No evidence of quantitative signal honesty across species of aposematic burnet moths (Lepidoptera: Zygaenidae)

Running title: Testing signal honesty across burnet moths

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Conflict of Interest

All the authors of this work declare no conflict of interest.

Abstract

Many defended species use conspicuous visual warning signals to deter potential predators from attacking. Traditional theory holds that these signals should converge on similar forms, yet variation in visual traits and the levels of defensive chemicals is common, both within and between species. It is currently unclear how the strength of signals and potency of defences might be related: conflicting theories suggest that aposematic signals should be quantitatively honest, or, in contrast, that investment in one component should be prioritised over the other, while empirical tests have yielded contrasting results. Here, we advance this debate by examining the relationship between defensive chemicals and signal properties in a family of aposematic Lepidoptera, accounting for phylogenetic relationships and quantifying coloration from the perspective of relevant predators. We test for correlations between toxin levels and measures of wing colour across 14 species of day-flying burnet and forester moths (Lepidoptera: Zygaenidae), protected by highly aversive cyanogenic glucosides, and find no clear evidence of quantitative signal honesty. Significant relationships between toxin levels and coloration vary between sexes and sampling years, and several trends run contrary to expectations for signal honesty. Although toxin concentration is positively correlated with increasing luminance contrast in forewing pattern in one year, higher toxin levels are also associated with paler and less chromatically salient markings, at least in females, in another year. Our study also serves to highlight important factors, including sex-specific trends and seasonal variation, that should be accounted for in future work on signal honesty in aposematic species.

Keywords: aposematism, defence, signal honesty, cyanogenic glucosides, *Zygaena*, insects, comparative studies

Introduction

Aposematic animals use conspicuous colours and patterns to warn potential predators of their unprofitability, linked to physical or chemical defences (Ruxton *et al*., 2004; Stevens & Ruxton, 2012). This strategy, first proposed by Alfred Russell Wallace to explain the colourful appearance of caterpillars (Wallace, 1867) is now recognised to occur in a wide range of taxa, from a host of invertebrates (e.g. Hemiptera [Exnerová *et al*., 2003], Lepidoptera [Rothschild, 1985]) and amphibians (e.g. poison frogs; Summers & Clough, 2001) to mammals (Stankowich *et al*., 2011) and birds (Dumbacher *et al*., 2008). Predators who encounter distasteful warningly-coloured prey should learn to associate the prey signal with their unpleasant experience and avoid attacking similar prey in the future. Bright and colourful patterns facilitate this process in a number of ways, enhancing the "efficacy" of aposematic signals by increasing their detectability, memorability and discriminability (Guilford & Dawkins, 1991; Ruxton *et al*., 2004). Moreover, traditional theory rooted in Fritz Müller's insights into mutually-beneficial mimicry between defended species (Müller, 1879), has held that warning signals should converge onto a limited number of common forms, to further speed up predator avoidance learning. Yet, there is extensive variation in warning coloration across aposematic taxa, which can be perceptible to their predators (Arenas & Stevens, 2017; Briolat *et al.* 2018a).

A key line of enquiry into this seemingly paradoxical variation explores the relationship between the strength of visual signals and levels of defences, which also vary greatly both between (e.g. Arenas *et al*., 2015) and within species (e.g. Brower *et al*., 1968). As conspicuous coloration incurs the cost of heightened detection by predators, it should often be too costly for undefended species, which would be captured and consumed (with the exception of Batesian mimics of aposematic species; Bates, 1862). Aposematic signals are

78 therefore generally considered to be qualitatively honest, reliably indicating the presence of a
79 defence (Sherratt, 2002; Ruxton et *al*., 2004). Whether they should also be expected to be
80 quantitatively honest, with the strength of visual signals reflecting the potency of the defences
81 they advertise, is more controversial.
82
83 Despite the cost of increased visibility to predators, early interpretations of aposematism as an
84 honest handicap signal (Grafen, 1990) have been criticised for the lack of a physiological link
85 between visual features and defensive chemistry (Guilford & Dawkins, 1993). This could be
86 provided by competition between traits for resources, leading to positive correlations between
87 signals and defences when these resources are limited (Blount et al., 2009; 2012). Yet some
88 theoretical models predict a disjunction between signals and defences, suggesting that prey
89 should prioritise investment in either signals, to which predators respond (Leimar e*t al*.,
90 1986), or defences, which do not incur detection costs (Speed & Ruxton, 2007). Overall,
91 considering the relative costs of signals and defences, quantitative honesty may be expected to
92 occur under certain conditions, depending on the economics of colour and toxin production
93 (Speed & Ruxton, 2007), predator behaviour (Guilford, 1994; Speed *et al*., 2010) and prey
94 resilience to attack (Sherratt, 2002). While most theoretical work focuses on single species,
95 several of these evolutionary mechanisms have been proposed to underpin signal honesty
96 across closely-related species too (Summers *et al.*, 2015). Coevolutionary dynamics with
97 mimics of defended prey (Franks *et al*., 2009), cautious or "go-slow" behaviour on the part of
98 predators (Guilford, 1994), exaptation through other functions of visual signals (Lee *et al*.,
99 2011), and resource allocation trade-offs (Blount *et al.*, 2009), are all thought to have the
100 capacity to lead to honest signalling between populations or species (Holen & Svennungsen,
101 2012; Summers *et al.*, 2015).
102

Most empirical studies of the relationship between signals and defences across clades of species have found positive correlations between measures of visual signal strength and measures of toxicity, suggesting quantitative honesty in signalling (Summers & Clough, 2001; Cortesi & Cheney, 2010; Santos & Cannatella, 2011; but see Darst *et al*., 2006, Winters *et al*. 2018). Work on ladybird beetles (Coccinellidae), combining toxin bioassays to field predation experiments with ladybird models presented to birds, has explicitly linked more conspicuous coloration and higher defence levels to greater survival in the wild (Arenas *et al*., 2015). However, these studies are restricted in taxonomic scope, primarily focusing on poison frogs (Dendrobatidae), ladybird beetles and to a lesser extent marine opisthobranchs (Cortesi & Cheney, 2010; Winters *et al*. 2018), so research in a wider range of taxa is needed before more general conclusions can be drawn (Stevens, 2015; Summers *et al.*, 2015). Existing studies can also be difficult to compare, as they employ a wide range of methods for quantifying defences, from bioassays (e.g. Darst *et al*. 2006, Arenas *et al*., 2015) to specific quantification of individual chemicals (e.g. alkaloids in the Dendrobatidae; Summers & Clough, 2001), and vary in their approaches to measuring coloration. Animal visual systems differ from human perception and are highly variable between species, so it is essential to consider visual signals from the perspective of the relevant receivers, which in the case of aposematism are potential predators (Stevens, 2007; 2011). Although this is not always the case (e.g. Summers and Clough, 2001; Dumbacher *et al*., 2000; 2008), studies of aposematism are increasingly considering predator perception (e.g. birds [Darst *et al*., 2006; Arenas *et al*., 2015] and fish [Cortesi & Cheney, 2010; Winters *et al*. 2018]), as our understanding of animal vision improves.

Aposematic burnet moths (Lepidoptera: Zygaenidae) are well-suited to testing the relationship between signals and defences across closely-related species. In the Western Palearctic, the

Zygaenidae are represented by three subfamilies: the Zygaeninae, Procridinae and Chalcosiinae. Of the 1,036 species of Zygaenidae recognised worldwide (van Nieukerken *et al*., 2011), all 45 tested so far, including members of all three relevant subfamilies (38 Zygaeninae, including 35 *Zygaena* spp., two Procridinae and five Chalcosiinae), possess potent chemical defences, in the form of cyanogenic glucosides (Davis & Nahrstedt, 1982, 1985; Zagrobelny *et al.*, 2004). The Zygaenidae synthesise the cyanogenic glucosides linamarin and lotaustralin *de novo*, from the amino acids valine and isoleucine respectively (Wray *et al*., 1983), but species in the Zygaeninae further have the apparently unique ability to simultaneously sequester the same compounds from their host plants (Zagrobelny *et al*., 2014). Cyanogenic glucosides, occurring in plants and several arthropod lineages (Zagrobelny *et al*., 2008), are bitter-tasting compounds, distasteful to avian predators, so are likely to facilitate taste-rejection during an attack (Skelhorn & Rowe, 2009). They are also toxic, releasing hydrogen cyanide upon enzymatic breakdown, due to enzymes either in the gut of predators or present in the prey themselves (Zagrobelny *et al*., 2008). In terms of coloration, there are dramatic differences in wing patterns between subfamilies of Zygaenidae, and more subtle variation within. Burnet moths in the genus *Zygaena* are characterised by classically conspicuous aposematic markings, with a typical pattern of black forewings with red spots, and red hindwings. Both within and between species, there can be extensive variation on this theme, with respect to the colour, size, shape and number of markings (Hofmann & Tremewan, 2017). By contrast, temperate species of Procridinae, or forester moths, are iridescent green or dull brown in colour (Drouet, 2016) and are generally considered cryptic (Efetov & Tarmann, 1999). The single representative of the Chalcosiinae in Western Europe, *Aglaope infausta* (L.), has brown forewings with discreet red markings, and red hindwings.

To test for evidence of quantitative signal honesty across the Zygaenidae, we measured signal and defence properties in 14 species, collected in 2015 and 2016 from a range of locations in Denmark, France and the UK. As the defences of the Zygaenidae have been extensively studied, we were able to accurately quantify the levels of cyanogenic glucosides in our samples, using a liquid chromatography – mass spectrometry (LC-MS) protocol specifically refined to identify linamarin and lotaustralin. In terms of signal receivers, birds are the most likely visually-driven predators of adult Zygaenidae. Experiments with captive birds, including *Cyanistes caeruleus* (blue tits) and *Parus major* (great tits) (Wiklund & Järvi, 1982) as well as *Sturnus vulgaris* (starlings; Rammert, 1992), suggest that they generally find burnet moths distasteful, yet observations in the wild reveal that several species, such as *Alauda arvensis* (skylarks), *Anthus pratensis* (meadow pipits) and even *S. vulgaris,* will nevertheless attack and in some cases partly or entirely consume these moths (Tremewan, 2006). Using visual modelling techniques, we measured multiple characteristics of zygaenid wing patterns, from the perspective of a potential avian predator, with a visual system modelled on the blue tit, *C. caeruleus*. In addition, molecular data and recent phylogenies of the Zygaenidae and the genus *Zygaena* are available (Niehuis *et al*., 2006a,b,c; 2007), enabling evolutionary relationships to be accounted for when analysing variation across species. This study is the first detailed exploration of the chemical defences and coloration of multiple species in this family of aposematic Lepidoptera. We test the idea of quantitative signal honesty in a new study system, using relevant and meaningful measures of signals and defences, to contribute to the debate over signal honesty across aposematic species.

Materials and Methods

Specimen collection and rearing

Individuals of 14 Zygaenidae species were collected in spring and summer 2015 and 2016, from locations in Denmark, France, and the UK (Table 1; see Supporting Information S1 for full details). Where possible, host plants were sampled at the same locations (see Supporting Information S2). To ensure that all Zygaenidae analysed were virgin, an important consideration as males and females exchange cyanogenic glucosides during reproduction (Zagrobelny *et al*., 2007a,b; 2013), specimens were collected at the larval or pupal stage, then reared to maturity in the laboratory. Larvae and pupae were kept in individual boxes with air-holes, inside an incubator at 20°C, with a 16:8h day:night cycle, following protocols from previous work on *Zygaena filipendulae* (Linnaeus, 1758) (Zagrobelny *et al.*, 2007a). The larvae were fed *ad libitum* with the same host plant as they were found on in the field (Table 1). After emergence, the adults were euthanised by placing them in a -80°C freezer. 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 1). 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.

Wing photography

Photographs of the moths' forewings were taken with a calibrated, UV-sensitive digital camera (Nikon D7000 fitted with a 105mm CoastalOptics quartz lens), in controlled conditions inside a dark room. Lighting was provided by an EYE Color Arc Lamp MT70 bulb (Iwasaki Electric Co. Ltd.), its UV-blocking coating removed by lightly scrubbing with a steel brush (Troscianko & Stevens, 2015), thus emitting a spectrum of light similar to D65 daylight conditions. The forewings were chosen for analysis as they are more visible to predators than the hindwings, which in the Zygaenidae are hidden from view when at rest. As these wings are iridescent, only the right-hand wings were photographed (to keep scale direction

consistent), and the light source and camera were held at constant angles relative to the wings (50° and 90° respectively). The wings were photographed flat against a background of grey ethylene-vinyl acetate (EVA, or craft foam). A scale bar and a set of two polytetrafluoroethylene (PTFE) 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, enabling calibration of the images with respect to lighting conditions (Troscianko & Stevens, 2015). Each specimen was photographed twice, using different filters (a UV/infrared blocking filter [Baader UV/IR Cut Filter], transmitting between 400 and 700 nm, and a UV pass and IR blocking filter [Baader U filter], transmitting between 300 and 400 nm). All photographs were taken in RAW format, with a constant aperture (f8) and manual white balance set to "cloudy".

Image analysis

All image analysis was performed in ImageJ (Schneider *et al*., 2012) using open access custom-made plugins in the Image Calibration and Analysis Toolbox (Troscianko & Stevens, 2015). Methods used for processing images and extracting colour metrics are summarised below; full details are provided in Supporting Information S3. To allow for objective colour measurements, images were linearised and normalised (Stevens *et al*., 2007), then scaled to 100 pixels/mm. Photographs taken with the two types of filter were combined using an automatic alignment tool, and the resulting multispectral images were mapped to avian vision, as previous observations show that birds are likely to be the most relevant visual predators of burnet moths (Tremewan, 2006). Each image was converted to the visual system of *C. caeruleus*, the model species for the ultraviolet-sensitive (UVS) avian visual system (Hart *et al*., 2000) using a highly-accurate polynomial mapping technique (Stevens & Cuthill, 2006; Stevens et al. 2007; Pike, 2011; Troscianko & Stevens, 2015) to produce a set of image layers

corresponding to the predicted cone catch values for each of the five avian cone types: long wavelength- (LW-), medium wavelength- (MW-), short wavelength- (SW-) and ultraviolet- (UV-) sensitive photoreceptors, and double cones. Relevant wing areas were selected using the freehand tool in Image J. Most species display red forewing markings, but for *Rhagades pruni* (Denis & Schiffermüller, 1775), the iridescent blue patch at the base of the wing was selected as the markings, while for *Theresimima ampellophaga* (Bayle-Barelle, 1808) the whole uniform wing was measured as a single patch. Cone catch values for every photoreceptor type were obtained from each selected patch, then averaged to obtain a single measure of colour per individual, for both the wing markings and wing background area.

Colour metrics

Based on the average cone catch values, several measures of coloration were calculated: luminance, saturation, and hue of the forewing marking colours, as well as both chromatic and luminance contrasts between markings and background colours. In brief, luminance (perceived lightness) was taken as the cone catch value for the double cones (Jones & Osorio, 2004; Osorio & Vorobyev, 2005), and saturation, measuring colour 'richness', was calculated by plotting wing colours in a tetrahedral colour space and measuring the Euclidian distance from each colour to the centre of the tetrahedron (after Endler & Mielke, 2005; Stoddard & Prum, 2008). Hue, representing the type or shade of a colour, was derived using principal component analysis (after Spottiswoode & Stevens, 2011) to obtain a ratio of cone catch values broadly inspired by the general principle of colour opponency, known to be relevant to avian vision (Osorio *et al*., 1999). In this study, hue is given by the following equation, such that higher hue values represent colours with relatively higher reflectance in the LW or UV channels, indicating redder colours, higher ultraviolet reflectance, or both:

$$\text{Hue} = (\text{LW}+\text{UV})/(\text{SW}+\text{MW}) \quad (1)$$

Chromatic and achromatic contrasts between the markings and background colours provide a sense of the salience of wing markings, and may be relevant to predator behaviour, although the relative importance of pattern contrast over colour *per se* in aposematic signals remains unclear (Svádová *et al*., 2009; Aronsson & Gamberale-Stille, 2008; 2012a,b). Internal contrasts were calculated using a log version of the Vorobyev-Osorio model (Vorobyev & Osorio, 1998) and relative cone abundance values for *Cyanistes caeruleus* as a model for the UVS avian visual system (Hart *et al.*, 2000), with a widely-used estimate of the Weber fraction (ω=0.05; Eaton, 2005; Håstad *et al*., 2005; Stevens, 2011) to calculate noise. Achromatic, or luminance, contrast was taken as the natural logarithm of the ratio between the mean double cone catch values of two colours, divided by the same Weber fraction (Siddiqi *et al*., 2004). Both contrasts are measured in "just-noticeable differences" (JNDs): values below 1 suggest that the two colours compared are indiscriminable, even in optimal lighting conditions, while values above 1 and higher indicate colours increasingly easy to discriminate (Siddiqi *et al*., 2004). Supporting Information S3 provides details on the calculations of all the metrics described above.

Quantification of chemical defences

After photography, each specimen, complete with its forewings, was preserved in 1ml 80% methanol in preparation for analysis of their cyanogenic glucoside content. Quantification of linamarin and lotaustralin in our samples was performed by liquid chromatography – mass spectrometry (LC-MS), following a protocol specifically refined to identify these compounds, and used in previous work on the chemistry of the Zygaenidae (Zagrobelny *et al*., 2004, 2007a,b; 2014; 2015; Fürstenberg-Hägg *et al*., 2014; Pentzold *et al*., 2015; 2016). Samples were prepared by grinding up the specimens in 1ml ice-cold 55% MeOH with 0.1% formic

acid then passing them through an Anopore 0.45µm filter (Whatman). The analytical LC-MS was performed with an Agilent 1100 Series LC (Agilent Technologies, Germany), and Bruker HCT-Ultra ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany), run in positive electrospray mode, with an oven temperature of 35°C. A Zorbax SB-C18 column (Agilent; 1.8µM, 2.1x50 mm) was used for chromatographic separation, running with a flow rate of 0.2 ml/min, increased to 0.3 ml/min from 11.2 to 13.5 min. The mobile phases A and B were composed respectively of H_2O with 0.1% (v/v) HCOOH, 50 µM NaCl, and MeCN with 0.1% (v/v) HCOOH, with a gradient 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. Sodium adducts of linamarin (retention time [RT] 2.6 min, $[M+Na]^+$ at *m/z* 270) and lotaustralin (RT 5.5 min, $[M+Na]^+$ at *m/z* 284) were detected and compared to authentic standards (Møller *et al*., 2016) using native analysis software. The total amount of each compound was estimated according to its Extracted Ion Chromatogram (EIC) peak areas, and quantified using calibration curves for the linamarin, lotaustralin, and amygdalin standards. Finally, the concentration of cyanogenic glucosides in each sample was determined by dividing the total amount of compounds in each sample by the specimen mass, recorded at the time of preservation. Samples of larval host plants were analysed similarly. To rule out that differences between samples from 2015 and 2016 were due to the LC-MS machine, a subset of 20 samples (5 *A. infausta* and 5 *Z. trifolii* from each year, both males and females) were run together a second time in 2017. Analysing the results with a mixed effects model including specimen ID as a random effect, we found no significant effect of the interaction between collection year and machine run (original, in 2015 or 2016, vs. second run in 2017) on the concentration of cyanogenic glucosides for either *A. infausta* (χ^2_1=1.73, df=1, p=0.19) or *Z. trifolii* (χ^2_1=0.64, p=0.43), suggesting that differences between years were not due to variation in the sensitivity of the LC-MS machine in 2015 and 2016.

301

302 *Phylogenetic reconstruction*

303 The phylogenetic tree was reconstructed using previously-published mitochondrial and
304 nuclear sequences, following existing studies of the evolutionary history of the Zygaenidae
305 (Niehuis *et al*., 2006a; 2007): complete sequences of the mitochondrial genes for NADH
306 dehydrogenase subunit 1 (ND1), tRNA-leucine (tRNA-Leu), the large subunit ribosomal
307 RNA (16S rRNA), tRNA-valine (tRNA-Val) and a large fragment of the sequence for the
308 mitochondrial small subunit of rRNA (12S rRNA), as well as two nuclear DNA fragments, an
309 almost complete sequence of the small subunit rRNA (18S rRNA) and the 5' end of the large
310 subunit rRNA (28S rRNA). A new phylogenetic tree was built from these sequences, as
311 previously-published phylogenies using all available sequences (Niehuis *et al*., 2006a; 2007)
312 did not include all our species of interest. *Sesia bembeciformis* (Lepidoptera: Sesiidae) was
313 used as an outgroup to root the tree (Niehuis *et al*., 2006a,b,c). Sequences for each species
314 photographed and the outgroup were downloaded from GenBank
315 (http://www.ncbi.nlm.nih.gov/; see Supporting Information S4). and aligned using MUSCLE
316 (Edgar, 2004), as implemented by the 'ape' package (Paradis *et al*., 2004) in R 3.3.1 (R
317 Development Core Team, 2015). The alignments for each sequence were then concatenated to
318 produce a single final alignment (5697 base pairs [bp] long) for phylogenetic reconstruction.
319

320 Phylogenetic relationships were assessed with maximum likelihood (ML), using the
321 'phangorn' package (Schliep, 2011) in R. The most appropriate model of evolution was
322 identified as a GTR+G+I model, allowing for variation in mutation rates between sites and the
323 presence of invariant sites, according to ML estimates calculated with the modelTest function
324 in 'phangorn'. Tree topology was then optimised by nearest-neighbour interchange (NNI),
325 using the optim.pml function. Finally, partitions allowing different rates of evolution for

nuclear and mitochondrial sequences or for every different gene were tested with the pmlPart function. Based on Akaike Information Criterion (AIC) scores, a partitioned model considering each gene separately was selected ($AIC_{no\ partition}$= 40049.83, $AIC_{nuclear/mitochondrial\ partition}$= 39575.70, $AIC_{partition\ by\ gene}$= 39405.41). The final rooted tree (Figure 1) was bootstrapped with 1000 replicates, and nodes with less than 70% support were collapsed into polytomies.

Statistical analyses

All analyses were carried out in R 3.3.1 (R Development Core Team, 2015). To test whether data collected in 2015 and 2016 could be analysed together, we examined differences in cyanogenic glucoside concentration and colour metrics (luminance, saturation, hue, internal contrasts and relative marking area on the forewing) between years, across the seven species collected in both (see Table 1). These were tested for each dependent variable, with a linear model and stepwise model simplification, allowing interactions between the independent variables of year, sex and species in the full model. Luminance, hue, and chromatic contrast were log-transformed to fit model assumptions.

As this investigation revealed significant effects of year and sex on both toxicity and colour metrics, we subsequently analysed the relationship between colour metrics and cyanogenic glucoside levels across species separately for each year. The data were also analysed across both sexes, and for males and females separately. To account for evolutionary relatedness between species, we used phylogenetic generalised least squares (PGLS) models, allowing λ to be fitted by maximum likelihood (Mundry, 2014), as implemented by the package 'caper' (Orme, 2013). We set out to test the relationship between cyanogenic glucoside concentration and all available colour metrics in a single model, but several of these variables were highly-

correlated. To deal with the problem of collinearity, we calculated variance inflation factors (VIFs) using the vif function in the 'car' package (Fox & Weisberg, 2011), and selected appropriate models 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. This yielded 3-4 different models per dataset (combination of sex and collection year; see Supporting Information S5). 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. Finally, small phylogenies suffer from a lack of power (Freckleton *et al*., 2002), making it difficult to accurately estimate parameters of phylogenetic signal, such as λ (Symonds & Blomberg, 2014; Arenas *et al*., 2015). We thus re-ran the same PGLS models with λ fixed to 1, corresponding to a Brownian model of evolution, to check whether our results were affected by a low estimate of phylogenetic signal.

With the exception of *Zygaena filipendulae*, for which quantitative signal honesty has already been investigated (Briolat *et al*., 2018b), sample sizes in this study are generally too low to explore intra-specific variation in toxin level and coloration, especially as the different collection years and localities used for each species would also have to be accounted for (see Table S1). However, we do investigate quantitative honesty in *Z. ephialtes*, a species for which all samples (N=21) originated from a single location in 2015 (see Supporting Information S6). Following Briolat *et al*. (2018b), we used multiple linear regression and stepwise model simplification to test the relationship between the concentration of cyanogenic glucosides in each sample and forewing coloration. As above, VIFs were used to determine

that models including saturation or hue should be run separately. Models included all other possible colour metrics (luminance, chromatic contrast, luminance contrast, relative marking area, and either hue or saturation), and sex was allowed to interact with every metric.

Results

Within species, signals and defences vary between years and between sexes

Analysing data from the seven species collected in both 2015 and 2016 revealed significant interactions between sex, year, and species when testing for differences in both cyanogenic glucoside concentration and measures of colour (Table 2). 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*; in males, a more complex picture emerged, with half the species showing an increase in toxicity between years, and half showing a decrease (Figure 2).

With regards to coloration, there was a significant interaction between year and species for all colour metrics analysed (Table 2). Individuals of all species collected in 2016 consistently displayed features suggesting that their markings would be more salient to predators (Figure 3). Specimens of species with red wing markings collected in 2015 had paler wing markings than those found in 2016, although the extent of the difference varied between species and sexes (Figure 3a; Table 2). They also displayed markings with higher saturation and hue values, more contrasting to the wing background colours, and occupying a larger proportion of the forewing (Figure 3b-f). This indicates that their markings had more intense colours, which were also relatively redder (or had higher UV reflectance), larger and more conspicuous. For *Rhagades pruni*, which displays iridescent blue markings, trends in luminance and hue were opposite to those seen in all other species (Figure 3a; 3d).

401 Nevertheless, this led to similar effects on marking saturation and internal contrasts in the
402 forewings, which were also higher in 2016 than 2015 in this species (Figures 3c; 3e).
403 Differences in the levels of signals and defences between years cannot be fully elucidated
404 with samples from only two years but may be linked to variation in climate and
405 environmental conditions (see Supporting Information S7). As sex and year do influence both
406 colour metrics and cyanogenic glucoside levels, these variables cannot be ignored in cross-
407 species analyses of signal honesty. Subsequent tests of the relationship between colour and
408 toxicity were thus carried out separately for each year and each sex.
409
410 In *Z. ephialtes*, for which sufficient samples were collected in a single year and location, some
411 significant associations were found between cyanogenic glucoside levels and measures of
412 coloration. Toxin levels increased with relative marking size in males, but decreased in
413 females (linear model, $F_{1,16}$=23.50, p=0.00018; Supporting Information S6). Moreover, across
414 both sexes, there was a negative relationship between the internal chromatic contrast of the
415 forewing and the concentration of cyanogenic glucosides (linear model, $F_{1,16}$=29.77,
416 p=0.000053; Supporting Information S6).
417
418 *Across species, there is no clear evidence of quantitative honesty*
419 Despite a small number of species sampled, our phylogenetic tree (Figure 1) is in broad
420 agreement with previously-published phylogenies of the Zygaenidae and the genus *Zygaena*
421 (Niehuis *et al*., 2006a; 2007). Using PGLS models to account for evolutionary relatedness, we
422 found very few correlations between cyanogenic glucoside concentration and any of our
423 measures of coloration (Supporting Information S5). While trends followed the same
424 direction whether males, females, or all specimens were considered, the significance of these

relationships did vary depending on sex (Table 3; Supporting Information S5). Moreover, significant correlations were not consistent between years (Table 3).

In addition, there were contrasting trends between luminance and colour, and most of the significant relationships between defences and certain signal properties were not indicative of quantitative honesty in the warning signals of these species. For samples collected in 2015, there was a positive correlation between luminance and cyanogenic glucoside concentration, suggesting that higher toxin levels were associated with paler markings (PGLS; across both sexes, $F_{1,7}$=13.41, p=0.0081; for females, $F_{1,6}$=14.98, p=0.0083; Figure 4a). This relationship was not significant for male samples, although the direction of the trend matched results in females and across both sexes (PGLS for males, $F_{1,7}$=5.92, p=0.051; Figure 4a). However, there was also a significant negative relationship, in females, between measures of colour (saturation, hue, and chromatic contrast between markings and background colours) and cyanogenic glucoside levels (PGLS; saturation, $F_{1,6}$=11.78, p=0.014; hue, $F_{1,6}$=15.68, p=0.0075; chromatic contrast, $F_{1,6}$=13.71, p=0.010; Figure 4b), indicating that higher toxin levels correlated with less intense, potentially less red, and less conspicuous markings, at least in terms of colour. In 2016, there was a positive correlation between internal luminance contrast and cyanogenic glucoside concentration, (PGLS; across both sexes, $F_{1,9}$=6.80, p=0.0029; in males, $F_{1,8}$=11.47, p=0.0095; Figure 5). This was not significant for females but the direction of the trend was consistent with those in males and across both sexes ($F_{1,6}$=3.96, p=0.094; Figure 5). 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 relationship between cyanogenic glucoside concentration and luminance, or any other colour metric in 2016 (Supporting Information S5).

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). When λ was set to 1, corresponding to a Brownian model of evolution, only one relationship, the positive correlation between luminance contrast and cyanogenic glucoside levels in males in 2016, remained significant ($F_{1,8}=11.61$, $p=0.0093$; Table 3).

Discussion

Overall, we found little evidence of quantitative signal honesty across the sampled species of Zygaenidae. Most colour metrics were not correlated with the concentration of defensive cyanogenic glucosides, whether male or female specimens were considered, and irrespective of the value of λ in phylogenetically-controlled analyses (Table 3, Supporting Information S7). The trends that did emerge from this dataset usually suggested a dishonest relationship between the strength of colour signals and defence levels, as higher toxin concentrations were associated with paler and less chromatically vibrant colours in 2015. Nevertheless, relationships between the concentration of cyanogenic glucosides and achromatic features could be seen to suggest quantitative honesty. When λ was estimated as a low value by maximum likelihood, some trends were significant in 2015, and, in particular, luminance was positively correlated with the concentration of cyanogenic glucosides across species. However, this did not lead to significant differences in achromatic contrast in the wings, and paler markings *per se* seem unlikely to constitute more salient markings. In terms of colour, only negative correlations with toxicity were found, suggesting dishonesty in signalling: saturation, hue, and chromatic contrast were all negatively correlated with cyanogenic glucoside levels in 2015, especially in females. Under a Brownian motion model of evolution, we found only one significant relationship, a positive correlation in 2016 between luminance contrast and cyanogenic glucoside concentration across males of these species. This could be

474 a potentially useful cue for predators, although there were no other significant correlations
475 between other measures of coloration and toxin levels in that year.
476
477 *Signal honesty across species – disentangling visual features*
478 Assessing the relevance of these correlations to predator behaviour is difficult, as determining
479 which aspects of signals and defences are most relevant to predators is not straightforward.
480 Chemical defences are generally assessed by measuring toxin levels, but these may vary
481 across body parts, total toxin amounts may be more relevant if prey are swallowed whole, and
482 distastefulness, inducing taste-rejection by predators (Skelhorn & Rowe, 2009; 2010) may not
483 covary with toxicity: in nudibranchs, similarly-distasteful red-spotted species were shown to
484 vary widely in their chemical profiles and lethality to brine shrimp (Winters *et al*., 2018). As
485 cyanogenic glucosides are bitter-tasting and can be dispensed to predators *via* defensive fluids
486 during an attack (Jones *et al*., 1962), measuring levels of linamarin and lotaustralin in burnet
487 moths should provide a relevant estimate of both unpalatability and toxicity. By contrast, the
488 question of which properties of warning signals predators most attend to is still somewhat
489 unresolved, and is poorly-studied in the context of the Zygaenidae.
490
491 Several lines of evidence suggest that chromatic features are the most important for avoidance
492 learning, at least for avian predators (Stevens & Ruxton, 2012). In the laboratory, learning
493 experiments, primarily with *Gallus gallus domesticus* chicks but also with *C. caeruleus* and
494 other passerines, suggest that chromatic features are generally more important than pattern for
495 avoidance learning, generalisation and memory in birds (Osorio *et al*., 1999a,b; Exnerová *et*
496 *al.*, 2006; Aronsson & Gamberale-Stille, 2008; Svádová *et al.*, 2009; Aronsson & Gamberale-
497 Stille, 2012a; Kazemi *et al*. 2014). These findings are broadly supported by several artificial
498 predation experiments in the wild, suggesting that colour is most critical in determining the

499 survival of model prey exposed to avian predators, although pattern can have an added effect
500 (Nokelainen *et al*., 2012; Finkbeiner *et al*., 2014; Arenas *et al*., 2015; Tan *et al*., 2016). As
501 such, colour generally seems more important than luminance in predator avoidance, and
502 several chromatic features are thought to be especially relevant to aposematic prey and their
503 predators. Field studies with model frogs and ladybirds have shown that chromatic contrast to
504 the natural background is particularly important (Hegna *et al*., 2011; Arenas *et al*., 2015),
505 while experiments presenting different species of Lycaeidae seed bug larvae to domestic
506 chicks suggest that prey with redder and more saturated signals are more strongly avoided
507 (Gamberale-Stille & Tullberg, 1999). Long-wavelength colours are also thought to be more
508 effective as warning signals, due to innate avoidance by some predators and their greater
509 stability under different lighting conditions (Arenas *et al.,* 2014). Finally, experiments with
510 artificial stimuli and natural prey items such as *Arctia plantaginis* (wood tiger moth) larvae
511 suggest that larger coloured markings generate greater avoidance (Forsman & Merilaita,
512 1999; Lindström *et al.*, 1999; Lindstedt *et al*., 2008; Smith *et al*., 2014). In an honest
513 signalling paradigm, we would thus expect stronger defences to be associated with stronger
514 signals, represented by more saturated, redder, larger and more conspicuous markings
515 (Stevens & Ruxton, 2012; Arenas *et al*., 2015). Yet, in our study, we found no association
516 between marking size and toxicity across species, and the few correlations between chromatic
517 features and toxicity we found in 2015 go against our expectations for quantitative honesty.
518
519 On the other hand, correlations between achromatic features, such as luminance and
520 luminance contrast to wing background colours, could also be utilised by predators.
521 Achromatic information may still be relevant to avian predators, potentially helping them to
522 distinguish small pattern elements (Stevens, 2007), triggering initial avoidance of aposematic
523 patterns (Sandre *et al*., 2010) and speeding up learning (Aronsson & Gamberale-Stille,

524 2012b). Luminance contrast in the pattern of prey items can also facilitate detection and
525 avoidance learning in experiments with mantids (Prudic *et al*., 2007), suggesting that it could
526 be a useful cue for some invertebrate predators, to which burnet moths are also exposed
527 (though note that mantids seem to lack colour discrimination, whereas many other
528 invertebrates have good colour vision). In 2016, we found that internal luminance contrast
529 was positively correlated with toxicity, so there is the potential for this signal property to act
530 as an honest signal. Yet it is also important to note that this trend was not linked to differences
531 in marking luminance, so was likely to be driven by changes in the luminance of the dark
532 background area of the moths' wings. As the dark pigment melanin is involved in many other
533 functions, from immune defences to thermoregulation (Solano, 2014), other selective
534 pressures besides avoiding predation could be responsible for the trends in wing background
535 luminance, and hence the relationship between luminance contrast and toxin levels. It would
536 be useful to know more about the response of avian predators to the different features of a
537 burnet moth-like pattern, to conclusively determine whether any of the correlations found
538 here could be relevant to predator behaviour in the wild. Across the board, comprehensively
539 examining variation in many aspects of their colour signals suggests a lack of quantitative
540 honesty across the zygaenid species studied here, but features such as luminance contrast
541 between wing markings and background colours may be worthy of further investigation.
542
543 The above conclusions across species are broadly supported by results found when testing
544 quantitative honesty within species in the Zygaenidae. In *Z. filipendulae*, few significant
545 associations emerged between measures of coloration and cyanogenic glucoside levels, and
546 the trends that were uncovered are more indicative of a negative relationship between signal
547 strength and toxicity: within some populations, higher cyanogenic glucoside concentrations
548 were associated with paler markings, while across populations, higher toxin levels were found

549 in females with smaller and paler markings (Briolat *et al*., 2018b). Within *Z. ephialtes*, we
550 found a negative correlation between toxin levels and internal chromatic contrast, similarly
551 suggesting a negative correlation between signal salience and defence levels. As in *Z.*
552 *filipendulae*, there is also a negative relationship between the relative size of the red markings
553 and cyanogenic glucoside concentration, such that more toxic females have smaller markings.
554 However, this relationship is reversed in males, raising the possibility that the area of red
555 markings could act as an honest signal of toxicity in males. Aside from this potentially
556 interesting difference between sexes, which may be related to the overall smaller size of
557 males, there is little evidence of quantitative honesty within the Zygaenidae studied so far. As
558 already discussed in the case of *Z. filipendulae* (Briolat *et al*., 2018b), the highly aversive
559 nature of cyanogenic glucosides and fluctuations in individual toxin content over a moth's
560 lifetime, depending on reproductive events, might limit the usefulness of quantitative honesty
561 in burnet moths. More data would be required to test within-species variation in a greater
562 number of zygaenid species, and determine whether this is a family-wide pattern.
563
564 Relatively few studies have explored the relationship between coloration and the levels of
565 chemical defences across species while accounting for phylogeny as we do here (but see
566 Summers & Clough, 2001; Cortesi & Cheney, 2010; Santos & Cannatella, 2011), so the
567 present study makes a rare contribution to the field. While some species have very small
568 sample sizes (N=1 or N=2), these were still included in the analysis as increasing the number
569 of species is key to greater reliability in phylogenetic analyses. The absence of signal honesty
570 in the Zygaenidae is contrary to the results of other studies of signal honesty across species, in
571 ladybirds (Arenas *et al*., 2015) and nudibranchs (Cortesi & Cheney, 2010), as well as some
572 work in poison frogs (Summers & Clough, 2001; Santos & Cannatella, 2011, but see Darst *et*
573 *al*., 2006). It 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 & Mappes, 2004; Nokelainen *et al.*, 2014) and life-history traits (Longson & 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 be expected (Speed & 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. Sampling host plants from collection sites wherever possible, we measured the cyanogenic glucoside content of plant tissues the larvae were likely to feed on (Supporting Information S2) to address this issue. Although not comprehensive, our results suggest that, among our samples, only *Z. filipendulae* and *Z. occitanica* were feeding on plants with high levels of cyanogenic glucosides. *Z. trifolii*, *Z. cynarae*, *R. pruni* and in some cases *A. infausta* may also have been able to both sequester the cyanogenic glucosides linamarin and lotaustralin from their hostplants as well as synthesise them themselves (Davis & Nahrstedt, 1986; Zagrobelny *et al*., 2014), while the other species appear to have relied entirely on *de novo* synthesis. Moreover, behavioural differences between the species in the *Zygaena* genus and the others will modulate their exposure to predators. The Procridinae behave more like cryptic species, flying rapidly and seeking to evade capture, while red-spotted burnet moths are much more sluggish (Hofmann & Tremewan, 2017) and highly visible. Finally, although many of these species do co-exist in the wild, our samples were collected from many different locations, so were not exposed to the same community of predators.

Considerations for cross-species studies of signal honesty

Sex-specific trends in quantitative honesty found for *Z. filipendulae* (Briolat *et al*., 2018) and *Z. ephialtes* suggest that differences between sexes should be considered in studies of signal

599 honesty. The costs and benefits of aposematic signalling may vary between males and females
600 of warningly-coloured species, due to size dimorphism, trade-offs related to sexual signalling,
601 and variation in habitat use and behaviour, modulating their exposure to predators. In
602 sexually-dimorphic seven-spot ladybirds (*Coccinella septempunctata*) an honest relationship
603 between elytra carotenoids and coccinelline levels was only found in females, a result
604 attributed to greater resource-limitation or greater benefits of aposematic signalling in the
605 larger sex (Blount *et al.*, 2012). Burnet moths are similarly sexually-dimorphic, with larger
606 females (Naumann *et al*., 1999), but other factors may also affect the economics of
607 aposematic signalling: while both sexes are highly visible at rest, males are generally more
608 active (Naumann *et al*., 1999), and there is some limited evidence that visual signals could
609 play a role in sexual signalling, at close range (Zagatti & Renou, 1984; Koshio, 2003;
610 Friedrich & Friedrich-Polo, 2005), and at certain times of day (Hofmann & Kia-Hofmann,
611 2010). Across species, trends were broadly similar between sexes in this study, but the
612 significance of these relationships varied, suggesting that ignoring differences between sexes
613 could mask interesting results. This is an important consideration, as no existing studies of
614 quantitative honesty across aposematic species and populations analyse males and females
615 separately, even in taxa in which males and females are known to differ (e.g. in ladybirds;
616 Arenas *et al*., 2015).
617
618 Our study also revealed considerable variation, in both coloration and toxicity, between
619 individuals collected in two different years. These differences are unlikely to be caused by
620 inconsistencies in our experimental procedures. While caterpillars were raised under natural
621 conditions during collection trips, subsequent rearing conditions were kept as consistent as
622 possible between specimens collected in 2015 and 2016. Moreover, differences in colour
623 between years were found even among *Z. trifolii* specimens, collected as pupae from the same

location and placed in an incubator with the same settings until eclosion, suggesting that conditions prior to euthanasia were not responsible for this variation. Preliminary experiments verified that the time that specimens were kept in the -80 °C freezer between termination and photography did not impact coloration. Methods and equipment used for image capture did not vary between years, and all images from both seasons were processed and analysed together. Finally, we verified that differences in toxin levels were not caused by variations in the sensitivity of the LC-MS machine and column used, by re-running a subset of samples from both years together. While existing studies of signal honesty in aposematic species do not consider temporal variation in signal and defence traits, our study suggests that seasonal variation may have an impact on these traits.

With only two years of data, it is difficult to explain the observed patterns of between-year variation, but environmental conditions, linked to variation in weather across years (see Supporting Information S7), are likely to impact investment in coloration and chemical defences in burnet moths. Variation in coloration in tiger moths (Erebidae) has been linked to fluctuations in local ecological conditions (Galarza *et al*., 2014), and in particular temperature (Goulson & Owen, 1997; Lindstedt *et al*., 2009). Climate may also indirectly affect resource allocation to signals and defences in aposematic species, via effects on their host plants. Cyanogenic plants possess highly variable levels of defensive chemicals, strongly affected by environmental conditions (Gleadow & Woodrow, 2002). The effects of temperature have been well-documented in both *Trifolium repens* (white clover; Daday, 1954a,b; 1958; De Aráujo, 1976; Stochmal & Oleszek, 1997; Richards & Fletcher, 2002 and *Lotus corniculatus* (bird's foot trefoil), a key host plant of several Zygaenidae (Ellis *et al*., 1977; Jones, 1977; Salgado *et al.*, 2016). For the species relying completely on *de novo* synthesis of cyanogenic glucosides, plant productivity may still be important. For example, nitrogen limitation will

lead to reduced investment in cyanogenic glucosides in burnet moths, due to trade-offs with other products, as suggested by the hypothesized breakdown of cyanogenic glucosides during pupation to fuel chitin synthesis (Zagrobelny *et al.*, 2007b). Interestingly, all the species in which cyanogenic glucoside levels decreased between years in males (*A. infausta*, *R. pruni* and *Z. sarpedon*) 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. Comparing host plant levels of cyanogenic glucosides and other nutritional resources to moth toxin levels and coloration across years would help elucidate the relationship between environmental conditions, host properties and aposematic phenotypes. This type of longitudinal study 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.

In conclusion, the present work deepens our understanding of the relationship between signals and defences across species, by contributing to the small number of studies testing signal honesty across closely-related aposematic species, with sophisticated methods for quantifying chemical defences, phylogenetic controls and measures of coloration accounting for predator vision. We find no clear evidence of quantitative signal honesty across the sampled species of Zygaenidae, especially not with regards to those aspects of appearance most likely to be salient to predators, a result likely attributable to varying costs of signal and defence production across species. Our study also highlights the importance of considering differences between sexes and temporal variation in analyses of signal honesty moving forward.

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<u>Table 1:</u> Number (N), species and host plants of photographed specimens.

Species	Country	Hostplant at collection site	N	
			2015	2016
Aglaope infausta (Linnaeus, 1767)	France	*Cotoneaster* sp., *Crateagus* sp., *Prunus* sp. (Rosaceae)	21	17
Rhagades pruni (Denis & Schiffermüller, 1775)	France	*Prunus spinosa* (Rosaceae)	8	8
Theresimima ampellophaga (Bayle-Barelle, 1808)	France	*Vitis* sp. (Vitaceae)	0	1
Zygaena cynarae (Esper, 1789)	France	*Peucedanum cervaria* (Apiaceae)	1	0
Zygaena ephialtes (Linnaeus, 1767)	France	*Securigera varia* (Fabaceae)	21	0
Zygaena erythrus (Hübner, 1806)	France	*Eryngium campestre* (Apiaceae)	0	11
Zygaena exulans (Hohenwarth, 1792)*	France	Polyphagous – host plant unknown	0	5
Zygaena filipendulae (Linnaeus, 1758)	Denmark, France, UK	*Lotus corniculatus, Dorycnium pentaphyllum, Hippocrepis comosa* (Fabaceae)	107	8
Zygaena lonicerae (Scheven, 1777)	France	*Trifolium* sp. (Fabaceae)	0	1
Zygaena minos (Denis & Schiffermüller, 1775)	France	*Pimpinella saxifraga* (Apiaceae)	1	1
Zygaena occitanica (Villiers, 1789)	France	*Dorycnium pentaphyllum* (Fabaceae)	0	2
Zygaena sarpedon (Hübner, 1790)	France	*Eryngium campestre* (Apiaceae)	6	2
Zygaena transalpina (Esper, 1780)	France	*Hippocrepis comosa*, *Securigera varia* (Fabaceae)	3	13
Zygaena trifolii (Esper, 1783)	UK	*Lotus pedunculatus* (Fabaceae)	9	14

*: collected as pupae only

Table 2: Results of stepwise simplification of 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	3.21	5, 192	0.0083	**
Luminance	Sex:Species:Year	2.35	5, 192	0.042	*
Saturation	Sex:Species:Year	1.42	5, 192	0.22	-
	Sex:Year	0.17	1, 197	0.68	-
	Sex:Species	1.49	5, 198	0.20	-
	Species:Year	4.17	6, 203	<0.001	***
	Sex	5.87	1, 203	0.016	*
Hue	Sex:Species:Year	0.82	5, 192	0.54	-
	Sex:Year	0.061	1, 197	0.80	-
	Sex:Species	1.53	5, 198	0.18	-
	Species:Year	27.95	6, 203	<0.001	***
	Sex	4.99	1, 203	0.027	*
Chromatic contrast	Sex:Species:Year	0.47	5, 192	0.80	-
(JNDs)	Sex:Year	0.0056	1, 197	0.94	-
	Sex:Species	3.08	5, 198	0.011	*
	Species:Year	3.32	6, 198	0.0039	**
Achromatic contrast	Sex:Species:Year	1.12	5, 192	0.35	-
(JNDs)	Sex:Year	2.06	1, 197	0.15	-
	Sex:Species	5.57	5, 198	<0.001	***
	Species:Year	10.67	6, 198	<0.001	***
Relative marking area	Sex:Species:Year	0.84	5, 192	0.35	-
(%)	Sex:Year	0.0013	1, 197	0.97	-
	Sex:Species	5.45	5, 198	<0.001	***
	Species:Year	2.97	6, 198	0.0085	**

Table 3: Results of stepwise simplifications of PGLS models testing the relationship between cyanogenic glucoside concentration ([CNGlc]) and colour metrics, yielding a significant result with λ estimated by maximum likelihood ($\lambda=1*10^{-6}$), and re-run with λ=1 (Brownian motion model of evolution).

Dataset	Model	Results with $\lambda=1*10^{-6}$	Results with λ=1
2015, overall	[CNGlc] ~ luminance	$F_{1,7}$=13.41, p=0.0081	$F_{1,7}$=5.45, p=0.052
2015, males	[CNGlc] ~ luminance	$F_{1,6}$=5.92, p=0.051	$F_{1,6}$=2.67, p=0.15
2015, females	[CNGlc] ~ luminance	$F_{1,6}$=14.98, p=0.0083	$F_{1,6}$=4.37, p=0.082
2015, females	[CNGlc] ~ saturation	$F_{1,6}$=11.78, p=0.014	$F_{1,6}$=3.56, p=0.11
2015, females	[CNGlc] ~ hue	$F_{1,6}$=15.68, p=0.0075	$F_{1,6}$=5.28, p=0.061
2015, females	[CNGlc] ~ chromatic contrast	$F_{1,6}$=13.71, p=0.010	$F_{1,6}$=4.58, p=0.076
2016, overall	[CNGlc] ~ luminance contrast	$F_{1,9}$=6.80, p=0.028	$F_{1,9}$=4.24, p=0.070
2016, males	[CNGlc] ~ luminance contrast	$F_{1,8}$=11.47, p=0.0095	$F_{1,8}$=11.61, p=0.0093
2016, females	[CNGlc] ~ luminance contrast	$F_{1,6}$=3.96, p=0.094	$F_{1,6}$=3.64, p=0.11

Figures:

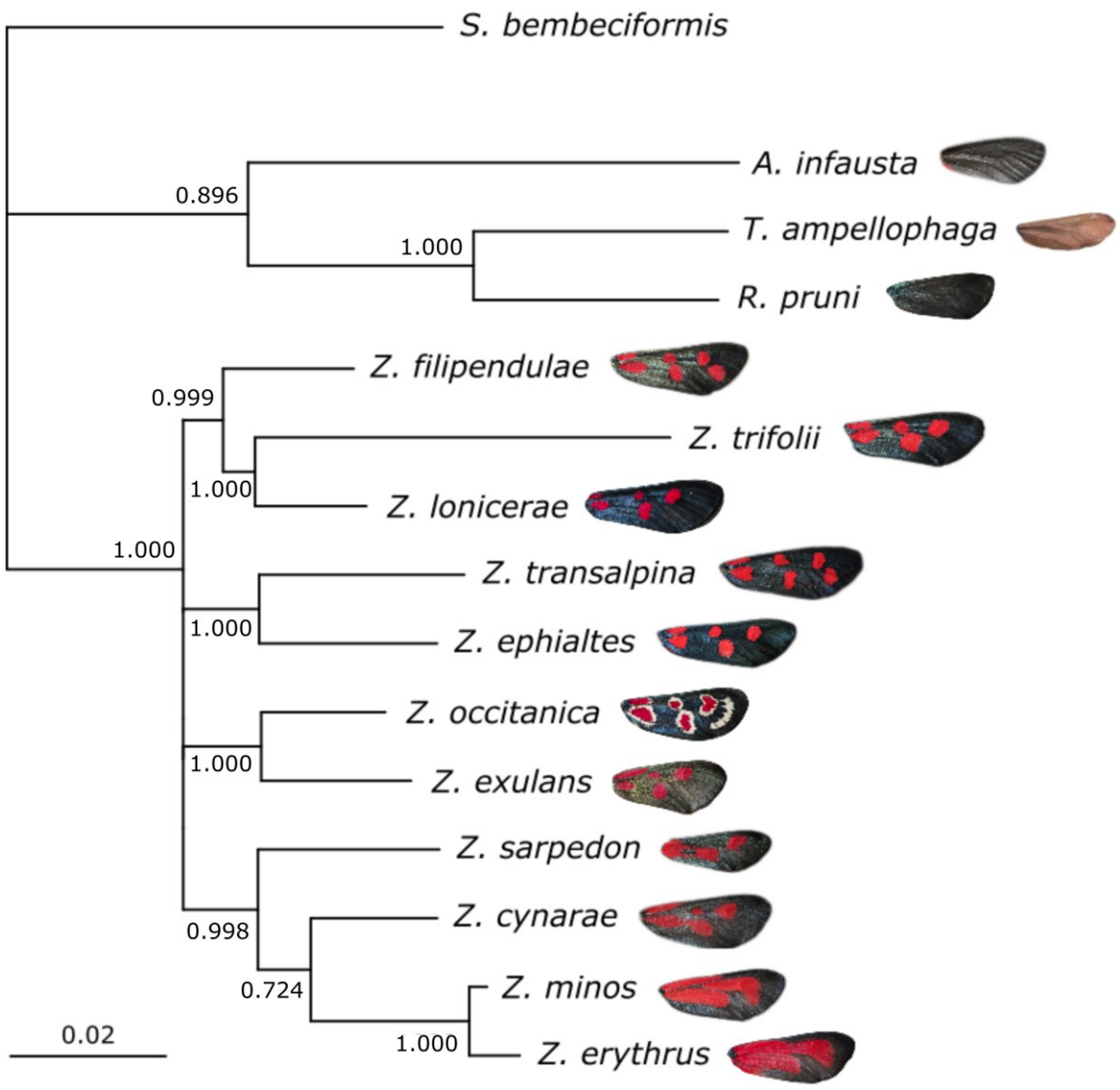

Figure 1: Phylogenetic tree of the Zygaenidae used in this study. Branch labels represent bootstrap values for 1000 replicates; the scale bar corresponds to genetic distances between sequences, along branch lengths. Image credits: *T. amphellophaga,* adapted from www.lepinet.fr/especes/nation/lep/index.php?id=02140, ©Daniel Morel; all other images authors' own.

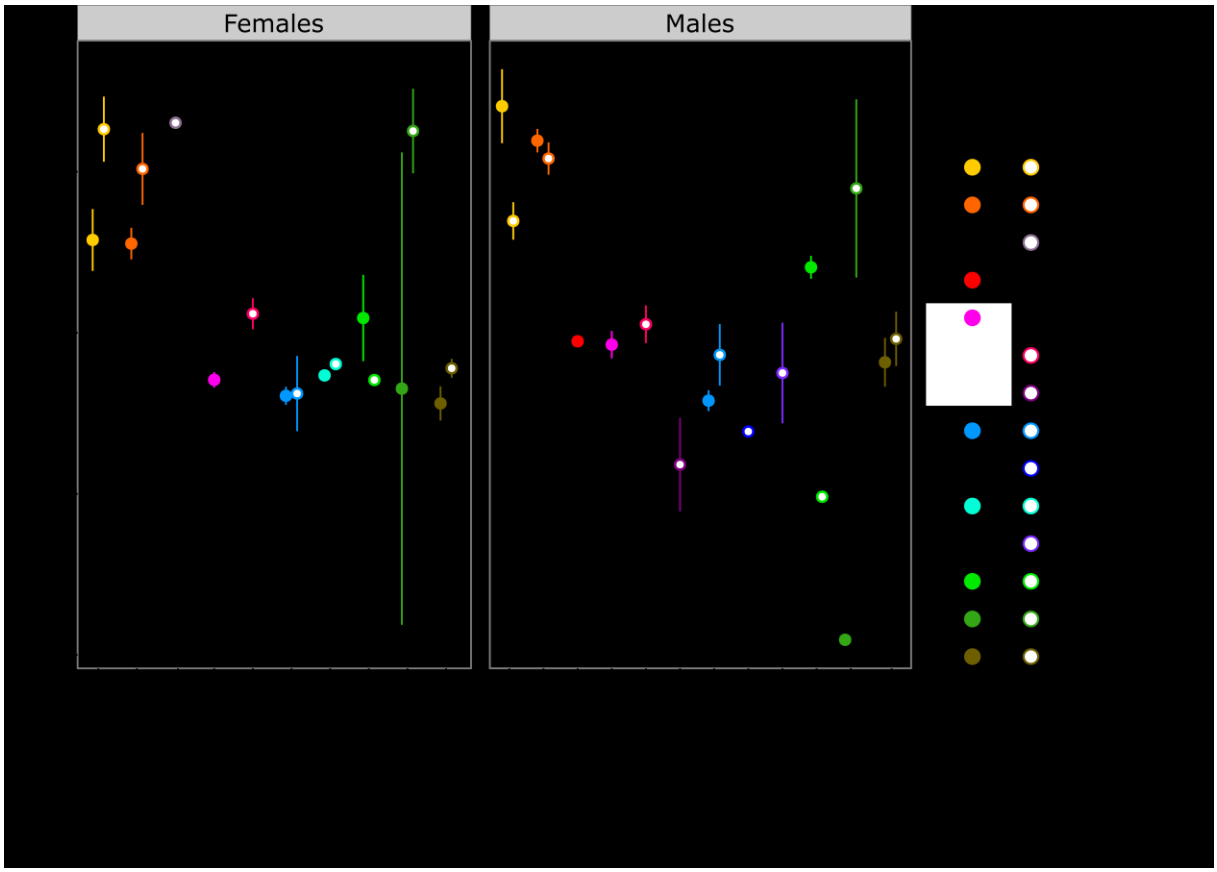

Figure 2: Mean and standard error of the concentration of cyanogenic glucosides (CNGlc) in males and females of each species. Filled circles = samples collected in 2015; open circles = samples collected in 2016.

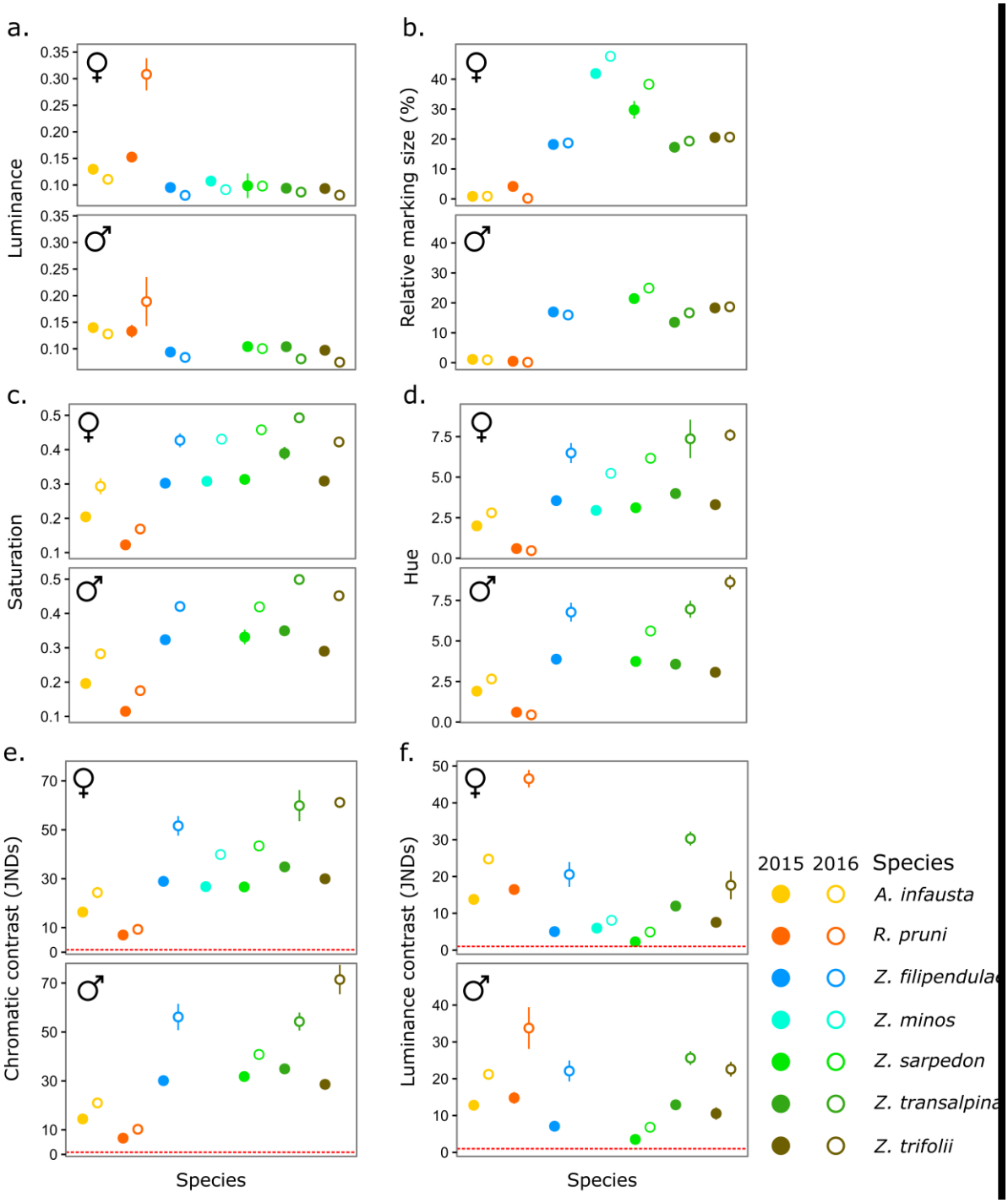

Figure 3: Mean values and standard errors of coloration for males and females of species collected in 2015 and 2016. Filled circles = samples collected in 2015; open circles = samples collected in 2016. In (b), relative marking size is measured as the percentage of the forewing area occupied by contrasting markings. In (e) and (f), the red dashed line represents the threshold for discrimination, JND = 1.

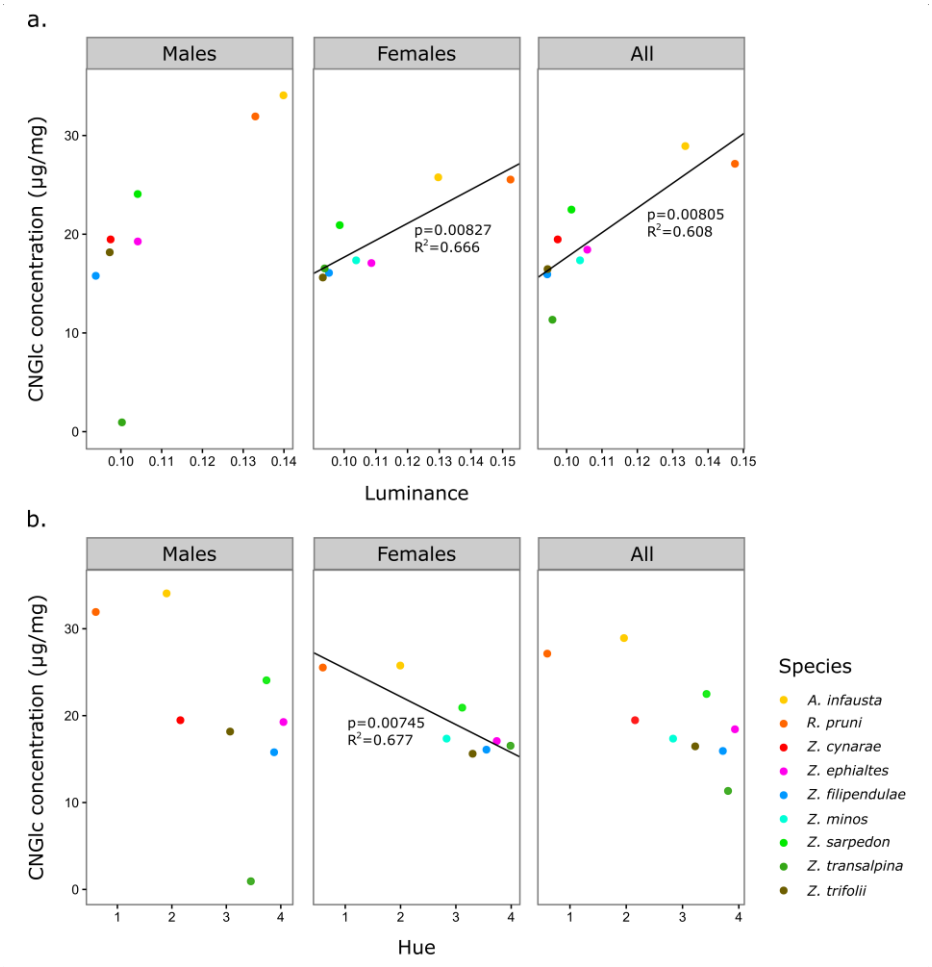

Figure 4: Mean cyanogenic glucoside (CNGlc) concentration and (a) luminance and (b) hue in species sampled in 2015, calculated in males, females and across both sexes. Lines represent the results of PGLS models.

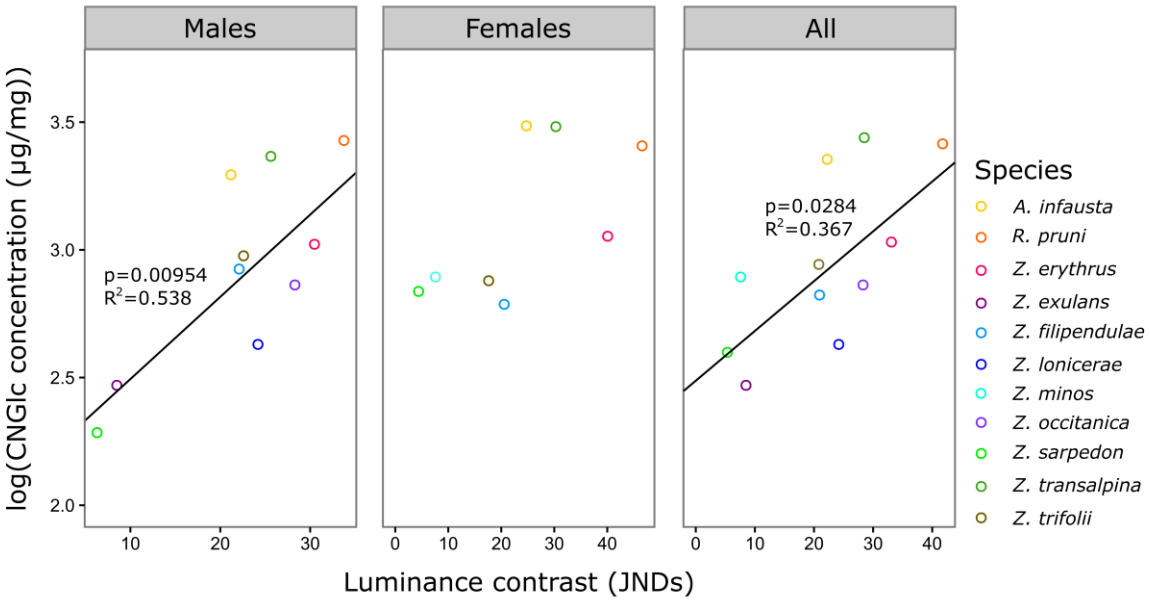

Figure 5: Mean log-transformed cyanogenic glucoside (CNGlc) concentration and luminance contrast in species sampled in 2016, calculated in males, females and across both sexes. Lines represent the results of PGLS models.