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Characterizing the pigment composition of a variable warning signal of *Parasemia plantaginis* larvae

Carita Lindstedt*[1], Nathan Morehouse[3,4], Hannu Pakkanen[2], Jérôme Casas[3], Jean-Philippe Christides[3], Kimmo Kemppainen[2], Leena Lindström[1] and Johanna Mappes[1]

[1]*Centre of Excellence in Evolutionary Research, Department of Biological and Environmental Sciences, P.O. Box 35, FI-40014 University of Jyväskylä, Jyväskylä, Finland;* [2]*Department of Chemistry, P.O. Box 35, FI-40014 University of Jyväskylä, Jyväskylä, Finland;* [3]*Institut de Recherche en Biologie de l'Insecte, IRBI UMR CNRS 6035, Université de Tours, 37200 Tours, France; and* [4]*School of Life Sciences, Arizona State University, Tempe, Arizona 85287-4501, USA*

Summary

1. Aposematic animals advertise their defences to predators via warning signals that often are bright colours combined with black patterns. Predation is assumed to select for large pattern elements and conspicuousness of warning signals because this enhances avoidance learning of predators. However, conspicuousness of the colour pattern can vary among individuals of aposematic species, suggesting that warning signal expression may be constrained by opposing selection pressures. If effective warning signals are costly to produce, variation in signal expression may be maintained via physiological trade-offs. To understand the costs of signalling that might underlay both physiological and ecological trade-offs, it is crucial to identify the pigments involved in aposematic traits, how they or their precursors are acquired and how their production and/or deposition interact with other physiological processes.

2. We characterized the pigments responsible for the genetically and phenotypically variable orange-black warning signal of the hairy larvae of an Arctiid moth, *Parasemia plantaginis*. We tested orange and black coloured hairs for the presence of six candidate pigment types using high-performance liquid chromatography, spectral and solubility analyses.

3. After excluding the presence of carotenoids, ommochromes, pterins and pheomelanins in orange hairs, our results suggest that tiger moth larvae produce their orange warning signal by depositing both diet-derived flavonoids and trace levels of synthesized eumelanin in their hairs. The nearby black hairs are coloured by eumelanin.

4. In light of previous studies, we conclude that although a large orange patch increases the 1larvae's antipredator efficacy, variation in the size of orange patches within a population can be driven by scarcity of flavonoids in diet. However, traces of eumelanin found in the orange hairs of the larvae may also play a significant role in the maintenance of the signal pattern on poor quality diets.

5. The goal of the future studies will be to test the condition dependence of pigment deposition in aposematic colour patterns by directly manipulating relevant nutritional parameters such as dietary flavonoid or nitrogen content (i.e. amino acid content).

Key-words: Arctiidae, melanins, flavonoids, warning colours, insects, aposematism

Introduction

Aposematic animals often use bright colouration to advertise their secondary defences to predators (Poulton 1890; Ruxton, Sherratt & Speed 2004). Selection by predators favours these conspicuous warning signals because large and bright patterns are more easily learnt and remembered by predators (Guilford & Dawkins 1991; Forsman & Merilaita 1999; Gamberale-Stille & Tullberg 1999; Lindström *et al.* 1999; Lindstedt, Lindström & Mappes 2008). Therefore, predator psychology and its relationship to the effectiveness of warning signals have gained a lot of attention in studies of warning colour evolution (review in Ruxton, Sherratt & Speed 2004). However, recent work has

*Correspondence author. E-mail: carita.lindstedt@jyu.fi

brought new perspectives to the evolution of aposematism by studying the prey's selective environment as a whole; e.g. it has been shown that environmental constraints (Grill & Moore 1998; Ojala, Lindström & Mappes 2007; Tullberg *et al*. 2008; Lindstedt, Lindström & Mappes 2009a; Lindstedt *et al*. 2009b) and population densities (Sword 1999, 2002) can affect warning signal expression in addition to selective pressures arising from predators.

Many studies considering sexual ornamentation in animals highlight that to be able to study ecological and evolutionary trade-offs between signal expression and other fitness components in detail, it is important to link them with the physiological mechanisms that affect signal expression (e.g. Hill & Montgomerie 1994; Hooper, Tsubaki & Siva-Jothy 1999; Grether, Hudon & Endler 2001). Extending these ideas to the study of warning signals suggests that if the pigments deposited in a warning colour pattern are expensive to acquire or produce, trade-offs between effective warning signal expression and other energetic or resource requirements may occur. To understand possible physiological (and ecological) trade-offs, one first has to know the biochemical composition of aposematic traits. However, the biochemical basis of aposematic colouration has been studied in detail in surprisingly few species (but see Bezzerides *et al*. 2007; Sandre *et al*. 2007). Here, we sought to characterize the pigments responsible for the variable orange-and-black aposematic colouration of larval wood tiger moths (*Parasemia plantaginis*), in the hope that our findings would provide some insight into possible ecological and physiological trade-offs associated with the production of this aposematic colour pattern.

Insects, and Lepidoptera in particular, utilize a variety of pigments (carotenoids, flavonoids, pterins, ommochromes and melanins being most common) to produce oranges and blacks such as those found in the trait in question (Fox 1976; Kayser 1985). These pigments have distinct biochemical properties and linkages to other physiological processes. For example, in previous studies of the chemical basis of warning colouration, bright orange, red and yellow colour elements have been found to be produced by carotenoids (Bezzerides *et al*. 2007; Sandre *et al*. 2007). Animals cannot synthesize carotenoids and must obtain carotenoid pigments directly from their diet (Fox 1976; Hill, Inouye & Montgomerie 2002). Therefore, their scarcity in diet can constrain colour expression (Hill & Montgomerie 1994; Carroll *et al*. 1997; Grill & Moore 1998) and maintain variation in signal production (Grether, Hudon & Millie 1999; Hooper, Tsubaki & Siva-Jothy 1999; Senar, Figuerola & Domenech 2002). Potential linkages to free-radical scavenging and immune support (Burton 1989; Olson 1989; Lozano 1994; Camplani, Saino & Møller 1999; Hõrak *et al*. 2001) may also produce competition between colouration and other physiological needs for carotenoid pigments.

Flavonoids and flavins are less well studied from this perspective, but they share a number of ecological and physiological characteristics with carotenoids. These classes of pigments are employed by many insects in orange, yellow and UV-absorbing colour patterns (Kayser 1985, Burghardt *et al*. 2000, Knuttel & Fiedler 2001), must be derived from dietary plant sources (Knuttel & Fiedler 2001), and may have similar antioxidant and immunological functions (Kayser 1985; McGraw 2005). Arguments paralleling those outlined above, and already extensively propounded elsewhere for carotenoids may also apply to the use of flavonoids and flavins in insect colouration.

While insects do employ diet-derived pigments in their colour patterns, they perhaps more commonly use pigments that they synthesize *de novo*. Pterins, ommochromes and melanins are pigments that can produce orange colouration and must be synthesized from basic precursors (Kayser 1985). Melanin is also by far the most common pigment used in black pigmentation (Fox 1976). Because these pigment types require access to pools of basic precursors, they are likely to compete directly with other physiological processes (Stoehr 2006). For example, melanins are synthesized from tyrosine, an essential amino acid that is required for dopamine and serotonin production (Sugumaran 2002). Ommochromes are derived from tryptophan, another essential amino acid linked to a handful of basic physiological processes (Linzen 1974). Pterins are purine-derivatives, requiring large amounts of guanosine diphosphate for their synthesis. Production of large quantities of any of these pigments is therefore likely to result in physiological trade-offs (Stoehr 2006). It is also worthwhile to note that many insects, including *P. plantaginis*, are herbivorous, feeding on plant material that is predominantly carbohydrate-based, and therefore poor in amino acids and purine-derivatives (reviewed in Mattson 1980; Chown & Nicolson 2004). This suggests that supply of required precursors may be limited by ecological availability (e.g. host-plant quality, Talloen, Van Dyck & Lens 2004; Freitak *et al*. 2005) offering one possible mechanism which may be responsible for producing variation in colour patterns. In addition, colour can be a result of more than just one pigment type. For example, Grether, Hudon & Endler (2001) found that male guppies (*Poecilia reticulate*) use both carotenoids and pteridine pigments in the orange spots that they display to the females, which may have important implications for the signalling utility of these colour patterns. Natural melanins may also often occur in conjunction with the pigment types mentioned above or may contain mixed polymers which produce intermediate colours (Kayser 1985).

Given the variety of potential connections between pigments and other physiological processes, we sought to characterize the pigments responsible for the colouration of the aposematic larvae of the wood tiger moth (*Parasemia plantaginis*). These larvaeare polyphagous herbivores which have an aposematic pattern consisting of an orange patch of hair on the dorsal side of their black and hairy body. This pattern is known to exhibit considerable phenotypic variation, influenced by both environmental and genotypic factors (Ojala, Lindström & Mappes 2007; Lindstedt, Lindström & Mappes 2008, 2009a; Lindstedt *et al*. 2009b) (Fig. 1). Thus, even though the frequency of intermediate orange signal sizes (proportion of orange approx. 40–50%) appears to be high, the

Fig. 1. Variation in the size of the orange patch and black pattern elements in aposematic *Parasemia plantaginis* larvae.

proportion of the orange in larval colouration can vary approximately from 20% to 80% (Ojala, Lindström & Mappes 2007; Lindstedt, Lindström & Mappes 2009a). Predators learn to avoid larvae with large orange patches (proportion of orange $>60\%$) faster, suggesting selection toward larger signal sizes (Lindstedt, Lindström & Mappes 2008). Larvae are also chemically defended by sequestered iridoid glycosides (Lindstedt *et al*. 2009b). Despite possible directional selection by predators, existing variation in the size of the orange patch indicates that opposing selection pressures may constrain effective warning signal expression (Ojala, Lindström & Mappes 2007; Friman *et al*. 2009; Lindstedt, Lindström & Mappes 2009a). Moreover, if the pigments deposited in the colour signal pattern are expensive to acquire or produce, physiological constraints may restrict optimal signal expression, thus maintaining the observed variation. In an earlier study Ojala, Lindström & Mappes (2007) found that larvae reared on an artificial diet poor in flavonoids and carotenoids (Ojala *et al*. 2005) expressed smaller orange patches compared to larvae reared on a high quality diet that was rich in flavonoids and carotenoids. This indicates that pigment deposition to the warning signal is susceptible to variation in environmental conditions and therefore effective signal expression may be constrained by environmental factors. To gain insight into the physiological mechanisms underlying warning signal variation in *P. plantaginis*, we sought to identify the orange and black pigments composing the signal using high-performance liquid chromatography (HPLC), solubility and fluorescence analyses, as well as characterization of the pigments *in vivo* and *in vitro* via spectrometry. More specific information regarding the biochemical composition of this warning signal should allow for future work to study the physiological aspects of warning signal expression in detail and address questions such as: How expensive are the pigments responsible for the warning signal to produce or acquire? Are the pigments known to be linked to any immunological or physiological functions? And what kind of resources (e.g. in diet) could limit their production?

Materials and methods

BIOLOGICAL MATERIAL

Larvae for the pigment samples were fed with a mixture of *Taraxacum* spp. leaves and lettuce to ensure high survival rate of the larvae during rearing. Because the colour pattern of *P. plantaginis* larvae is based on the colour of hairs, orange and black hairs from the larvae were collected with forceps and stored in Kimax-tubes from frozen late instar larvae. Hairs were stored at −20 °C and sample amounts used for the analyses were 20 mg per analysis. We tested the orange and black hairs for presence of six candidate pigment groups (carotenoids, melanins, flavonoids and flavins, pteridines and purine derivatives, ommochromes, and papiliochromes).

FLUORESCENCE AND SOLUBILITY TESTS

Fluorescence and solubility of the hair pigments were tested first to shorten the list of possible pigment types. Pterins and purine derivatives, papiliochromes, flavonoids and flavins are fluorescent when stimulated with ultraviolet (UV) light, whereas melanins, ommochromes and carotenoids do not fluoresce (Fox 1976; Kayser 1985; Umebachi 1985). We subjected orange and black hair samples to short- and long-wave UV light (254 and 366 nm) using a Mineralight lamp (model UVGL-58; UVP Inc., San Gabriel, CA, USA), and noted any fluorescence visible to the naked eye. In addition, extracted pigments in solution were evaluated for fluorescence.

Pterins and purine derivatives, papiliochromes and flavonoids are all soluble in strong acids and bases (Fox 1976; Kayser 1985; Umebachi 1985). Therefore, to test for the presence of these pigments, we micronized hair samples using a ball-mill, and placed the ground samples in 0·1 M NaOH overnight. Flavonoids and papiliochromes are also soluble in aqueous and organic (alcohol) solvents (Kayser 1985; Umebachi 1985; Burghardt, Proksch & Fiedler 2001). We therefore micronized an additional set of samples and placed them in 90% methanol overnight. The following day, both sets of samples were microcentrifuged for 20 min at 13 000 **g**, and the resulting supernatant measured for the presence of extracted pigmentary material using an absorbance spectrophotometer across a wavelength range from 190 to 900 nm (DU 520; Beckman Coulter, Fullerton, CA, USA). Resulting spectra were compared to published spectral profiles of each pigment class. Similar protocols were also followed using acidified methanol to screen for ommochromes (see below).

CHARACTERIZATION BY REFLECTANCE SPECTROMETRY

We evaluated the spectral properties of the pigments *in vivo* by taking spectral reflectance measurements from samples of *P. plantaginis* hairs mounted on black cardstock following methods outlined in Rutowski *et al*. (2005). Samples were illuminated using an pulsed-xenon light source (Model PX-2; Ocean Optics, Dunedin, FL, USA) positioned at the zenith above the sample and oriented normal to the sample's surface. Spectra were collected from the illuminated area using an optical fibre positioned at 45° below the zenith and connected to a spectrophotometer (USB2000; Ocean Optics). All spectra were normalized in relation to a magnesium oxide white standard. These spectral measures gave us the opportunity to compare the reflectance spectra of both orange and black hairs to known spectra of different pigments. In particular, this allowed us to compare carotenoids and melanins, which are both non-fluorescent under short-wavelengths,

but have distinctive spectral signatures. Carotenoids have their strongest absorbance peaks in the mid-wavelength ranges (450–550 nm), but absorb little if any UV light (300–400 nm), resulting in characteristic UV reflectance off of carotenoid-containing integumentary structures (e.g. Andersson & Prager 2006). Melanins, on the other hand, absorb strongly at all visible and UV wavelengths, producing reflectance spectra that increase slowly and monotonically from UV to long wavelengths (Sarna & Sealy 1984).

HPLC-ANALYSIS

Carotenoid analysis

Carotenoid analysis was carried out as in McGraw *et al.* (2004, 2005) with modifications. About 5 mg of hair sample was placed into a screw-capped tube and 2 mL acidified pyridine (three drops of 12 N HCl in 50 mL of pyridine) was added. Tubes we filled with argon gas and then closed tightly. Sample tubes were placed in an oven where they were kept for 3 h at 95 °C. After cooling to room temperature, 2 mL of water and 1 mL of hexane: tert-butyl methyl ether (TBME) (1:1) was added into the tubes. Tubes were shaken for 5 min and centrifuged at 1619 **g** for 5 min. Non-polar organic phase (upper layer) was removed and placed into HPLC tubes and the hexane: TBME solution was evaporated with nitrogen gas. Dried samples were redissolved in 250 μL of HPLC mobile phase (acetonitrile:methanol, 1:1, v/v). The bottom layer consisting of water and pyridine was evaporated to dryness with nitrogen gas and analysed for pheomelanin (see below).

The HPLC system (Waters, Milford, MA, USA) consisted of a Waters 510 HPLC pump with a flow rate set to 0·5 mL min^{-1}. A sample of 50 μL was injected with Waters 717 plus autosampler. Samples were run through a Gemini reversed phase C18 column of 150 mm length × 3 mm i.d. particle size 5 μm (Phenomenex, Torrance, CA, USA) analytical column, maintained at 32 °C. Data were acquired between 250 and 600 nm with Waters 996 Photodiode Array Detector. Processing of the data was done with Millenium32 software (Waters Corp., Milford, MA, USA).

Ommochrome analysis

Several synthetic ommochromes were obtained in the laboratory by the method of Butenandt (Butenandt & Schaefer 1962) and identified by HPLC coupled with mass spectrometry. These ommochromes were then used as standards in the following analysis. The hair samples were crushed in 2 mL acidified methanol using a glass tissue grinder. Each sample was transferred to a 2 mL Eppendorf tube and incubated overnight at room temperature. The method of separation of the different compounds was based on a technique of ion-pairing described previously (Arnault *et al.* 2003). Separations studies were carried out using a second Waters HPLC system, consisting of a 600E quaternary gradient pump, a 996 diode array detector and a 717 autoinjector. The UV absorbance spectral data were collected from 200 to 500 nm. Compounds were separated on a Hypurite Elite reversed phase C18 column of 150 mm length × 3 mm i.d. particle size 3 μm (Thermo Hypersil; Keystone, Bellafonte, PA, USA). The gradient was based upon a binary solvent system. Solvent A consisted of 20 mM sodium phosphate monobasic and 10 mM heptanesulphonate, pH 2·1 (adjusted with orthophosphoric acid 85%) and solvent B consisted of 50% acetonitrile/50% solvent A (v/v), pH 2·1. The data were acquired and processed using Millenium 3·1 software (peak identification based on retention time combined with a spectral library).

Pheomelanin analysis

Pheomelanin analysis was performed according to Kolb *et al.* (1997) and Borges *et al.* (2001) with slight modifications. To identify peaks in the pheomelanin analysis we used 3-amino-4-hydroxyphenylalanine (AT; Aeros Organics, Geel, Belgium) as a standard. Hypophosphorous acid (50% H_3PO_2; Sigma-Aldrich, St. Louis, MO, USA) and hydriodic acid (HI, Fluka/Sigma-Aldrich, St. Louis, MO, USA) were used to hydrolyse the samples. As an ion-pair we used 1-octanesulphonic acid (Sigma). We used AT, red human hair samples (received from a female colleague) and feather samples [4–8 mg, originating from the head of a male domestic chicken (*Gallus domesticus*)] to calibrate the method. They were placed into screw-capped tubes to which 20 μL 50% H_3PO_2, 500 μL 57% HI and 100 μL UHQ-water was added. Tubes were tightly closed and samples were hydrolysed in oven at 130 °C for 16 h. After that we dried samples in the oven at 60 °C for 3–6 h under stream of nitrogen. Dried samples were redissolved in 1 mL of 0·05 M Li-phosphate buffer (pH 4·0) similar to Borges *et al.* (2001).

Extraction of the compounds was done with an aromatic sulphonic acid solid-phase extraction (SPE) column (Strata SCX, 100 mg mL^{-1}, Phenomenex, Torrance, CA, USA).

The SPE columns were first washed two times with 1 mL of methanol. Columns were then washed three times with 1 mL of Li-phosphate buffer before addition of the samples. After washing twice with 1 mL of water, AT and AHP were eluted with 2 mL of 0·3 M KCl, pH 8·5.

For the HPLC analysis we used a GS50 gradient pump, LC25 chromatographic oven and ED50 electrochemical detector (all units Dionex, Sunnyvale, CA, USA). The detector was set to 350 mV. Separation was done by a Gemini 5-μm C18, 150 × 3·0 mm i.d. analytical column (Phenomenex). The column was used at room temperature with an injection volume of 25 μL of SPE treated sample solution.

The data were acquired and processed with Chromeleon 6·50 SP6 Build 1032 software.

The mobile phase consisted of a mixture of 1:99 (v/v) methanol:potassium phosphate buffer (0·01 M, pH 5·7), 1-octanesulphonic acid sodium salt (1 mM) as an ion-pair reagent and disodium EDTA (0·1 mM). The flow rate was 0·5 mL min^{-1}.

Eumelanin analysis

Eumelanin analysis was done according to Borges *et al.* (2001) with modifications. Samples (7–9 mg) were placed in a screw-capped tube to which 820 μL 0·5 M NaOH, 80 μL 3% H_2O_2 and an internal standard (48 nmol phthalic acid) were added. Samples were then heated in boiling water for 20 min. After cooling 20 μL 10% Na_2SO_3 and 250 μL 6 M HCl was added. Samples were then extracted twice with 7 mL ethyl acetate. Ethyl acetate was dried at 45 °C under a stream of air. The residue was dissolved into 1 mL of starting HPLC mobile phase.

HPLC analysis was carried out with an HPLC, equipped with a GS50 gradient pump, AS50 autosampler and UVD 170U UV/Vis detector (Dionex). 50 μL of sample was injected into a Spherisorb S5 C8, 250 × 4·6 mm i.d. analytical column (Waters, Milford, MA, USA). The column was set to 40 °C. Analytes were detected at wavelength 280 nm and the data was collected with Chromeleon 6·50 SP6 Build 1032 software. HPLC mobile phase consisted of 0·01 M potassium phosphate buffer (pH 2·1) and methanol. Flow rate was 1·0 mL min^{-1} and the used gradient was as follows: 99:1 buffer:MeOH (v:v) ramped evenly from time 0 to 15 min to 40:60 buffer:MeOH (v:v),

held at 40:60 for 6 min and ramped evenly back to initial eluent composition over 5 min. We compared chromatograms obtained from *P. plantaginis* to chromatograms obtained from black human hair.

Results

ORANGE HAIRS

The orange pigments found in the hairs were not fluorescent in vivo, but were soluble in 0·1 M NaOH and methanol solutions, and fluoresced faint yellow in solution. Absorbance spectra of the extracted material most closely resembled the general properties of flavonoids (Mabry, Markham & Thomas 1970), with absorbance strongest in the far-UV (in methanol: λ_{max} = 281 nm, shoulders at 234 nm, 311 nm). In addition to the strong match between solubility, fluorescence and absorbance properties of the extracted pigment and known properties of flavonoids, several lines of evidence help to exclude carotenoids, pterins and ommochromes. Carotenoids can easily be excluded due to the fluorescent and UV-absorbing properties of the extracted pigment. Results from our HPLC analyses also confirm the absence of carotenoids in the orange hairs. Ommochromes and pterins can also be ruled out, as they are not expected to be soluble in methanol. This is also supported by our HPLC analyses as we did not find any matched peaks with the ommochrome standards tested. In addition, ommochromes have characteristically strong absorbance peaks in the near-UV, violet, blue or green, whereas the pigment(s) isolated from the orange hairs absorbs most strongly in the far-UV.

The HPLC analyses revealed that orange or black hairs did not contain pheomelanins. This is in accordance with the scientific literature, where pheomelanins have only been described from vertebrates (Fox 1976; Kayser 1985). As such, pheomelanins were excluded from further analyses. However, we found eumelanin in the HPLC analyses of orange hairs, as the peaks in HPLC chromatograms of human black hair matched with the peaks of *P. plantaginis* (Fig. 2). This was also supported by the reflectance measurements. Eumelanin itself is not equally light absorbing across all visible wavelengths as one might suppose from its often very black hue in animal tissues. Instead, melanin is strongly absorbent across all light wavelengths, but is more strongly absorbent in the short wavelengths (Sarna & Sealy 1984). Therefore, tissues that contain small amounts of eumelanin should gradually increase in light reflectance when moving towards longer wavelengths. This feature is rarely seen in melanin patterns, as eumelanin has such a high extinction coefficient across visible wavelengths (Fox 1976; Kayser 1985). However, at trace amounts one can begin to see this difference between long and short wavelengths. The spectra shown in Fig. 3 may therefore also be consistent with what would be expected from very small amounts of melanin deposited in these hairs. Such a contribution of trace amounts of melanin to the orange-colouration of these hairs would also be consistent with our fluorescence results, where melanin may be quenching the fluorescence of flavonoids *in vitro* which is visible in solution. Taken together, we conclude that the orange hair colour is based on flavonoids and traces of eumelanin pigments.

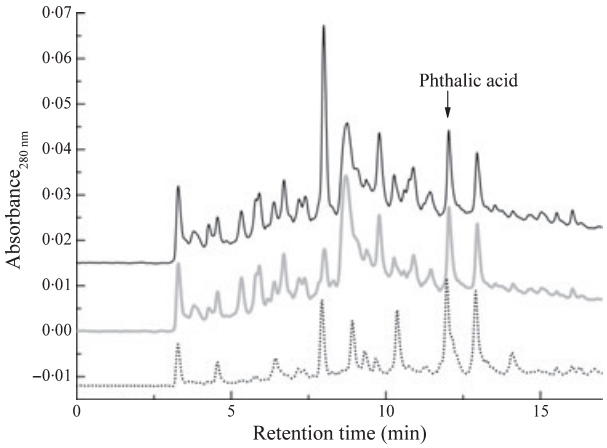

Fig. 2. HPLC chromatograms of the *Parasemia plantaginis* black hairs (black line), orange hairs (grey line) and human black hair (dotted black line) in eumelanin analysis.

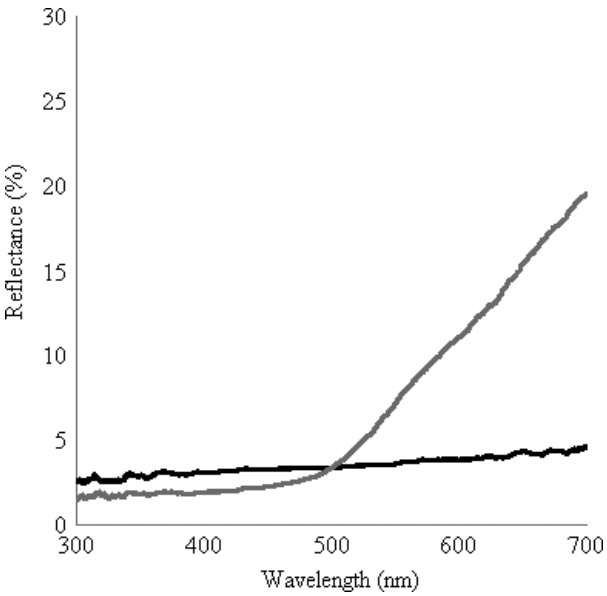

Fig. 3. Lines show mean spectra of orange hairs (grey line) and black hairs (black line) of *Parasemia plantaginis* larvae. All values are percents scaled relative to complete dark and a magnesium dioxide white standard.

BLACK HAIRS

Pigments in the black hairs were not soluble in any solvents tested and did not fluoresce, excluding all alternatives except melanins (Fig 3). HPLC analyses revealed that the black hairs of *P. plantaginis* contained eumelanins. The HPLC chromatograms of black and orange hairs of *P. plantaginis* were almost identical with the exception of the peak at 8 min representing in all likelihood PTCA. The ratio of this peak between

black and orange hairs was 4:1, which is similar to the ratio of PTCA in human black and red hair analysed by comparable H_2O_2/HO-oxidation (Liu *et al.* 2005). However, the relative peak size of likely PTCA peak in black hairs of *P. plantaginis* was triple to that of human black hair. On the other hand the chromatograms of *P. plantaginis* seemed to be more complex compared to chromatograms of human black hair indicating a more heterogeneous structure of the eumelanin present.

Discussion

Much of the empirical efforts to understand the costs of bright colouration have focused on carotenoid-based colouration in vertebrates (e.g. Endler 1980; Hill & Montgomerie 1994; Grether, Hudon & Millie 1999; McGraw & Hill 2000, Hill, Inouye & Montgomerie 2002). However, there is accumulating evidence that other pigment groups such as melanins, flavonoids and pterins may impose significant acquisition and/or production costs resulting in physiological trade-offs (Saffranek & Riddiford 1975, Windig 1999; True 2003; Talloen, Van Dyck & Lens 2004, McGraw 2005; Griffith, Parker & Olson 2006). In this study our results suggest that aposematic *P. plantaginis* larvae produce the colour of their orange warning signal by depositing flavonoids and trace elements of eumelanin into the hairs. The black pattern elements are formed of hairs containing high levels of eumelanin.

The results of our pigment analyses are consistent with the study of Ojala, Lindström & Mappes (2007). According to the results of that study, the *P. plantaginis* larvae reared on a diet high in flavonoids (*Rumex* sp., Ojala *et al.* 2005) expressed larger signal sizes than larvae reared on artificial diet that included only traces of flavonoids (Ojala *et al.* 2005). Although large signal size is beneficial against predators (Lindstedt, Lindström & Mappes 2008), our findings combined with the results of Ojala, Lindström & Mappes (2007) offer indirect evidence that flavonoid concentration of the diet could constrain the size of the orange warning signal. However, even on an artificial diet which had only trace amounts of flavonoids (Ojala *et al.* 2005) *P. plantaginis* larvae were able to express rather large signals. This suggests that the warning signal sizes of *P. plantaginis* larvae are not purely dependent on the flavonoids in diet. That is, larvae may be able to compensate for the variation in diet quality and its consequences on warning signal expression by using two kinds of pigments to produce their warning signal pattern (see also Grether, Hudon & Endler 2001). This indicates that traces of eumelanin found in the orange hairs of the larvae may also play a significant role in the maintenance of the signal pattern on poor quality diets. More work to understand how the pigment composition of orange hairs may respond to changes in diet is clearly needed.

Interestingly, results from the study by Ojala, Lindström & Mappes (2007) also suggest that investment in the melanic components of the aposematic signal may be costly. Darker *P. plantaginis* larvae with higher amounts of melanin in their hairs had lower survival and lower growth rates compared to larvae with larger orange patterns irrespective of diet quality. This offers indirect evidence for the 'costly melanin' hypothesis (i.e. the investment in black hairs on the body could incur costs). Trade-offs between melanization and life history traits have also been shown in other insect studies between adult melanization and development time (Talloen, Van Dyck & Lens 2004) or adult size (Saffranek & Riddiford 1975) or both (Windig 1999; Ma *et al.* 2008) further supporting the idea that melanin-based black colour patterns can be expensive to produce.

Why then does the warning signal pattern of *P. plantaginis* vary if production of smaller orange patches and larger black patterns are associated with higher production (melanin) and predation costs (smaller orange patterns, Lindstedt, Lindström & Mappes 2008)? Our suggestion is (see also Ojala, Lindström & Mappes 2007, Friman *et al.* 2009; Lindstedt, Lindström & Mappes 2009a) that opposing selection pressures on signal size could in certain situations make production of more extensive black pigmentation advantageous. Even though larger orange warning signals are more effective against predators (Lindstedt, Lindström & Mappes 2008), this benefit in *P. plantaginis* larvae may trade off against benefits arising from larger black pattern elements, such as thermoregulatory benefits (Lindstedt, Lindström & Mappes 2009a) and better immunity against pathogens (Friman *et al.* 2009). In general, darker colouration can also protect individuals from UV-radiation (Hessen 1996; but see Gunn 1998). However, even if larger melanic pattern elements are favoured in a particular selective regime, variation in the size of black pattern elements may be maintained if important dietary precursors are scarce (e.g. Morris, Yu & Rogers 2002). Thus, a more robust understanding of the extant variation in this aposematic colour pattern will need to incorporate information regarding the selective benefits and dietary limitations simultaneously acting on both the orange (flavonoid- and eumelanin-based) and black (eumelanin-based) components and their relative sizes. Since black melanin based pattern elements are part of the protective colour pattern in many herbivorous insects, costs involved with melanin production are a likely if little studied factor in insect colour production.

In addition to Ojala, Lindström & Mappes (2007), few studies have addressed condition dependence of warning signal expression so far (e.g. Grill & Moore 1998; Sword 1999; Sandre *et al.* 2007). Based on the few studies that have probed this interesting question, it seems that pigment deposition and its susceptibility to environmental conditions may vary among aposematic species depending on ecological specifics, such as diet characterisitcs and importance of other selection pressures. For example, Sandre *et al.* (2007) found that production of carotenoid based yellow colouration was not influenced by diet manipulations in the polyphenic *Orqyia antiqua* larvae. This is not very surprising because carotenoids are often plentiful in the diet of herbivores (e.g. Ojala *et al.* 2005). However, variation in the carotenoid or flavonoid content of diet could be more important for non-herbivorous species

whose diet contains smaller quantities of these compounds. For instance, in aposematic ladybirds (Marples, Van Veelen & Brakefield 1994) the intensity of carotenoid based orange-to-red colour in their elytra is increased with increasing amount of aphids containing carotenoids in their diet (Britton *et al*. 1977; Grill & Moore 1998; Bezzerides *et al*. 2007). Therefore, in the future, more studies are needed where the condition dependence of pigment deposition and possible physiological trade-offs are directly tested by manipulating relevant dietary constituents such as carotenoid, flavonoid or nitrogen content (i.e. amino acid content) of a diet. Before these more targeted empirical tests are possible, more information about the chemical composition of the pigments used by aposematic species is needed. As shown in the *P. plantaginis* case, chemical analysis of aposematic colouration can propose new lines of research into the complex interactions involved in expression of warning signals.

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