
Phragmatobia fuliginosa	BBLPE563-09;BBLPE566-09;BBLPE584-09;BBLPE585-09;BBLPE598-09;CNEII643-13;FBLMU563-09;GBLAB635-13;GWORA2563-09;GWOTK543-12;GWOTK559-12;GWOTL158-13;GWOTL189-13;IBLAO543-12;LEATC151-13;LEATD090-13;LEATD256-13;LEFIA116-10;LEFIA117-10;LEFIB218-10;LEFIC784-10;LEFID430-10;PHLAV029-12;RWWA953-09;RWWC514-11;ABKWR039-07;BLTIB957-08;CGUKA110-09;CGUKA234-09;CGUKB553-09;CGUKB562-09;CGUKC084-09;CGUKC157-09;CGUKC431-09;CGUKD546-09;GBLAA1771-15;GBLAA395-14;GBLAC277-13;GBLAC583-13;GBLAD209-14;GBLAD413-14;GBLAF238-14;IBLAO1034-14;LEATD531-13;RDLQ105-05;RDLQ106-05;RRGCO250-15;RRGCO304-15;RRGCO501-15;RWWC1019-12;CGUKB049-09;RDLQ107-05;RDLQB511-05;RDLQB740-05;RDLQF686-06;RDLQG269-06;RDMAB319-05;RDMAB389-05;RDMAB390-05;RDNM109-05;RDNMB305-05;RDNME827-08;RDNMG136-08;TMNBB015-06;TMNBB016-06;TMNBB017-06;TMNBB018-06;TMNBB019-06;TMNBB020-06;TMNBB021-06;XAJ854-06;XAJ924-06;XAJ931-06;XAK014-06;XAK064-06;GBLGC182-12;GBLGC228-12;GWOR3848-09;GWOR4179-09;GWORK358-09;LON561-08;MNBB399-05;MNBB618-05;NLLEA792-12;NLLEA794-12;PMG015-03;RRGCO487-15;RRINV3907-15;SMTPR10336-16;XAB035-04;XAB480-04;XAB481-04;XAD765-05;XAG012-05;XAG231-05;XAG427-05;XAG444-05
Setina irrorella	GBLAB171-13;GWORL290-09;LEATB559-13;LEATD479-13;LEFID143-10;LEFIF133-10;LEFIJ572-10;LENOA1179-11;LENOA1180-11;ODOPE707-11;ODOPE708-11;PHLAA006-09;PHLAA034-09;PHLAA036-09;PHLAA121-09;PHLAA266-09;PHLAA293-09;PHLAA294-09;PHLAB1148-10;PHLAF321-11;PHLSA603-11;PHLSA664-11;CGUKD808-09 ;PHLAA037-09
Smerinthus ocellata	CGUKA041-09;CGUKA286-09;CGUKA702-09;CGUKB059-09;CGUKC394-09;CGUKD1000-09;CGUKD310-09;CGUKD495-09;GBLAB043-13;GBLAB163-13;GBLAC270-13;GBLAF480-14;LEFIA060-10;LEFIA061-10;LEFIF631-10;ODOPE734-11;SOWC551-06
Sphinx pinastri	CGUKA328-09;CGUKB793-09;FBLMU940-09;GBLAA1450-15;GBLAA1667-15;GBLAC024-13;GBLAC025-13;GBLAC1061-13;GBLAC572-13;GBLAD108-14;GBLAF278-14;GWORA2546-09;GWOSZ102-11;LEATA347-13;LEATD055-13;LEFIA065-10;LEFIA066-10;LEFIA933-10;LEPPK004-13;LEPPK053-13;LON854-11;PHLAC523-10;PHLAC659-10;PHLAG906-12;PHLAV002-12;CGUKC278-09;GBGL16246-14;GBLGC206-12;GBLGC250-12;GWOR4157-09;GWOR4201-09;SOWA393-06
Spilosoma lubricipeda	CGUKA021-09;FGMLB654-13;GBLAA1601-15;GBLAA356-14;GBLAA889-14;GBLAD259-14;GBLAF330-14;GBLAF784-14;GWORE2014-09;GWORE2039-09;GWOTL159-13;IBLAO975-14;IBLAO976-14;LASTS426-14;LEATB515-13;LEATH732-14;LEFIA114-10;LEFIA115-10;LEFIC685-10;LTOLB330-09;LTOLB331-09;LTOLB409-09;LTOLB411-09;PHLAG926-12;PHLSA704-11;CGUKA233-09;CGUKA445-09;CGUKA562-09;CGUKA630-09;CGUKA769-09;CGUKB238-09;CGUKB724-09;CGUKB931-09;CGUKD284-09;GBGL16251-14;GBGL17523-15;GBGL17524-15;GWOR4143-09;GWOR4155-09;GWOR4223-09;GWOR4224-09;GWORK360-09;GWORK361-09;GWORK408-09;GWORK532-09;LEFIA1369-10;NLLEA132-12;NLLEA160-12;NLLEA161-12;NLLEA316-12;GBGL16252-14;GBGL16253-14;GBGL16254-14;GBGL17525-15;GBLGC192-12;GBLGC204-12;GBLGC272-12;GBLGC273-12
Spilosoma lutea	CGUKA042-09;CGUKA455-09;CGUKA759-09;CGUKA784-09;CGUKB062-09;CGUKB175-09;CGUKB742-09;CGUKB926-09;CGUKC165-09;CGUKC341-09;GBLAA1711-15;GBLAB175-13;GBLAC576-13;GBLAD768-14;GBLAF262-14;GWORZ111-10;IBLAO973-14;LEATB492-13;LEATD088-13;LEATD089-13;LEATG177-14;LEFIA112-10;LEFIA113-10;LEFIC884-10;PHLAH461-12;CGUKD285-09;GWORK359-09
Tyria jacobaeae	CGUKB1020-09;GWOSK783-11;IBLAO837-12;IBLAO870-12;LEFIF138-10;LEFIL049-10;LEFIL050-10;LON1108-12;LON960-12;NARCA183-09;NARCA184-09;NARCA185-09;NARCA186-09;NARCA187-09;NARCA188-09;NARCA189-09;NARCA455-10;NARCA456-10;NARCA457-10;ODOPE714-11;PHLAD763-11;PHLAD764-11;RWWA094-09;RWWA134-09;RWWB830-10;CGUKA053-09;CGUKA235-09;CGUKB177-09;CGUKB552-09;CGUKC346-09;CGUKD338-09;GBGL16402-14;GBLAB174-13;LEATG175-14;LEATG176-14;LEATJ1107-15;LON217-08;LOWCE193-06;NLLEA235-12;NLLEA236-12;NLLEA325-12;RDMAB921-06;RDNMG966-08;RWWC599-11
Xestia c-nigrum	BBLEC424-09;BBLEC430-09;BBLEC444-09;BBLEC448-09;BBLEC466-09;BBLEC684-09;BBLEC849-09;BBLPB824-10;BBLPB825-10;BBLPB826-10;BBLPB827-10;BBLPB828-10;BBLPB829-10;BBLPB830-10;BBLPC030-09;BBLPC050-09;BBLPC058-09;BBLPC060-09;BBLPC061-09;BBLPC063-09;BBLPC085-09;BBLPC093-09;BBLPC347-09;BBLPC407-09;BBLPC430-09;BBLPE424-09;CNRMH349-12;FBLMV030-09;GMLC1040-12;GMLC1130-

Species	Sequence accession numbers
	12;GWORL297-09;GWORZ215-10;GWOSZ111-11;GWOTF337-12;GWOTG524-12;GWOTG525-12;GWOTL113-13;GWOTL114-13;IBLAO323-12;IBLAO333-12;LALPA1249-11;LALPA638-10;LALPA714-10;LEFIC673-10;LEFID794-10;LEFID939-10;LEFIJ659-10;LENOA181-11;LENOA182-11;LGSMG898-10;LGSMG902-10;MAMOT2169-12;MAMOT2266-12;MAMOT2269-12;MAMOT2281-12;MAMOT2293-12;MAMOT2294-12;MAMOT2298-12;MAMOT2299-12;MAMOT2300-12;MAMOT2301-12;MAMOT2303-12;MAMOT2306-12;MAMOT2313-12;MAMOT2335-12;MAMOT2343-12;MAMOT2347-12;MAMOT2370-12;MAMOT2623-12;MAMOT2896-12;MAMOT471-10;MAMOT629-10;MAMOT824-10;PHLAV017-12;RWWA055-09;GBLAB604-13;LEATA195-13;LEATA289-13;LEATB748-13;LOCBF391-13;MAMOT3624-13;RWWA374-09;RWWA501-09;RWWB090-09;RWWB333-09;RWWB343-09;RWWB352-09;RWWB353-09;RWWB378-09;RWWB405-09;RWWB411-09;RWWB420-09;RWWB445-10;RWWB747-10;RWWB809-10;RWWC140-10;RWWC858-12;TRLEP030-13;UAMIC528-13;UAMIC529-13;ABKWR122-07;BLTIB269-08;BLTIB315-08;BLTIB339-08;CGUKA025-09;CGUKA299-09;CGUKA367-09;CGUKA437-09;CGUKA564-09;CGUKA758-09;CNSIA005-15;CNSIA006-15;CNSIA007-15;CNSIA013-15;CNSIA028-15;GBLAA1112-15;GBLAA1598-15;GBLAA1716-15;GBLAC324-13;GBLAC335-13;GBLAF240-14;LEATD170-13;LEATD250-13;RRMPG942-15;RRSSA4287-15;CGUKA789-09;CGUKA811-09;CGUKB043-09;CGUKB249-09;CGUKB727-09;CGUKB936-09;CGUKD240-09;LBCC287-05;LBCC288-05;LBCD029-05;LBCD030-05;LBCD493-05;LHLEP427-06;LOCBC163-06;LOWC794-05;LOWC795-05;LOWC796-05;LOWC797-05;LOWC798-05;LOWC799-05;LOWC800-05;LOWC801-05;LOWC802-05;LOWC803-05;LOWC804-05;LOWC912-05;LOWCD783-06;LOWCE806-06;LOWCE810-06;LOWCE812-06;LOWCE840-06;LOWCE858-06;LPABC910-09;LPMNB442-09;LPMNB443-09;LPSOB715-08;LPSOB716-08;LPSOB972-08;LPSOB978-08;LPSOB979-08;LPSOC095-08;LPSOC103-08;LPSOC104-08;LPSOC105-08;LPSOC108-08;LPSOC109-08;LPSOC112-08;LPSOC114-08;LPSOC115-08;LPSOC116-08;LPSOC291-08;LPSOC292-08;LPSOC293-08;LPSOC294-08;LPSOC295-08;LPSOC296-08;LPSOC297-08;LPSOC298-08;LPSOC299-08;LPSOC300-08;LPSOC301-08;LPSOC302-08;LPSOC303-08;LPSOC304-08;LPSOC305-08;LPSOC326-08;LPSOC332-08;LPSOC345-08;LPSOC346-08;LPSOC363-08;LPSOD970-09;MMNA085-08;RDLQ726-07;RDLQ727-07;RDLQB639-05;LBCB890-05;LBCB891-05;LBCB892-05;LBCB893-05;LGSM045-04;LPVIC114-08;MNBB119-05;MNBB169-05;MNBB170-05;RDLQB707-05;RDLQF885-06;TTMNB440-06;TTMNB441-06;TTMNB442-06;TTMNB443-06;TTMNB444-06;TTMNB445-06;TTMNB446-06;TTMNB447-06;TTMNB448-06;TTMNB449-06;TTMNB566-06;XAJ549-06;XAK337-06;XAK434-06;MNBB171-05;MNBB386-05;MNBB387-05;MNBB422-05;MNBB423-05;MNBB424-05;MNBB425-05;MNBB427-05;MNBB521-05;MNBB522-05;MNBB523-05;MNBB524-05;MNBB560-05;MNBB590-05;MNBB593-05;MNBB599-05;MNBB600-05;MNBB608-05;MNBB666-05;MNBB668-05;PHMNB003-03;PHMNB178-04;PHMNB222-04;PHMNB247-04;PHMNB745-05;GBGL16455-14;GBGL16459-14;GBGL16461-14;GBGL16462-14;GBGL16463-14;GWOR3951-09;LON272-08;NLLEA313-12;NLLEA513-12;NLLEA514-12;PHMO085-03;RRINV3941-15;XAB150-04;XAB363-04;XAD244-04;XAD434-04;XAD500-04;XAF549-05;XAF780-05;XAH087-05;XAH223-05;XAH530-05;XAH549-05;XAH562-05;XAH605-05;GBGL16421-14;GBGL16423-14;GBGL16425-14;GBGL16427-14;GBGL16429-14;GBGL16430-14;GBGL16432-14;GBGL16435-14;GBGL16436-14;GBGL16438-14;GBGL16439-14;GBGL16440-14;GBGL16442-14;GBGL16443-14;GBGL16445-14;GBGL16449-14;GBGL16453-14;GBGL16457-14;GBGL16458-14;NOCTU038-13;NOCTU040-13;NOCTU158-13;NOCTU159-13;NOCTU162-13;NOCTU163-13;GBGL16416-14;GBGL16417-14;GBGL16418-14;GBGL16420-14;GBGL16424-14;GBGL16428-14;GBGL16431-14;GBGL16433-14;GBGL16444-14;NOCTU039-13;NOCTU041-13;NOCTU160-13;NOCTU166-13;NOCTU167-13;NOCTU171-13;NOCTU172-13;NOCTU174-13;NOCTU175-13;NOCTU176-13;NOCTU179-13;NOCTU180-13;NOCTU183-13;NOCTU184-13;NOCTU186-13;NOCTU188-13;GBGL16415-14;GBGL16419-14;GBGL16422-14;GBGL16426-14;GBGL16434-14;GBGL16437-14;GBGL16441-14;GBGL16446-14;GBGL16447-14;GBGL16448-14;GBGL16450-14;GBGL16451-14;GBGL16452-14;GBGL16454-14;GBGL16456-14;GBGL16460-14;GBGL16464-14;GBGL16465-14;GBGL16466-14;NOCTU161-13;NOCTU165-13;NOCTU169-13;NOCTU173-13;NOCTU177-13;NOCTU189-13;GBGL16467-14;GBGL17834-15;NOCTU157-13;NOCTU164-13;NOCTU168-13;NOCTU170-13;NOCTU178-13;NOCTU181-13;NOCTU182-13;NOCTU185-13;NOCTU187-13;QMA842-13;SMTPR3045-16;ZMBN361-16
Yponomeuta cagnagella	CGUKB760-09;CGUKC624-09;CGUKD062-09;CGUKD1057-09;CGUKD613-09;FBLMW082-10;FBLMW495-10;LASTS608-14;LEATA486-13;LEATD650-13;LEATE427-13;LEATE428-13;LEATE744-13;LEATE827-13;LEATJ028-15;LEFIK196-10;LEFIL258-10;ODOPE338-11;ODOPE352-11;ODOPE355-11;PHLAC180-10;PHLAF113-11;PHLAG280-12;PHLSA315-11;TMNBC324-06;XAC731-04;XAC875-04;XAC906-04;XAD038-04;XAD189-04;XAD411-04;XAG168-05;XAG244-05;XAG433-05;XAK497-07;XAK504-07;XAK595-07;XAK596-07;XAK613-07

Species	Sequence accession numbers
Zygaena exulans	ABOLB061-15;ABOLB062-15;FBLMX109-11;GWORT468-10;GWOSA767-10;GWOTF950-12;LEATC566-13;LEATD135-13;LEATD136-13;LEFIG929-10;LEFIG930-10;LEFIK831-10;LON173-08;LON194-08;PHLAA113-09;PHLAA345-09;PHLAB1027-10;PHLAB1028-10;PHLAB1214-10;PHLAC877-10;PHLAC878-10
Zygaena filipendulae	ABOLB034-15;ABOLB252-15;ABOLB253-15;ABOLB254-15;ABOLB258-15;CGUKD513-09;FBLMT340-09;FBLMT341-09;FBLMX112-11;GWORK325-09;LEATH708-14;LEATH709-14;LEATJ219-15;LEATJ222-15;LEATJ255-15;LEFID817-10;LEFIE570-10;LEFIE571-10;LEFIE584-10;LON658-09;LON659-09;PHLAH747-12;PHLAH748-12;ZYGMO186-09;ZYGMO187-09;GWORK326-09;LEFIA1430-10
Zygaena lonicerae	FBLMX116-11;LEATA411-13;LEATD157-13;LEATH470-14;LEATJ277-15;LEATJ278-15;LEFID715-10;LEFID716-10;LEFIK833-10;LENOA1521-11;LON933-12;ZYGMO188-09;ZYGMO189-09;ZYGMO190-09
Zygaena loti	ABOLB256-15;FBLMT333-09;FBLMT334-09;FBLMX108-11;LEATD151-13;LEATD470-13;LEATG105-14;LEATH746-14;PHLAH705-12;ZYGMO177-09;ZYGMO178-09;ZYGMO179-09;ZYGMO180-09
Zygaena purpuralis	ABOLB245-15;ABOLB246-15;FBLMU285-09;FBLMV691-09;FBLMX105-11;GWORK323-09;GWORZ078-10;LEATA412-13;LEATD468-13;LEATD469-13;LEATH468-14;LEATH673-14;LEATH714-14;PHLSA442-11;ZYGMO166-09;ZYGMO167-09
Zygaena trifolii	FBLMT342-09;LENOA1506-11;ZYGMO474-10
Zygaena viciae	ABOLB250-15;ABOLB251-15;FBLMT335-09;FBLMT336-09;FBLMV690-09;FBLMX110-11;LEATH443-14;LEATH571-14;LEATH785-14;LEFID717-10;LEFID718-10;LEFIJ1381-12;LEFIJ1382-12;LEFIJ1383-12;LEFIK832-10;LENOA1486-11;PHLAW026-13;ZYGMO173-09;ZYGMO174-09;ZYGMO175-09;ZYGMO176-09

Appendix 3.8: Results of analyses of wing colours, for the violet-sensitive (VS, peafowl) visual system.

Supplementary table 3.8.1: Results of linear mixed effects models, testing the effect of category on luminance

Wing area	(X^2)	df	p
Forewing background	4.859	2	0.0881
Forewing markings	3.801	2	0.150
Hindwing background	12.478	2	*0.00195*
Hindwing markings	5.146	2	0.0763

Supplementary table 3.8.2: Results of linear mixed effects models, testing the effect of category on saturation

Wing area	(X^2)	df	p
Forewing background	2.852	2	0.240
Forewing markings	8.546	2	*0.0139*
Hindwing background	9.0912	2	*0.0106*
Hindwing markings	3.078	2	0.215

Supplementary table 3.8.3: Results of linear mixed effects models, testing the effect of category on hue values

Wing area	(X^2)	df	p
Forewing background	4.175	2	0.124
Forewing markings	5.419	2	0.0666
Hindwing background	10.083	2	*0.00646*
Hindwing markings	1.561	2	0.458

Supplementary table 3.8.4: Results of linear mixed effects models, testing the effect of category on chromatic contrast

Wing area	(X^2)	df	p
Forewings	13.669	2	*0.00108*
Hindwings	3.200	2	0.202

Supplementary table 3.8.5: Results of linear mixed effects models, testing the effect of category on luminance contrast

Wing area	(X^2)	df	p
Forewings	0.483	2	0.785
Hindwings	0.427	2	0.808

Supplementary table 3.8.6: Results of linear mixed models testing the effect of category on conspicuousness to natural backgrounds.

Contrast	Plant type	Wing area	(X^2)	df	p
Chromatic	Average herbaceous	Forewing background	8.337	2	*0.0155*
		Forewing markings	7.589	2	*0.0225*
	Average tree bark	Forewing background	4.851	2	0.0885
		Forewing markings	11.713	2	*0.00286*
	Average host plant foliage*	Forewing background	0.126	2	0.939
		Forewing markings	4.724	2	0.0942
Luminance	Average herbaceous	Forewing background	5.144	2	0.0764
		Forewing markings	5.121	2	0.0773
	Average tree bark	Forewing background	3.424	2	0.181
		Forewing markings	3.662	2	0.160
	Average host plant foliage*	Forewing background	0.540	2	0.764
		Forewing markings	2.706	2	0.259

* or lichen for Lithosiinae

Supplementary table 3.8.7: Volumes occupied by the colours of all the moths in each category in the avian tetrahedral colour space.

Category	Palatable	Toxic diurnal	Toxic nocturnal
Volume	0.000957	0.0328	0.0237

Comparison of volumes for each species, between categories: $(X^2)_2$=7.419, p=0.0245.

Appendix 4.1: Full results of model simplification for multiple regression between cyanogenic glucoside concentration, sex and colour metrics in three populations of *Z. filipendulae*. Significant results are highlighted in italics.

a. Forewings

Holywell Bay							
Model including saturation				**Model including hue**			
Factor	**F**	**df**	**p**	**Factor**	**F**	**df**	**p**
Saturation:Sex	0.112	1,11	0.745	Chromatic contrast:Sex	0.0957	1,11	0.764
Chromatic contrast:Sex	0.0049	1,12	0.945	Hue:Sex	0.0037	1,12	0.952
Luminance:Sex	0.223	1,13	0.645	Luminance:Sex	0.215	1,13	0.651
Proportion red:Sex	0.424	1,14	0.526	Proportion red:Sex	0.380	1,14	0.547
Luminance contrast:Sex	0.394	1,15	0.540	Luminance contrast:Sex	0.402	1,15	0.536
Sex	0.0001	1,16	0.991	Sex	0.0007	1,16	0.979
Saturation	0.0504	1,17	0.825	Hue	0.132	1,17	0.721
Luminance contrast	0.439	1,18	0.516	Luminance contrast	0.439	1,18	0.516
Proportion red	1.671	1,19	0.212	Proportion red	1.671	1,19	0.212
Luminance	4.358	1,20	*0.0499*	Proportion red	4.358	1,20	*0.0499*
Chromatic contrast	5.645	1,20	*0.0276*	Chromatic contrast	5.645	1,20	*0.0276*
Lamorna Cove				**Lamorna Cove**			
Model including saturation				**Model including hue**			
Factor	**F**	**df**	**p**	**Factor**	**F**	**df**	**p**
Saturation:Sex	0.0267	1,11	0.873	Hue:Sex	0.0142	1,11	0.907
Chromatic contrast:Sex	0.614	1,12	0.449	Chromatic contrast:Sex	0.533	1,12	0.480
Luminance:Sex	1.676	1,13	0.218	Luminance:Sex	1.733	1,13	0.211
Luminance contrast:Sex	1.193	1,14	0.293	Luminance contrast:Sex	1.015	1,14	0.331
Proportion red:Sex	2.024	1,15	0.175	Proportion red:Sex	1.844	1,15	0.195
Proportion red	1.273	1,16	0.276	Proportion red	1.428	1,16	0.250
Saturation	1.786	1,17	0.199	Hue	2.035	1,17	0.172
Chromatic contrast	0.409	1,18	0.531	Chromatic contrast	0.409	1,18	0.531
Sex	1.316	1,19	0.266	Sex	1.316	1,19	0.266
Luminance contrast	0.973	1,20	0.336	Luminance contrast	0.973	1,20	0.336
Luminance	1.506	1,21	0.233	Luminance	1.506	1,21	0.233
Taastrup				**Taastrup**			
Model including saturation				**Model including hue**			
Factor	**F**	**df**	**p**	**Factor**	**F**	**df**	**p**
Chromatic contrast:Sex	0.0032	1,13	0.956	Chromatic contrast:Sex	0.0103	1,13	0.921
Luminance:Sex	0.0064	1,14	0.937	Luminance:Sex	0.0175	1,14	0.897
Proportion red:Sex	0.0832	1,15	0.777	Proportion red:Sex	0.098	1,15	0.759
Luminance contrast:Sex	0.740	1,16	0.402	Luminance contrast:Sex	0.711	1,16	0.412
Saturation:Sex	0.0686	1,17	0.797	Hue:Sex	0.0792	1,17	0.782
Chromatic contrast	0.775	1,18	0.390	Chromatic contrast	0.974	1,18	0.337
Saturation	0.141	1,19	0.712	Hue	0.310	1,19	0.584
Sex	1.203	1,20	0.286	Sex	1.203	1,20	0.286
Proportion red	0.924	1,21	0.347	Proportion red	0.924	1,21	0.347
Luminance contrast	3.435	1,22	0.0773	Luminance contrast	3.435	1,22	0.0773
Luminance	6.768	1,23	*0.0160*	Luminance	6.768	1,23	*0.0160*

b. Hindwings

Holywell Bay							
Model including saturation				**Model including hue**			
Factor	**F**	**df**	**p**	**Factor**	**F**	**df**	**p**
Saturation:Sex	0.0116	1,17	0.916	Hue:Sex	0.0001	1,17	0.991
Luminance:Sex	0.584	1,18	0.455	Luminance:Sex	0.626	1,18	0.439
Luminance	0.675	1,19	0.421	Luminance	0.656	1,19	0.428
Saturation	0.928	1,20	0.347	Hue	0.772	1,20	0.390
Sex	1.525	1,21	0.231	Sex	1.525	1,21	0.231
Lamorna Cove				**Lamorna Cove**			
Model including saturation				**Model including hue**			
Factor	**F**	**df**	**p**	**Factor**	**F**	**df**	**p**
Saturation:Sex	2.780	1,17	0.114	Hue:Sex	4.125	1,17	0.0582
Luminance:Sex	2.232	1,18	0.153	Luminance:Sex	2.293	1,18	0.147
Luminance	0.029	1,19	0.958	Hue	0.0231	1,19	0.881
Saturation	0.0195	1,20	0.890	Luminance	0.016	1,20	0.901
Sex	0.467	1,21	0.502	Sex	0.467	1,21	0.502
Taastrup				**Taastrup**			
Model including saturation				**Model including hue**			
Factor	**F**	**df**	**p**	**Factor**	**F**	**df**	**p**
Saturation:Sex	0.0007	1,19	0.980	Hue:Sex	0.0001	1,19	0.992
Luminance:Sex	0.180	1,20	0.676	Luminance:Sex	0.174	1,20	0.681
Saturation	0.592	1,21	0.450	Hue	0.627	1,21	0.437
Sex	0.780	1,22	0.387	Sex	0.780	1,22	0.387
Luminance	1.576	1,23	0.222	Luminance	1.576	1,23	0.222

Appendix 4.2: Full results of mixed models testing the effects of sex and diet on colour metrics in *Z. filipendulae*. Significant results are highlighted in italics.

Factor	$(\chi^2)_1$	p	$(\chi^2)_1$	p	$(\chi^2)_1$	p
	FW luminance		FW saturation		FW hue	
Sex:Diet	0.369	0.544	1.181	0.277	1.0323	0.310
Diet	0.955	0.329	0.0082	0.928	0.0062	0.937
Sex	0.340	0.560	0.0552	0.814	0.0449	0.832
	FW proportion red		Chromatic contrast		Luminance contrast	
Sex:Diet	0.675	0.411	0.728	0.394	0.501	0.479
Diet	2.651	0.104	0.0717	0.789	1.880	0.170
Sex	17.911	*<0.001*	2.975	0.0846	2.270	0.132
	HW luminance		HW saturation		HW hue	
Sex:Diet	0.266	0.606	0.166	0.684	0.0537	0.817
Diet	0.976	0.323	0.379	0.538	0.0452	0.832
Sex	0.197	0.657	0.364	0.546	0.495	0.482

Appendix 4.3: Full results of mixed models testing the relationship between cyanogenic glucoside concentration and colour, dietary treatment and sex in *Z. filipendulae*. Significant results are highlighted in italics.

Factor/Interaction	$(\chi^2)_1$	p	$(\chi^2)_1$	p	$(\chi^2)_1$	p
	FW luminance		FW saturation		FW hue	
Colour:Diet:Sex	*4.715*	*0.0299*	0.114	0.736	0.0977	0.755
Colour:Sex	-	-	0.0324	0.857	0.0765	0.782
Diet:Sex	-	-	1.907	0.167	2.026	0.155
Colour:Diet	-	-	0.297	0.586	0.184	0.668
Colour	-	-	1.929	0.165	1.599	0.206
Sex	-	-	0.0324	0.857	0.0324	0.857
Diet	-	-	*4.241*	*0.0395*	*4.241*	*0.0395*
	FW proportion red		Chromatic contrast		Luminance contrast	
Colour:Diet:Sex	2.233	0.135	0.891	0.345	0.0003	0.987
Colour:Sex	0.659	0.417	0.247	0.619	0.643	0.423
Diet:Sex	0.0067	0.935	1.930	0.165	3.102	0.0782
Colour:Diet	*5.099*	*0.0239*	0.391	0.532	0.0136	0.907
Colour	-	-	*4.075*	*0.0435*	0.003	0.956
Sex	0.105	0.746	0.422	0.516	0.0324	0.857
Diet	-	-	*5.311*	*0.0212*	*4.241*	*0.0395*
	HW luminance		HW saturation		HW hue	
Colour:Diet:Sex	3.812	0.0509	0.0008	0.977	0.0018	0.966
Colour:Sex	1.866	0.172	0.0878	0.767	0.0925	0.761
Diet:Sex	2.974	0.0846	2.190	0.139	2.393	0.122
Colour:Diet	2.004	0.157	0.537	0.464	0.287	0.592
Colour	0.773	0.379	0.169	0.681	0.110	0.740
Sex	0.0324	0.857	0.0324	0.857	0.0324	0.857
Diet	*4.241*	*0.0395*	*4.241*	*0.0395*	*4.241*	*0.0395*

Appendix 4.4: Results of analyses for *Z. filipendulae*, with colour metrics based on the violet-sensitive (VS) visual system. The same transformations were applied to the data as for the analyses based on the ultraviolet-sensitive (UVS) visual system. Significant results are highlighted in italics.

a. Colour and toxicity between populations

Using the VS model revealed the same correlations between colour and cyanogenic glucosides as for the UVS data, although, in addition, forewing chromatic contrast was significantly negatively correlated, and luminance contrast positively correlated, with toxin levels in females.

Supplementary table 4.4a: Correlations between colour metrics and cyanogenic glucoside concentrations across populations. Significant results are highlighted in italics. FW=forewing, HW=hindwing.

Colour metric	**Males**	**Females**
FW luminance	No correlation, $F_{1,7}$=1.036, p=0.343	*Positive correlation, $F_{1,9}$=15.485, p=0.00343*
FW saturation	No correlation, $F_{1,7}$=0.868, p=0.382	Trend towards negative correlation, $F_{1,9}$=3.386, p=0.0989
FW hue	No correlation, $F_{1,7}$=0.865, p=0.383	No correlation, $F_{1,9}$=2.868, p=0.125
FW chromatic contrast	No correlation, $F_{1,7}$=0.567, p=0.476	*Negative correlation, $F_{1,9}$=6.580, p=0.0304*
FW luminance contrast	No correlation, $F_{1,7}$=0.671, p=0.440	*Positive correlation, $F_{1,9}$=13.785, p=0.00482*
Proportion of red area in FWs	No correlation, $F_{1,7}$=0.0034, p=0.955	*Negative correlation, $F_{1,9}$=5.252, p=0.0477*
HW luminance	No correlation, $F_{1,7}$=0.686, p=0.435	No correlation, $F_{1,9}$=1.806, p=0.212
HW saturation	No correlation, $F_{1,7}$=0.725, p=0.423	No correlation, $F_{1,9}$=0.0061, p=0.939
HW hue	No correlation, $F_{1,7}$=0.753, p=0.414	No correlation, $F_{1,9}$=0.0047, p=0.947

b. Colour and toxicity within populations

i. Relationship between colour and toxicity in Holywell, Lamorna and Taastrup

Multiple regressions yielded qualitatively identical results to those using the UVS visual model, with one exception: the negative correlation between chromatic contrast and toxicity was no longer significant in the Holywell Bay population.

Supplementary table 4.4b: Results of multiple regressions exploring the relationship between cyanogenic glucoside concentration and colour metrics in the forewings (i) and hindwings (ii). Results are presented for models including saturation only, as results of models with hue are similar.

a. Forewings

Factor	**F**	**df**	**p**
Holywell Bay			
Proportion red:Sex	0.015	1,11	0.905
Luminance contrast:Sex	0.110	1,12	0.746
Saturation:Sex	0.242	1,13	0.631
Chromatic contrast:Sex	0.0468	1,14	0.832
Luminance:Sex	0.234	1,15	0.636
Saturation	0.0001	1,16	0.993
Proportion red	0.0003	1,17	0.987
Chromatic contrast	0.799	1,18	0.383
Sex	2.902	1,19	0.105
Luminance contrast	2.942	1,20	0.102
Luminance	4.472	1,21	*0.0466*
Lamorna Cove			
Saturation:Sex	0.0582	1,11	0.814
Proportion red:Sex	0.0796	1,12	0.783
Luminance:Sex	1.780	1,13	0.205
Luminance contrast:Sex	1.444	1,14	0.250
Chromatic contrast:Sex	1.344	1,15	0.265
Luminance contrast	0.154	1,16	0.700
Proportion red	0.651	1,17	0.431
Saturation	1.042	1,18	0.321
Sex	0.859	1,19	0.366
Chromatic contrast	0.843	1,20	0.369
Luminance	1.401	1,21	0.250

Factor	F	df	p
Taastrup			
Chromatic contrast:Sex	0.0019	1,13	0.966
Proportion red:Sex	0.0339	1,14	0.857
Luminance:Sex	0.0923	1,15	0.765
Luminance contrast:Sex	0.476	1,16	0.5
Saturation:Sex	0.204	1,17	0.657
Chromatic contrast	0.138	1,18	0.715
Saturation	0.0435	1,19	0.837
Luminance contrast	0.767	1,20	0.392
Sex	1.304	1,21	0.266
Proportion red	0.928	1,22	0.346
Luminance	6.505	1,23	*0.0179*

b. Hindwings

Factor	F	df	p
Holywell Bay			
Saturation:Sex	0.125	1,17	0.728
Luminance:Sex	0.227	1,18	0.640
Luminance	0.684	1,19	0.418
Sex	1.309	1,20	0.266
Saturation	1.956	1,21	0.177
Lamorna Cove			
Saturation:Sex	3.120	1,17	0.0953
Luminance:Sex	1.854	1,18	0.190
Saturation	0.0036	1,19	0.953
Luminance	0.118	1,20	0.735
Sex	0.467	1,21	0.502
Taastrup			
Saturation:Sex	0.0607	1,19	0.808
Luminance:Sex	0.178	1,20	0.678
Saturation	0.341	1,21	0.565
Sex	0.906	1,22	0.352
Luminance	1.458	1.23	0.240

ii. Sex differences in coloration

Population and sex differences in colour metrics were very similar between visual models. However, with the VS visual model, population-level differences in forewing luminance, saturation and hue became significant: markings in the Taastrup population were significantly lighter ($p_{Holywell-Lamorna}$=0.524, $p_{Holywell-Taastrup}$=0.356, $p_{Lamorna-Taastrup}$=0.0365), less saturated ($p_{Holywell-Lamorna}$=0.790, $p_{Holywell-Taastrup}$=0.190, $p_{Lamorna-Taastrup}$=0.0316) and with lower hue values ($p_{Holywell-Lamorna}$=0.856, $p_{Holywell-Taastrup}$=0.160, $p_{Lamorna-Taastrup}$=0.0338) than those from

Lamorna Cove. In addition, there were no significant differences in luminance contrast.

Supplementary table 4.4c: Results of linear models testing for sex and population-level differences in colour metrics across Holywell Bay, Lamorna Cove and Taastrup. Unlike the UVS data, luminance contrast was not logit-transformed.

Factor	**F**	**df**	**p**	**F**	**df**	**p**	**F**	**df**	**p**
	FW luminance			FW saturation			FW hue		
Sex:Population	1.101	2,67	0.340	1.224	2,67	0.301	1.392	2,67	0.256
Population	3.681	2,69	*0.0303*	3.464	2,69	*0.0369*	3.464	2,69	*0.0369*
Sex	1.266	1,69	0.264	4.372	1,69	*0.0402*	4.378	1,69	*0.0401*
	Proportion red			Chromatic contrast			Luminance contrast		
Sex:Population	1.509	2,67	0.229	1.361	2,67	0.264	0.0511	2,67	0.950
Population	2.283	2,69	0.110	4.249	2,69	*0.0182*	1.153	2,69	0.322
Sex	17.766	1,71	*<0.001*	19.293	1,69	*<0.001*	0.286	1,71	0.651
	HW luminance			HW saturation			HW hue		
Sex:Population	0.444	2,67	0.644	1.415	2,67	0.250	1.505	2,67	0.229
Population	7.787	2,69	*<0.001*	2.409	2,69	0.0975	2.615	2,69	0.0804
Sex	16.062	1,69	*<0.001*	30.375	1,71	*<0.001*	7.313	1,71	*<0.01*

c. Contrast between wing and natural background colours

Results were qualitatively identical to those based on the UVS model for chromatic contrast to plants, and very similar for luminance contrast. However, population-level differences in luminance contrast became significant with the VS model (markings in Taastrup were less contrasting than those in Lamorna Cove).

Supplementary table 4.4d: Results of mixed models testing differences in contrast between forewing markings in Holywell Bay, Lamorna Cove and Taastrup, and natural backgrounds. Lc=*Lotus corniculatus*, Ka=*Knautia arvensis.*

Factor	**χ^2**	**df**	**p**	**Tukey's post-hoc tests**
Chromatic contrast				
Sex:Population	3.211	2	0.201	-
Sex	4.884	1	*0.0271*	-
Population	15.566	2	*<0.001*	$p_{Holywell-Lamorna}$=0.323, $p_{Holywell-Taastrup}$=0.0476, $p_{Lamorna-Taastrup}$*<0.001*
Plant type	638.07	2	*<0.001*	$p_{Lc\ leaves-Lc\ flowers}$ *<0.001,* $p_{Lc\ leaves-Ka\ flowers}$*<0.001,* $p_{Lc\ flowers-Ka\ flowers}$ *<0.001*
Luminance contrast				
Sex:Population	2.356	2	0.308	-
Sex	1.268	1	0.260	-
Population	6.316	2	*0.0425*	$p_{Holywell-Lamorna}$=0.535, $p_{Holywell-Taastrup}$=0.347, $p_{Lamorna-Taastrup}$*=0.0325*
Plant type	852.92	2	*<0.001*	$p_{Lc\ leaves-Lc\ flowers}$ *<0.001,* $p_{Lc\ leaves-Ka\ flowers}$*<0.001,* $p_{Lc\ flowers-Ka\ flowers}$ *<0.001*

d. Dietary manipulations

Results for the dietary experiment were qualitatively identical for both visual models.

i. Differences in coloration between diets

Supplementary table 4.4e: Results of mixed models testing for coloration differences between dietary treatments.

Factor	**$(\chi^2)_1$**	**p**	**$(\chi^2)_1$**	**p**	**$(\chi^2)_1$**	**p**
	FW luminance		FW saturation		FW hue	
Sex:Diet	0.280	0.596	1.217	0.270	1.066	0.302
Diet	0.574	0.449	0.0353	0.851	0.0033	0.954
Sex	0.270	0.604	0.132	0.717	0.162	0.687
	FW proportion red		Chromatic contrast		Luminance contrast	
Sex:Diet	0.675	0.411	0.470	0.493	2.025	0.155
Diet	2.651	0.104	0.001	0.974	0.0011	0.974
Sex	17.911	*<0.001*	2.530	0.112	1.157	0.282
	HW luminance		HW saturation		HW hue	
Sex:Diet	0.0636	0.801	1.015	0.314	0.717	0.397
Diet	0.0884	0.766	0.149	0.7	0.0145	0.904
Sex	0.496	0.481	0.0064	0.936	0.0257	0.873

ii. Diet and the relationship between colour and toxicity

Supplementary table 4.4f: Results of mixed models testing the relationship between colour and cyanogenic glucoside concentration as affected by dietary treatment.

Factor/Interaction	**$(\chi^2)_1$**	**p**	**$(\chi^2)_1$**	**p**	**$(\chi^2)_1$**	**p**
	FW luminance		FW saturation		FW hue	
Colour:Diet:Sex	*4.004*	*0.0454*	0.263	0.608	0.321	0.571
Colour:Sex	-	-	0.0202	0.887	0.0451	0.832
Diet:Sex	-	-	1.843	0.175	1.927	0.165
Colour:Diet	-	-	0.435	0.510	0.345	0.557
Colour	-	-	2.566	0.109	2.505	0.114
Sex	-	-	0.0324	0.857	0.0324	0.857
Diet	-	-	*4.241*	*0.0395*	*4.241*	*0.0395*
	FW proportion red		Chromatic contrast		Luminance contrast	
Colour:Diet:Sex	2.233	0.135	0.609	0.435	0.186	0.667
Colour:Sex	0.659	0.417	0.125	0.724	3.646	0.0562
Diet:Sex	0.0067	0.935	1.687	0.194	1.130	0.288
Colour:Diet	*5.099*	*0.0239*	0.875	0.350	1.281	0.258
Colour	-	-	3.653	0.0560	0.914	0.339
Sex	0.105	0.746	0.0324	0.857	0.0324	0.857
Diet	-	-	*4.241*	*0.0395*	*4.241*	*0.0395*
	HW luminance		HW saturation		HW hue	
Colour:Diet:Sex	3.233	0.0722	0.518	0.472	0.567	0.452
Colour:Sex	1.190	0.275	0.0001	0.994	0.0113	0.915
Diet:Sex	2.423	0.120	1.654	0.199	1.722	0.189
Colour:Diet	2.948	0.0860	0.579	0.447	0.564	0.453
Colour	0.829	0.363	1.408	0.235	1.634	0.201
Sex	0.0324	0.857	0.0324	0.857	0.0324	0.857
Diet	*4.241*	*0.0395*	*4.241*	*0.0395*	*4.241*	*0.0395*

Appendix 5.1: Details of field collections: localities, sample numbers and collectors.

Species	Year emerged	Origin	Country	Latitude	Longitude	Altitude (m)	N	Collectors
A. infausta	2015	Antigny	France	47.0454	4.3524	420	3	b,f
	2015	La Faurie	France	44.3401	5.4419	820	2	e
	2015	Le Fournas	France	44.0753	5.9722	480	5	b,e
	2015	Nolay	France	46.9293	4.6656	455	4	b,f
	2015	Sully	France	47.1738	4.3748	490	7	f
	2016	Antigny	France	47.0454	4.3524	420	15	b
	2016	Andon	France	43.4534	6.5140	1110	2	a,b,d,
R. pruni	2015	Antigny	France	47.0454	4.3524	420	7	b,f
	2015	Lardier-et-Valenca	France	44.2512	5.5689	830	1	e
	2016	Lusigny-sur-Ouche	France	47.0831	4.6744	400	2	b
	2016	Nolay	France	46.9293	4.6656	455	6	b
T. ampellophaga	2016	Biot	France	43.6269	7.0981	60	1	a
Z. cynarae	2015	Mouans-Sartoux	France	43.6204	6.9725	150	1	a,b,d
Z. ephialtes	2015	Corzé	France	47.5395	-0.3413	50	21	i
Z. erythrus	2016	Garriguet-Ste- Eulalie	France	43.9866	4.3052	90	8	e
	2016	Le Fournas	France	44.0753	5.9722	480	3	b
Z. exulans	2016	Molines-en-Queyras	France	44.7018	6.8208	2600	5	e
Z. filipendulae	2015 2016	*See Appendix 2.1*	-	-	-	-	107	a-e,h, j, k
Z.lonicerae	2016	Roubion	France	44.0529	7.0301	1270	1	b,d
Z. minos	2015	Le Cialancier	France	44.2091	6.9781	1000	1	a,b,d
	2016	Le Cialancier	France	44.2091	6.9781	1000	1	a,b
Z.occitanica	2016	Mouans-Sartoux	France	43.6204	6.9725	150	1	a,d
	2016	Callian	France	43.3829	6.4630	430	1	a,b,d
Z.sarpedon	2015	La Faurie	France	44.3401	5.4419	820	6	e,g
	2016	Le Fournas	France	44.0753	5.9722	480	2	b
Z. transalpina	2015	Mazaugues	France	43.3486	5.9225	690	1	e
	2015	Mouans-Sartoux	France	43.6204	6.9725	150	2	a,b
	2016	Mazaugues	France	43.3486	5.9225	690	12	e
	2016	Roubion	France	44.0529	7.0301	1270	1	b,d
Z. trifolii	2015	Bostraze Bog	UK	50.1304	-5.6501	160	7	b,j
	2015	Loggans Moor	UK	50.2021	-5.3979	20	2	b,j
	2016	Bostraze Bog	UK	50.1304	-5.6501	160	14	b,j

a: Alain Bourgon, b: Emmanuelle Briolat, c: David Demergès, d: Pierre Desriaux, e: Eric Drouet, f: Claude Dutreix, g: Anne Filosa, h: Alain Migeon, i: Marc Nicolle, j: W. G. Tremewan, k: Mika Zagrobelny

Appendix 5.2: Verifying the reliability of LC-MS analyses across years.

For logistical and funding reasons, it was not possible to analyse the toxicity of all samples from both field seasons at the same time. However, to check that this did not affect the results, I decided to re-run a subset of samples of two species, *A. infausta* and *Z. trifolii*, collected in 2015 and 2016 (N_{total}=20, N=5 for each combination of year and species) together in June 2017. Total cyanogenic glucoside content was analysed with a linear model, allowing run (original data or 2017 re-run), species and year of collection to interact. There were no significant effect of any interactions with run (linear model: Run:Year:Species: $F_{1,32}$=0.101, p=0.753 ; Run:Year: $F_{1,33}$=0.104, p=0.749; Run:Species: $F_{1,35}$=0.167, p=0.685), suggesting that running the samples separately in 2015 and 2016 did not affect the results. Overall, the measurements of total cyanogenic glucoside content in each sample appeared slightly lower in this new run than in the original data, suggesting that the equipment, which had recently been less regularly used and maintained, was slightly less sensitive (the overall effect of run on cyanogenic glucoside content was close to significance: $F_{1,37}$=3.845, p=0.0575).

Supplementary figure 5.2: Total cyanogenic glucoside content in *A. infausta* and *Z. trifolii* samples, as measured in the original dataset, and in a 2017 re-run. Boxplots show the median and interquartile range.

Appendix 5.3: Results of Phylogenetic Generalised Least Squares (PGLS) models testing the relationship between toxicity and coloration in Zygaenidae, in each subset of data (combination of collection years and sex). Significant relationships are highlighted in italics. ILC=Internal Luminance Contrast, ICC=Internal Chromatic Contrast. In all cases, maximum likelihood found $\lambda=1*10^{-6}$.

2015, Males: Variables included in models of cyanogenic glucoside concentration

Luminance, Marking size, ILC, ICC		Luminance, Marking size, ILC, Saturation		Luminance, Marking size, ILC, Hue	
ILC	$F_{1,3}$=0.0007, p=0.980	ILC	$F_{1,3}$=0.0021, p=0.967	ILC	$F_{1,3}$=0.0538, p=0.814
ICC	$F_{1,4}$=1.172, p=0.340	Saturation	$F_{1,4}$=1.739, p=0.258	Hue	$F_{1,4}$=0.472, p=0.530
Marking size (%)	$F_{1,5}$=3.879, p=0.106	Marking size (%)	$F_{1,5}$=3.879, p=0.106	Marking size (%)	$F_{1,5}$=3.879, p=0.106
Luminance	$F_{1,6}$=5.916, p=0.051	Luminance	$F_{1,6}$=5.916, p=0.051	Luminance	$F_{1,6}$=5.916, p=0.051

2015, Females: Variables included in models of cyanogenic glucoside concentration

Marking size, ILC, Luminance		Marking size, ILC, Hue		Marking size, ILC, Saturation		Marking size, ILC, ICC	
ILC	$F_{1,4}$=0.734, p=0.440	ILC	$F_{1,4}$=0.108, p=0.759	ILC	$F_{1,4}$=0.0031, p=0.958	ILC	$F_{1,4}$=0.0576, p=0.822
Marking size (%)	$F_{1,5}$=1.217, p=0.320	Marking size (%)	$F_{1,5}$=0.751, p=0.426	Marking size (%)	$F_{1,5}$=0.130, p=0.733	Marking size (%)	$F_{1,5}$=0.183, p=0.687
Luminance	*$F_{1,6}$=14.975 p=0.00827*	Hue	*$F_{1,6}$=15.68, p=0.00745*	Saturation	*$F_{1,6}$=11.78, p=0.0139*	ICC	*$F_{1,6}$=13.713, p=0.0101*

2015, Overall: Variables included in models of cyanogenic glucoside concentration

Luminance, Marking size, ILC, Hue		Luminance, Marking size, ILC, ICC		Luminance, Marking size, ILC, Saturation	
Hue	$F_{1,4}$=0.0379, p=0.855	ICC	$F_{1,4}$=0.0252, p=0.882	Saturation	$F_{1,4}$=0.0001, p=0.993
Marking size (%)	$F_{1,5}$=0.819, p=0.407	Marking size (%)	$F_{1,5}$=0.819, p=0.407	Marking size (%)	$F_{1,5}$=0.819, p=0.407
ILC	$F_{1,6}$=4.355, p=0.0820	ILC	$F_{1,6}$=4.355, p=0.0820	ILC	$F_{1,6}$=4.355, p=0.0820
Luminance	*$F_{1,7}$=13.409, p=0.00805*	Luminance	*$F_{1,7}$=13.409, p=0.00805*	Luminance	*$F_{1,7}$=13.409, p=0.00805*

2016, Males: Variables included in models of cyanogenic glucoside concentration

Luminance, Marking size, ILC, Hue		Luminance, Marking size, ILC, Saturation		Luminance, Marking size, ILC, ICC	
Luminance	$F_{1,5}$=0.0009, p=0.978	Marking size (%)	$F_{1,5}$=0.0467, p=0.836	Luminance	$F_{1,5}$=0.138, p=0.726
Marking size (%)	$F_{1,6}$=0.285, p=0.613	Saturation	$F_{1,6}$=0.236, p=0.644	Marking size (%)	$F_{1,6}$=0.308, p=0.600
Hue	$F_{1,7}$=1.176, p=0.314	Luminance	$F_{1,7}$=1.190, p=0.311	ICC	$F_{1,7}$=1.658, p=0.239
ILC	*$F_{1,8}$=11.474, p=0.00954*	ILC	*$F_{1,8}$=11.474, p=0.00954*	ILC	*$F_{1,8}$=11.474, p=0.00954*

2016, Females: Variables included in models of cyanogenic glucoside concentration

Luminance, Marking size, ILC, Hue		Luminance, Marking size, ILC, Saturation		Luminance, Marking size, ILC, ICC	
Luminance	$F_{1,3}$=0.0956, p=0.777	Luminance	$F_{1,3}$=0.0207, p=0.895	Luminance	$F_{1,3}$=0.154, p=0.721
Hue	$F_{1,4}$=0.0389, p=0.853	Saturation	$F_{1,4}$=0.0567, p=0.824	ICC	$F_{1,4}$=0.057, p=0.823
Marking size (%)	$F_{1,5}$=1.141, p=0.334	Marking size (%)	$F_{1,5}$=1.141, p=0.334	Marking size (%)	$F_{1,5}$=1.141, p=0.334
ILC	$F_{1,6}$=3.957, p=0.0938	ILC	$F_{1,6}$=3.957, p=0.0938	ILC	$F_{1,6}$=3.957, p=0.0938

2016, Overall: Variables included in models of cyanogenic glucoside concentration

Luminance, Marking size, ILC, Hue		Luminance, Marking size, ILC, ICC		Luminance, Marking size, ILC, Saturation	
Hue	$F_{1,6}$=0.0001, p=0.911	Luminance	$F_{1,6}$=0.0416, p=0.845	Saturation	$F_{1,6}$=0.315, p=0.595
Marking size (%)	$F_{1,7}$=0.259, p=0.626	Marking size (%)	$F_{1,7}$=0.0872, p=0.776	Marking size (%)	$F_{1,7}$=0.259, p=0.626
Luminance	$F_{1,8}$=0.722, p=0.420	ICC	$F_{1,8}$=1.031, p=0.340	Luminance	$F_{1,8}$=0.722, p=0.420
ILC	*$F_{1,9}$=6.803, p=0.0285*	ILC	*$F_{1,9}$=6.803, p=0.0285*	ILC	*$F_{1,9}$=6.803, p=0.0285*

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