

# Carotenoid-based colour polyphenism in a moth species: search for fitness correlates

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## Abstract

Carotenoid-based integumental coloration is often associated with individual performance in various animals. This is because the limited amount of the pigment has to be allocated to different vital functions. However, most of the evidence for the carotenoid-based trade-off comes from vertebrate studies, and it is unclear if this principle can be applied to insects. This possibility was investigated in *Orgyia antiqua* L. (Lepidoptera: Lymantriidae). The larvae of this species are polyphenic in their coloration, varying from a highly conspicuous combination of yellow hair tufts on black background to cryptic appearance with brown hair tufts. The conspicuous larvae are aposematic, advertising their aversive hairiness. The maintenance of different colour morphs in *O. antiqua* requires explanation, as an aposematic signal is expected to evolve towards monomorphism. Chromatographic analysis showed that the yellow coloration of the hair is based on the carotenoid pigment lutein ( $\alpha$ -carotene-3,3'-diol). The colour of hair tufts was dependent on their carotenoid content. This justifies an expectation of carotenoid-based physiological trade-offs between aposematic coloration and individual performance. To test this hypothesis, we monitored life histories of differently coloured larvae reared on various host plants, recording their body sizes, growth rates, and mortalities in each instar. There was a significant but relatively low heritability of tuft coloration, which allowed us to expect environmental effects. We found no phenotypic associations between hair tuft colour and performance indices in *O. antiqua* larvae, neither did the quality of host plant affect the frequency of colour morphs. However, the frequency of colour morphs differed between larval instars. Our results suggest that carotenoid-mediated physiological trade-offs are not involved in the maintenance of colour morphs in *O. antiqua* larvae, and factors other than individual condition should be responsible for the observed variability.

## Introduction

Recent studies in evolutionary ecology have revealed various cases of associations between coloration of an animal and its life-history parameters. These connections are hypothesized to arise via trade-offs between colour and individual performance indices, typically being mediated by pigments with multiple functions, the carotenoids in particular (Lozano, 1994; Grether et al., 1999; Hill et al., 2002; Saks et al., 2003; Blount, 2004; Peters et al., 2004).

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Carotenoids are pigments that are predominantly responsible for conspicuous yellow, red, and orange colours of animal integuments. On the other hand, these compounds are considered to enhance immune functions (Alexander et al., 1985; Hughes, 2001; McGraw & Ardia, 2003) and scavenge harmful free oxygen radicals (Mortensen et al., 1997; Bertrand et al., 2006). Such radicals arise in normal metabolic processes, during immune challenge (Von Schantz et al., 1999 and references therein; for insects, see Whitten & Ratcliffe, 1999), and may also originate from other sources, for example, some ingested plant secondary metabolites (Ahmad, 1992). Due to their multiple physiological roles and the inability of animals to synthesize this class of compounds, carotenoids often constitute a limited

resource. It is possible, however, that carotenoids are not always limited for phytophagous organisms, in terms of availability in their food, but they may still be physiologically limited, for example, because of constraints related to absorption or modification of these substances (Olson & Owens, 1998).

The primary evidence of correlations between colour and performance comes from birds: bright carotenoid-dependent integuments are often positively correlated with fitness, and colour often functions as a signal trait in sexual selection (Hörak et al., 2001; Blount et al., 2003; Faivre et al., 2003). Analogous relationships have been found in other taxa such as fish (Houde & Torio, 1992) and reptiles (Shine & Harlow, 1998; Svensson et al., 2001). Notably, however, carotenoid-mediated relations between coloration and fitness have not been studied in insects, although carotenoid-based colour patterns are also characteristic of many species in this principal taxon (Wigglesworth, 1972).

In lepidopteran larvae, colour polymorphism (or polyphenism, when at least partly caused by environmental factors) is widespread and a variety of mechanisms have been proposed to explain its maintenance. As a proximate explanation, the existence of different morphs may be directly connected to pigment concentration in larval host plants (Burghardt et al., 2001; Eichenseer et al., 2002). The ultimate causes of maintenance of variable coloration may be related to the purpose of camouflage (Greene, 1989; Merilaita et al., 1999) or thermoregulation (Gunn, 1998; Hazel, 2002; Solensky & Larkin, 2003). The ultimate causes of the frequently observed effect of larval crowding remain unclear (Wilson & Reeson, 1998; Wilson et al., 2001). In addition to these mechanisms, the insights obtained from vertebrate studies could thus well justify the expectation of an additional reason for colour differences in lepidopteran larvae, that is, a carotenoid-mediated polyphenism that is causally related to individual condition.

The problem of variable coloration acquires a further dimension when aposematic colour morphs are involved. There are doubtless advantages of being aposematic in terms of avoidance of predation (Ruxton et al., 2004). As aposematic prey is best protected when common, predation pressure is expected to create purifying frequency-dependent selection, which leads to monomorphism in signal (e.g., Müller, 1879; Turner, 1987; Alatalo & Mappes, 1996; Mallet & Joron, 1999). Therefore, other things being equal, aposematic monomorphism should invariably represent an evolutionarily stable situation. The occasional co-existence of aposematic vs. non-aposematic colour morphs within a population, however, implies that there should be some counterbalancing costs of the aposematic coloration (Joron

et al., 1999; Mappes et al., 2005). The above discussion makes it reasonable to expect that these may have a physiological basis that is related to individual performance.

In the present study, we asked whether the well-expressed colour polyphenism in the larvae of the vapourer moth [*Orgyia antiqua* L. (Lepidoptera: Lymantriidae)] could be related to variation in individual growth performance or individual differences in allocation patterns of carotenoids. In this species, hair tuft colour varies from the classical warning combination of yellow against a dark background (Cott, 1940; Ruxton et al., 2004) to inconspicuous brown. The conspicuous morphs can be considered aposematic, with the warning coloration signalling the irritating hair cover that adversely affects palatability to predators. Indeed, aviary experiments showed that the birds' wariness was the highest when they were offered larvae with the strongest aposematic signal (SL Sandre, unpubl.).

In this study, we showed that the yellow pigment in *O. antiqua* hair tufts is, indeed, a carotenoid. Further, we demonstrate that the colour of the hair tufts is correlated with carotenoid concentration, which allows one to expect trade-offs between coloration and individual growth performance. As the second step, the existence of phenotypic correlations between colour pattern and performance indices is examined in laboratory-reared larvae.

## Materials and methods

### Study species

*Orgyia antiqua* is a capital breeding (Tammaru & Haukioja, 1996) moth species with a broad holarctic distribution. The species is a highly suitable object for life-history studies, as female body size provides a rather unequivocal measure of fitness (Tammaru et al., 2002). This is because the highly variable body size (150–600 mg) strongly correlates with fecundity, and the flightless females lay their eggs unselectively. The solitary polyphagous larvae typically pass through 4–5 (males) or 5–6 (females) instars. The larvae are hairy with blackish background colour. From the fourth instar onwards they have four conspicuous dorsal hair tufts on their fourth to seventh body segments. The colour patterns of the hair tufts were classified into one of the three classes: (i) two black anteriorly and two yellow posteriorly, (ii) all four bright yellow, or (iii) all four brown. Henceforth, these morphs will be labeled as pied, bright, and dull, respectively. The first two phenotypes are quite monomorphic but within the dull class there is considerable continuous variation from dark yellow to dark brown.

When addressing the question if the putative correlation between coloration and individual performance arises from different allocation patterns, it is not of primary

**Table 1** Rearing experiments with *Orgyia antiqua* larvae to study phenotypic correlations between tuft colour and performance indices. Absolute frequencies of colour morphs are presented for each larval instar in all experiments

Experiment	Year	Temperature	Food plant	Number of broods	Absolute frequency of colour morphs in different instars							
					Fourth instar			Fifth instar			Sixth instar	
					Bright	Pied	Dull	Bright	Pied	Dull	Bright	Dull
1	2002	Ambient, May–July <sup>1</sup>	<i>Betula pendula</i>	6	142	92	10	88	–	87	–	3
2	2003	Ambient, May–June <sup>1</sup>	<i>Betula pendula</i>	6	25	31	19	12	1	23	1	2
3	2002	Constant (22 °C), July–August	Six different <sup>2</sup>	4	101	50	2	86	16	26	8	30
4	2003	Constant (22 °C), May–June	Six different <sup>2</sup>	4	98	29	–	70	–	28	6	12

<sup>1</sup>Temperature dynamics in the study site (Tartu, Estonia): <http://meteo.physic.ut.ee>

<sup>2</sup>*Betula pendula* Roth, *Quercus robur* L., *Cotoneaster lucidus* Schltld., *Salix viminalis* L., *Salix caprea* L., and *Salix myrsinifolia* Salisb.

importance whether the individual differences are genetic or environmental. However, the expectation of an effect of host-plant species on the colour of the larvae can only rely on the premise of a certain degree of environmental influence. To our knowledge, the degree of genetic determination of hair colour has not been systematically studied in this species. Nevertheless, various observations indicate a relatively minor role of genotype over that of the environment, for example, there is some evidence of a considerable effect of larval crowding and rearing temperature on hair colour (SL Sandre, unpubl.). Furthermore, as a general rule, offspring of any individual female are polyphenic with respect to colour pattern. We also obtained an estimate of heritability of larval colour on the basis of rearing experiments, reported below.

#### Chemical analysis of hair pigments

The pigment of larval dorsal tufts was extracted (each sample three times) with acetone from the crushed tuft hair of each individual larva. Acetone from the extract was removed under the flow of nitrogen, and the sample was dissolved into methanol. The sample was analysed by high-performance liquid chromatograph (HPLC) (Agilent Series 1100; Agilent, Santa Clara, CA, USA) using isocratic methanol runs in 2003 and gradient runs in 2004 [A: acetonitrile; methanol and B: hexane; gradient: 0–4 min B was 0%, 4–7 min B was increased to 100% according to Gilmore & Yamamoto (1991)].

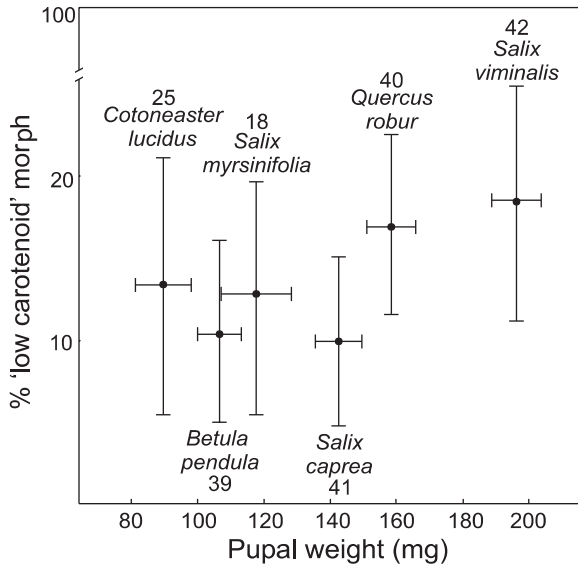
In 2003, the mean peak retention time of the main carotenoid at 440.4 nm was 1.03 min, and  $\beta$ -carotene (Fluca,  $\geq 95\%$  HPLC) was used as the standard for quantification (the identity of the carotenoid was not yet known in 2003). Due to the use of  $\beta$ -carotene as the standard, in 2003 the concentrations of carotenoid were interpreted as relative ranks only, not in absolute terms. After the carotenoid

was identified as lutein ( $\alpha$ -carotene-3,3'-diol) in 2004, the analysis was repeated at 440.4 nm and the gradient run gave the retention time of 2.2 min for lutein. A commercial analytical sample of lutein [BioChemica ~90% (HPLC) from alfalfa] was used as the standard.

In 2003, the larvae for hair pigment analysis were reared on artificial diet (a modified version of diet for Lymantriidae from Moore, 1985) and lettuce (*Lactuca sativa* L.) until the end of their fifth instar and then were frozen at  $-40$  °C. In 2004, larvae were reared on silver birch (*Betula pendula* Roth) and similarly frozen at the end of the fifth instar. The number of individuals tested from each colour morph was equal in 2003 (12 bright and 12 dull individuals), in 2004 samples from 14 dull, 11 bright, and four pied (only black hair) larvae were analysed. All four coloured hair tufts were removed and used in chemical analysis. Black hair samples, however, consisted of only two tufts per individual: no larvae with four black dorsal tufts were available.

#### Coloration and performance indices

Data used for the analysis of associations between hair tuft coloration and individual performance indices were obtained from four rearing experiments. These were carried out in spring and summer of 2002 and 2003 (Table 1), in Tartu, Estonia. Individuals used in this study represented out-bred laboratory lines originating from Estonia and Finland. In each of the experiments, the larvae (599 in total) were reared from hatching to pupation in 50-ml vials and they were fed ad libitum with host plant leaves, which were renewed regularly. In two of the experiments, the larvae were reared on different plant species starting from their third (2002) or fourth (2003) instar (they were reared on silver birch before being assigned to different plant treatments). The host-plant species (Table 1) were chosen, on the basis of pilot experiments, so that they differed in



**Figure 1** The percentage of the larvae representing the 'low carotenoid' group (dull and pied morphs are combined, as opposed to the 'high carotenoid' bright morph) in their last instar in samples of *Orgyia antiqua* reared on different host species. Host quality is expressed in terms of least square means for pupal weight. Values of both variables were corrected for brood, initial weight of the last instar, and sex (the LSMEANS option of SAS PROC GLM and PROC GENMOD; Littell et al., 2002). Horizontal bars denote standard errors of mean pupal weight, vertical bars show standard errors of the mean percentage of the 'low carotenoid' group, and figures indicate the number of individuals having reached pupation.

nutritional quality (the index of plant quality used was mean pupal weight attained on a plant species; Figure 1), and also with respect to the spectrum and the content of secondary metabolites. In particular, total content of phenolic glycosides has been shown to be as low as  $1.3 \text{ mg g}^{-1}$  (dry weight) in *Salix caprea* L. and *Salix viminalis* L., and as high as  $41.5 \text{ mg g}^{-1}$  in *Salix myrsinifolia* Salisb. (Julkunen-Tiitto, 1986). Two of the rearing experiments (Table 1) were carried out under constant temperature of  $22 \text{ }^{\circ}\text{C}$ ; the development of the larvae was synchronized at the beginning of the third and fourth instars, respectively. Such maximally standardized conditions allowed 'noise-free' recording of growth parameters. In contrast, the two other experiments were conducted under ambient conditions (Table 1), the hatching of the larvae being manipulated to cover a period of about 1 month. Such a setting was complementary to the standardized trials in providing more generality at the cost of increased levels of environmental noise.

Members of each brood (= offspring of an individual female) were distributed evenly between the treatments,

and positions of rearing vials were randomized with respect to host plant and brood. The larvae were weighed daily starting from the third instar [except in Experiment 4 (Table 1), in which only the initial and final weights of each instar were recorded]. Pupae were weighed and sexed on the first day after pupation. Starting from the fourth instar, colour of the hair tufts was visually recorded on the third day of each instar (each larva assigned to one of the three morphs, see above). In the rearings, nearly 30% of individuals died during the larval stage, mostly showing signs of a bacterial infection. It was therefore possible to analyse the potential significance of aposematic coloration in terms of disease resistance.

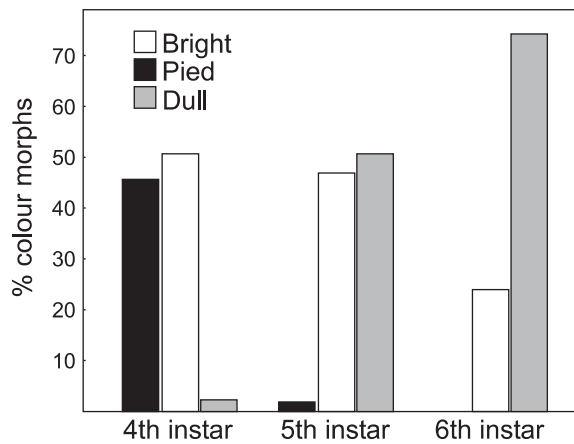
One of the performance indices, the final body size of each instar, was recorded during the inactivity period prior to moulting into the subsequent instar. Such a measurement reflects weight-accumulation efficiency in the course of an instar if the effect of the initial weight of the instar is accounted for statistically. Instantaneous relative growth rate (RGR) on the third day of each instar was used as another index of growth performance. The timing was chosen so as to capture the period of maximal growth in the middle of each instar (Esperk & Tammaru, 2004). Relative growth rate was calculated as  $\ln(\text{final weight}/\text{initial weight})/\text{time}$  (Scriber & Slansky, 1981).

#### Statistical analysis

The effect of colour morph on lutein concentration of hair tufts was analysed separately for each year with Kruskal–Wallis analysis of variance (ANOVA). A non-parametric test was chosen because the assumption of homogeneity of variances was violated: there was no variation in pigment concentration in the black hair. Separate analysis of years was necessary, because the data of the 2 years were, due to a methodological difference (see Materials and methods), not quantitatively comparable.

To explore the relative role of environmental vs. genetic effects as determinants of hair colour, heritability of this trait was estimated as based on the sample of larvae in the rearing trials (Table 1). To facilitate the application of the standard methods designed for binomial traits, the variable studied, hair tuft colour, was transformed into a variable with two levels, pooling the dull and pied morphs into the 'low carotenoid' class. The heritability of the resulting binary trait (= liability) was calculated on observed scale on the basis of the ratio of the component of variance attributable to broods (estimated with SAS PROC MIXED), and the total phenotypic variance, calculated as the product of frequencies of the low- and high-carotenoid classes (Lynch & Walsh, 1998, pp. 727–744).

To reveal possible costs associated with the aposematic coloration, data from Experiments 3 and 4 (Table 1) were



**Figure 2** Percentages of colour morphs in different instars of *Orgyia antiqua* ( $\chi^2 = 348$ ,  $P < 0.001$ ,  $n = 1097$ ).

analysed to detect a dependence of larval coloration on host-plant species. A generalized linear model for binomial traits was fitted for this purpose. Once again, to be able to apply an ordinary logistic regression, larval colour was treated as a binary trait (low- vs. high-carotenoid classes). To account for the effect of other potential determinants of larval coloration – experiment identity, brood, initial weight of the instar, sex and colour in previous instar – were included as additional factors. Such analyses were performed separately for fourth, fifth, and sixth instars.

To estimate the effect of hair tuft colour on individual performance, ANOVAs with performance indices as dependent variables and colour morph as the independent variable were performed separately for each instar, as well as for the total sample (including instar as an additional factor). Brood, host-plant species, sex, final body size of the previous instar, and experiment identity were included as additional effects in the models. Analysis of the effect of colour on instantaneous growth rate was performed on the data from Experiments 1, 2, and 3 (Table 1).

To analyse the association between colour and mortality, combined data of all rearing experiments were used. Dependence of survival on colour morph in each instar, with experiment, initial body size, and brood as additional factors, was analysed with the logistic regression (SAS PROC GENMOD). In all ANOVAs, type 3 sums of squares were used. Statistical analyses were conducted using the SAS system for Windows V8 ([www.sas.com](http://www.sas.com)).

## Results

### Identification of hair pigment

The identity of the carotenoid was determined by comparing the retention time and the spectrum of the substance in

*O. antiqua* hair tuft samples with that of the standard. The pigment was identified as the carotenoid lutein ( $\alpha$ -carotene-3,3'-diol).

The quantitative analysis on both 2003 and 2004 data showed that hair of different colour differed in carotenoid concentration (2003:  $\chi^2 = 2.31$ , d.f. = 2,  $P = 0.02$ ; 2004:  $\chi^2 = 7.94$ , d.f. = 2,  $P = 0.02$ ). Yellow hair had a higher concentration of carotenoid than brown hair in both years. In 2004, when the analysis allowed to obtain estimates in absolute terms, mean lutein concentration in the yellow hair was  $6.5 \pm 1.1 \text{ mg g}^{-1}$  (dry weight  $\pm$  SE), as opposed to  $3.1 \pm 1.0 \text{ mg g}^{-1}$  in the brown hair. In the black hair, no lutein could be detected.

### Colour morphs in rearing experiments

In all rearing experiments, there was a considerable variation in larval hair tuft colour. When pooled over experiments and instars, there were 58% bright, 20% pied, and 22% dull larvae ( $n = 1098$ ; see Table 1, for distribution of colour morphs within instars and experiments). None of the 20 broods were monomorphic in any of the instars. Even if the effect of brood on coloration was statistically significant in the fourth and the fifth instars ( $P < 0.001$ , but remaining non-significant in the sixth instar,  $P = 0.58$ ), the heritability of tuft coloration (as a binary trait, see Materials and methods) was estimated to be relatively low (0.176 for the fourth instar, 0.185 for the fifth instar), leaving thus ample space for the environmental factors to operate.

Colour morph frequency was significantly affected by larval age: the pied morph was associated with younger instars whereas the sixth instars tended to be dull (Figure 2). Typically, an individual larva was pied in the fourth, bright in the fifth, and brown in the sixth instar, but nearly all other combinations (there were no dull larvae in the fourth, and no pied ones in the sixth instar) were also present. Moreover, there was a lot of variation in the performance indices, that is, weights and growth rates (Tables 2 and 3), allowing for a meaningful analysis to be performed.

Host plant had no effect on hair tuft colour (fourth instar:  $\chi^2 = 2.83$ ,  $P = 0.73$ ,  $n = 204$ ; fifth instar:  $\chi^2 = 6.67$ ,  $P = 0.25$ ,  $n = 173$ ; sixth instar:  $\chi^2 = 1.27$ ,  $P = 0.93$ ,  $n = 44$ ). There appeared to be no systematic association between host-plant quality (in terms of mean pupal weight achieved on a host) and mean hair tuft colour on host plant (Figure 1). There was also no evidence of brood-specific effects of different hosts (brood\*plant interaction:  $\chi^2 = 54.1$ ,  $P = 0.12$ ,  $n = 193$ ).

### Life-history parameters

Colour had no effect on the body size attained by the end of any instar, after the effects of other potential determinants

**Table 2** Effect of hair tuft colour on body weight of *Orgyia antiqua* at the beginning of the subsequent instar (or pupa, for the last instar) as analysed by general linear model (GLM) with experiment identity, brood, and body weight in the beginning of the instar as additional factors. The analyses are performed separately for larvae that passed through a different number of instars.  $R^2$  is calculated as  $SS_{\text{effect}}/SS_{\text{total}}$  for the effect of colour morph

Instar	Number of instars before pupation	d.f.	F	P	$R^2$	Least square means (SE) for body weights (mg)					
						Female			Male		
						Bright	Pied	Dull	Bright	Pied	Dull
Fourth	4	1,123	0.83	0.4	0.94%	–	–	–	92.8 (2.9)	110.5 (16.7)	97.3 (5.8)
Fourth	5	2,301	1.11	0.4	0.21%	116.1 (2.3)	114.5 (2.0)	92.8 (10.7)	76.7 (2.6)	76.8 (2.9)	75.8 (10.2)
Fourth	6	1,57	1.99	0.1	3.7%	62.2 (3.0)	64.7 (3.1)	89.6 (13.8)	–	–	–
Fifth	5	2,303	0.55	0.7	0.11%	250.3 (9.3)	205.9 (57.7)	259.5 (7.1)	154.1 (9.7)	–	156.8 (25.1)
Fifth	6	1,55	0.52	0.6	0.72%	162.6 (5.9)	161.2 (12.2)	148.5 (13.3)	–	–	–
Sixth	6	1,48	0.86	0.4	1.1%	306.4 (34.5)	–	269.3 (26.0)	–	–	–

were accounted for in a multi-way ANOVA. This was the case when the instars were analysed separately (Table 2) as well as in an analysis of the data of all experiments combined (colour nested in instar:  $F_{3,466} = 2.28$ ,  $P = 0.14$ ). In an analogous analysis for instantaneous RGR, hair tuft colour did not explain a significant amount of variation in any of the instars (Table 3), neither was this the case when all the instars were all analysed together ( $F_{3,304} = 1.38$ ,  $P = 0.3$ ). The negative conclusion is strengthened by the consistently low proportion of variance attributable to the colour morph in all experiments (both under standardized and ambient conditions) and for all instars (Tables 2 and 3). Moreover, the directions of the differences revealed no consistent patterns.

Combined over the four rearing experiments, 104 of 599 (17%) larvae died during their fourth instar, as did 70 (16%) fifth instars out of 436. Colour morph had no influence on mortality risk in any of the instars (fourth instar:  $\chi^2 = 1.32$ ,  $P = 0.3$ ; in fifth instar:  $\chi^2 = 1.04$ ,  $P = 0.3$ ).

## Discussion

It was shown that the yellow coloration in the hair tufts of *O. antiqua* larvae is based on a carotenoid. The substance was identified as lutein, which is a pigment previously known to occur, along with various other carotenoids, in Lepidoptera. However, there was previously no data on the larval stage: a study of 114 species of Lepidoptera (imagoes only) had shown that lutein was the main carotenoid in two moth species, one of which represented the family Lymantriidae (Czeczuga, 1986).

To our knowledge, the present study is the first to show that variation in larval colour in Lepidoptera is based on a variable carotenoid content. Yellow hair of the bright morph of *O. antiqua* had a higher concentration of lutein than the brown hair of the dull morph. It has previously been demonstrated that larval coloration can also be based on carotenoids in Lepidoptera (Wigglesworth, 1972). Clark (1971) showed that *Hyalophora cecropia* (L.) (Lepidoptera:

**Table 3** Effect of hair tuft colour on the relative growth rate (RGR) of *Orgyia antiqua* in the beginning of an instar (see Table 2 for further details)

Instar	Number of instars before pupation	d.f.	F	P	$R^2$	Least square means (SE) for RGR (mg per mg per day)					
						Female			Male		
						Bright	Pied	Dull	Bright	Pied	Dull
Fourth	4	1,41	1.91	0.2	3.8%	–	–	–	0.27 (0.021)	–	0.20 (0.042)
Fourth	5	4,90	0.55	0.7	1.4%	0.36 (0.041)	0.32 (0.037)	0.31 (0.073)	0.26 (0.032)	0.29 (0.031)	0.34 (0.069)
Fourth	6	2,37	1.03	0.4	3.3%	0.41 (0.029)	0.37 (0.025)	0.40 (0.092)	–	–	–
Fifth	5	3,149	2.86	0.04 <sup>1</sup>	4.0%	0.23 (0.025)	0.30 (0.10)	0.29 (0.019)	0.25 (0.019)	–	0.15 (0.061)
Fifth	6	2,48	2.32	0.1	8.6%	0.31 (0.022)	0.32 (0.042)	0.20 (0.049)	–	–	–
Sixth	6	1,43	0.5	0.5	1.3%	0.20 (0.031)	–	0.23 (0.018)	–	–	–

<sup>1</sup>Not significant after Bonferroni correction.

Saturniidae) larvae reared on artificial diet lacking carotenoids were abnormally coloured, but, nevertheless, concentration of the pigment has never been directly measured in different colour morphs of a species.

Lutein is an efficient quencher of oxygen radicals (Ahmad & Pardini, 1990). This implies that it is, indeed, justified to expect the existence of trade-offs between aposematic coloration and physiological functions affecting individual performance. This is because, in *O. antiqua*, larval colour morphs appear to have different allocation patterns of carotenoids. Contrary to our expectations, however, we found no differences between the morphs in any aspect of larval performance studied, neither within nor between environments. Adult body size in *O. antiqua* is a reliable predictor of realized fecundity, representing thus a close equivalent to fitness. However, pupal weight was not associated with colour. Another correlate of fitness, instantaneous growth rate, was similarly unaffected by colour. Neither did mortality depend on coloration, so there was no support for the hypotheses of an interaction between colour and immune system. As a conclusion, unlike in, for example, various birds (review in Blount, 2004), coloration appears not to correlate with individual performance in the insect studied.

No associations between host-plant species and larval coloration were revealed in our study. As there were clear differences between the plant species in their quality for *O. antiqua* larvae, this result may indicate that neither the sequestration nor deposition of pigments is costly enough to be affected by diet quality. Otherwise, we should have expected the prevalence of larvae with less brightly coloured hair tufts on hosts of inferior quality. Besides their overall nutritional quality, the six host-plant species differed with respect to the biochemical composition of their foliage. Nevertheless, the frequency distribution of the colour morphs did not differ between the hosts, thus providing no evidence of a direct mechanistic effect of plant secondary metabolites on larval colour either (see Ahmad & Pardini, 1990; Ojala et al., 2005, for positive examples). In the case of an effect, we should have expected a lower proportion of bright-tufted larvae on hosts with high concentration of defensive secondary metabolites, as carotenoids are used for scavenging reactive oxygen radicals. It has to be noted, however, that the limited effect of environmental variables cannot be generalized beyond the conditions evaluated in the present study.

Larval coloration did not show any within-environment correlations with life-history traits. There was thus no evidence of a variation of hair tuft colour that could be caused by different allocation strategies between individuals. If, for instance, investing more carotenoids in hair had resulted in lower RGR or smaller final size, this could have

served as evidence in favor of a trade-off between investing in defence against bird predators, and other fitness-related traits. A trade-off might have been expected because growth is accompanied by high metabolic rate, and therefore leads to a high free-radical production (Van't Hof & Martin, 1989; Gouveia et al., 2000). These free radicals need to be neutralized by carotenoids, which could then not be deposited in hair tufts. Moreover, investment of the pigment in the immune system might draw the reserves of carotenoids from allocation to hair tufts. Larvae that had invested more in aposematic coloration could thus be more susceptible to pathogens. Higher susceptibility could then be reflected in a higher mortality of the more aposematic larvae, or their smaller body size, as metabolic resources are spent on fighting the infection (Freitak et al., 2003).

There are several possible reasons for the failure to detect a cost of aposematic coloration in our study. First, the allocation polymorphism may still exist, but the cost might not have been looked for in the right place. For example, it has been shown in a noctuid moth that there is no genetic correlation between antibacterial activity (lysozyme like) and hemolymph phenoloxidase activity (Cotter et al., 2004). Therefore, our results, which deal with defence against unicellular pathogens and thus largely with antibacterial activity, might not reflect the possible cost of aposematic coloration in terms of phenoloxidase activity, which has a major role in defence against parasitoids. Furthermore, as phenoloxidase-related melanized encapsulation of foreign objects (also observed in *O. antiqua*, SL Sandre, unpubl.) is a process in which many free radicals arise (Nappi & Vass, 1998), carotenoids would be especially important for this aspect of immunity.

Nevertheless, our results suggest that the evolution of coloration in *O. antiqua* is not likely to be constrained by biochemically mediated trade-offs but is solely driven by ecological factors instead. The likely mechanisms could be related to thermoregulation (Gunn, 1998; Hazel, 2002), or varying predation pressure against different colour morphs in space and time (Endler & Mappes, 2004). The association of colour morph with larval age (Figure 1) supports the idea of a decisive role of bird predation: it is reasonable to assume that the effectiveness of any colour pattern in defence against visually searching predators is dependent on the size of the prey (Gamberale & Tullberg, 1996). As larval colour appears neither to have a strong genetic background, nor does it seem to represent a straightforward response to the environmental parameters studied, the variation might be interpreted as coin-flipping plasticity, that is, a bet-hedging strategy in which a genotype randomly produces different morphs (Cooper & Kaplan, 1982; Meyers & Bull, 2002). The random production of

different larval colour morphs might then be an adaptation for prevention of frequency-dependent predation by birds. This may be crucial because the warning colours of *O. antiqua* do not provide an absolute protection for the larvae: birds trained to consume them do so efficiently (SL Sandre, pers. obs.). Studies on the role of bird predation as a selective factor behind the maintenance of colour polyphenism and the size dependence of coloration are currently underway.

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