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Weak and inconsistent associations between melanic darkness and fitness-related traits in an insect

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Abstract

The idea that the fitness value of body coloration may be affected by biochemically mediated trade-offs has received much research attention. For example, melanization is believed to interact with other fitness-related traits via competition for substrates, costs associated with the synthesis of melanin or pleiotropic effects of the involved genes. However, genetic correlations between coloration and fitness-related traits remain poorly understood. Here, we present a quantitative-genetic study of a coloration trait correlated to melanin-based cuticular darkness ('darkness', hereafter) in a geometrid moth, *Ematurga atomaria*. This species has considerable variation in larval appearance. We focus on correlations between larval darkness and fitness-related growth performance traits. Both a half-sib analysis and an 'animal model' approach revealed moderately high heritabilities of larval darkness and indices of growth performance. Heritability estimates of darkness derived from the animal model were, however, considerably higher than those based on the half-sib model suggesting that the determination of coloration includes genetic interactions and epigenetic effects. Importantly, on the host plant with the largest sample size, we found no evidence for either genetic or environmental correlations between darkness and growth parameters. On an alternative host plant, there was some indication of positive genetic and negative environmental correlation between these traits. This shows that respective relationships are environment-specific. Nevertheless, the overall pattern of weak and inconsistent correlations between larval coloration and growth parameters does not support universal trade-offs between these traits and suggests that physiological costs of producing colour patterns do not necessarily interfere with adaptive evolution of coloration.

Introduction

Evolutionary explanations of animal coloration have traditionally relied on the benefits provided by different appearances during visually mediated behavioural interactions, such as sex recognition (Rutowski, 1981; Schultz & Fincke, 2009) or escaping predator attack (Estrada & Jiggins, 2008). The idea that the fitness value of body coloration may also be affected by biochemically mediated trade-offs is more recent (Hill, 1990; Milinski & Bakker, 1990) but has received much research attention during the past two decades (e.g. McGraw, 2005; Svensson & Wong, 2011; Roulin, 2016). Pigments displayed on the animal integument are thought to often correlate with the bearers' physiological state due to their multiple roles in the organisms. As an example, common pigments such as carotenoids, pterins, flavonoids and melanins are known to have antioxidant functions (McGraw, 2005; Galván *et al.*, 2014) which may link them to individual

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differences in processes associated with oxidative stress, such as growth or immune response. Moreover, correlations between pigmentation and other traits of the organism may arise due to pleiotropic effects of the genes involved in determining colour patterns (Ducrest *et al.*, 2008; Wittkopp & Beldade, 2009; Roulin, 2016).

The degree of melanization has been found to show phenotypic correlations with fitness-related traits in various animal taxa (Roff & Fairbairn, 2013; Roulin, 2016). These correlations have been proposed to primarily reflect the cost of synthesizing melanin (Talloen *et al.*, 2004; Stoehr, 2006; Gonzalez-Santoyo & Cordoba-Aguilar, 2012): synthesis of melanin generates reactive oxygen and nitrogen species which are potentially harmful and need to be neutralized with antioxidants (Nappi & Ottaviani, 2000; True, 2003). In addition, for many animals, the availability of melanin precursors may be limiting (Veiga & Puerta, 1996; True, 2003; Poston *et al.*, 2005), and this limitation can be exacerbated by trade-offs between the multiple physiological roles of melanin in the organism (e.g. immune defence in insects, True, 2003). If the trade-off is based on limiting precursors, among-trait relationships may become environment-specific, as the limiting role of the precursors inevitably varies across environments (Morehouse, 2014). Naturally, however, such trade-offs may differ among environments also for a variety of other reasons (e.g. Gonzales *et al.*, 1999; Almasi & Roulin, 2015).

To date, much of respective literature has relied on phenotypic correlations between coloration and other fitness-related traits. However, deeper understanding of the evolutionary significance of these patterns requires investigating whether they are underlain by genetic correlations (Roulin, 2016). Such correlations have, however, remained poorly characterized. This applies to insects in particular, despite the fact that these animals are especially suitable for respective studies due to both the widespread within-species variation in coloration (e.g. Hazel, 2002; Gotthard *et al.*, 2009; Singh *et al.*, 2009; Välimäki *et al.*, 2015), and short generation times. The few existing quantitative-genetic studies on insects which have involved coloration-related traits (Wilson *et al.*, 2001; Armitage & Siva-Jothy, 2005; Cotter *et al.*, 2008; Roff & Fairbairn, 2013) are primarily concerned with correlations between melanization and immunological parameters, at the expense of paying attention to the more general relationships between coloration and physiologically based correlates of fitness.

In the present paper, we report results of a quantitative-genetic study designed to investigate correlations between coloration and growth performance in *Ematurga atomaria*, a geometrid moth with remarkable within-species variation in larval appearance (Porter, 1997; Sandre *et al.*, 2013). Three generations of the moths were reared in the laboratory, with a half-sib design being used in the F2 generation. Our quantitative-genetic analysis focussed on both genetic and environmental correlations between a melanization-related trait and indices of growth performance, all of which we consider informative in the context of evolutionary ecology of coloration. For example, a positive genetic correlation between larval melanization and growth rate would imply that there may be variability in 'overall genetic quality' between the genotypes, with the 'better' ones possessing more resources to invest in both better growth performance and melanization. A negative genetic correlation would be expected if the genotypes primarily differed in the way how they allocate resources between growth and pigment synthesis. In contrast, a positive environmental correlation would indicate that favourable environmental conditions also facilitate a higher level of investment in melanization, and would thereby indicate costliness of the latter. A negative environmental correlation between melanization and growth performance would be more problematic to interpret, but might, as one alternative, point towards an adaptive decision to allocate more resources to melanin-based immunological defences when environmental quality is poor. In contrast, absence of correlations between coloration and growth parameters would support the view that coloration traits are relatively autonomous evolutionarily, not being tightly integrated into physiologically based trade-offs determining offspring production of the moths.

Materials and methods

Study species

Ematurga atomaria (Lepidoptera: Geometridae) is a medium-sized (about 2.5 cm in wing span) diurnal moth common in various habitats across the Palaearctic zone. The solitary larvae are highly polyphagous external feeders of the leaves of their host plants (Leraut, 2009). In the study area, the species is most abundant in coniferous forests and on peat bogs where, due to their abundance, dwarf shrubs from the family Ericaceae must serve as primary host plants. There is no evidence of geographical differences in host preference (Meister *et al.*, 2017).

Larval development consists of five instars and lasts for about 1.5 months (Vellau *et al.*, 2013), from June to August in northern Europe, with individuals subsequently overwintering as pupae. The larvae are cryptic, mimicking parts of their host plants. There is a considerable variation in patterning and darkness of the larvae: they range from light green to dark brown in their overall appearance. The variation is continuous, but with some combinations of traits being more common than others (Sandre *et al.*, 2013). Part of this variation has been found to be based on plastic responses to host plant-related environmental factors (Sandre *et al.*, 2013).

Spectral characterization of pigmentary coloration

Investigation of the pigments responsible for darkening larval coloration was conducted by comparing spectra of larval cuticular reflectance with the expected absorbance of known insect pigment types. Although the coloration of *E. atomaria* larvae, as with many lepidopteran larvae, is likely to be the product of multiple pigment types, including bile pigments, carotenoids and melanins (Kayser, 1985), we were specifically interested in comparing the spectral properties of the lighter forms to that of the darker forms. To do so, reflectance measurements were taken similar to those in Hõrak *et al.* (2010). The reflectance of 12 (six light, six dark) frozen full-grown larvae was measured in three different positions from the dorsal side on 1 mm^2 using a spectrophotometer (USB2000, Ocean Optics Inc., Dunedin, FL, USA). Larval cuticle was illuminated by a collimated beam of light oriented normal to the cuticle surface from a quartz optical fibre positioned at the zenith above the larvae and connected to a full spectrum light source (DH2000, Ocean Optics Inc., Dunedin, FL, USA). Light reflected from the cuticle surface was then collected by a quartz fibre-optic collector positioned at an azimuth of 45° below the zenith and transmitted to the spectrophotometer. Data from the spectrophotometer were digitalized and passed into a computer with appropriate software (OOIBase, Ocean Optics Inc., Dunedin, FL, USA). The spectrophotometer was calibrated for diffuse reflectance measurements using a white, diffusely scattering PTFE-based standard (WS-2 tile, Avantes BV, Apeldoorn, Netherlands). Each measurement provided a measure of per cent reflectance for each 1-nm interval in the range of 400–700 nm. Average reflectance curves were calculated based on measurements of the light ($n = 6$) and dark larvae ($n = 6$). The shape of the resulting averaged spectra was then evaluated based on known absorbance profiles of insect pigment types (e.g. melanin, carotenoids; Kayser, 1985).

Pedigree of the moths and rearing design

The individuals used in this study were offspring of 75 female moths caught from the wild population around Tartu (58°22′ N 26°43′ E), Estonia in 2008. The adults of the F1 generation were mated so that sib matings were avoided: 46 males were successfully mated with two females and 15 males with one female, resulting in 107 F2 broods (offspring of an individual female). The larvae of the F1 generation (530 larvae reached the pupal stage) were equally divided between three host plants from the family Ericaceae: bilberry *Vaccinium myrtillus*, L., blueberry *V. uliginosum*, L. and heather *Calluna vulgaris* L. For the F2 generation, 12 individuals from each full-sib brood were reared on *V. myrtillus* and three on *C. vulgaris* (1601 larvae in total). This design was a compromise between the needs of several different subprojects relying on the same data set (this study vs. Sandre *et al.*, 2013; Vellau *et al.*, 2013; V. Sõber, S. -L. Sandre, T. Esperk, T. Teder & T. Tammaru, in preparation).

The larvae were reared individually in transparent 50-mL plastic vials at 22 °C with identical humidity (close to 100% in the closed vials containing fresh food) and light–dark cycle (16L : 8D) for all larvae. Rearing vials were arranged on rearing trays in a randomized order with respect to host plant and brood. Leaves of their assigned host plant were available to the larvae *ad libitum*, with fresh leaves provided every third day.

Traits recorded

Several traits related to coloration and growth performance of the larvae were recorded for all individuals in both years (F1 and F2). Coloration-related traits were recorded from digital photographs taken on the 2nd day of the 5th instar (for details of digital photography, see Sandre *et al.*, 2013). In particular, three categorical pattern traits and two colour traits, each with 2–5 levels, were visually scored. To simplify the analysis of coloration, multiple correspondence analysis (MCA) was used to reduce the set of categorical multilevel coloration traits to two continuous dimensions. The first dimension captures differences in the degree of patterning, whereas the second dimension correlates with cuticular darkness (for details of the analysis, see Sandre *et al.*, 2013). The subsequent analysis focusses on the second dimension (hereafter called *darkness*) because of (1) a presumably higher relevance of this dimension in the context of the present study (see Introduction) and (2) much more even distribution of the experimental individuals along this axis (figure 2 in Sandre *et al.*, 2013).

To obtain indices of growth performance, all larvae were weighed when they had finished their growth in their 4^{th} (penultimate) instar (during the intermoult growth stasis, Esperk & Tammaru, 2004), and as pupae (a few weeks after pupation). The dates of moulting into 5th instar and that of pupation were recorded, allowing us to calculate the duration of last larval instar. Growth ratio in the last larval instar was then calculated as pupal mass/final mass of the 4th instar. Growth ratio was included in the analysis to specifically focus on growth performance in the final larval instar. Special attention to final instar was justified because colour polymorphism is most clearly expressed in this developmental stage, and it is also technically challenging to handle younger larvae. Pupae were sexed by morphological inspection when being weighed.

The indices of growth performance used should function as rather straightforward fitness correlates. Indeed, in capital breeding insects (Davis *et al.*, 2016), realized

fecundity of females is largely determined by adult body mass, for which pupal mass is a reasonable proxy (Honěk, 1993; Tammaru *et al.*, 1996, 2002; Rhainds *et al.*, 1999). *Ematurga atomaria* is clearly a capital breeder (Javoiš *et al.*, 2011) and shows a strong correlation between body size and fecundity (Meister *et al.*, 2018). Moreover, the principle of L-shaped reaction norms for body size and development time at maturation (see Teder *et al.*, 2014; for insect data) also allows one to reliably associate long developmental periods with inferior fitness. In particular, for the univoltine *E. atomaria*, there is no reason to assume that the effects of seasonality (Gotthard, 2008) would interfere with the long development/low fitness relationship (see Vellau *et al.*, 2013, for discussion).

Statistical analyses

Quantitative-genetic analyses were performed with the following four focal variables: (1) pupal mass (live weight, mg), (2) duration of the development in the 5th (final) larval instar (days), (3) growth ratio in the final instar (explained above) and (4) the coloration variable *darkness* (the second dimension from the MCA, Sandre *et al.*, 2013 and above). These variables could be recorded for 1987 F1 and F2 individuals who reached the pupal stage (1380 reared on *V. myrtillus*, 416 reared on *C. vulgaris* and 191 reared on *V. uliginosum*).

As the values of all four focal variables are known to be affected by host plant in *E. atomaria* (Sandre *et al.*, 2013; Vellau *et al.*, 2013), we analysed the data separately by the plants the larvae had been reared on. We primarily focussed on the larvae reared on *V. myrtillus,* that is, the treatment group with the largest sample size. Nevertheless, we preformed some of the analyses in parallel also for the smaller sample of larvae reared on *C. vulgaris*. The sample reared on *V. uliginosum* was too limited to be analysed separately but the data on those individuals were still used in the pedigree-based analyses.

First, the estimates of quantitative-genetic parameters for the 4 focal variables were calculated using only phenotypic records of F2 individuals relying on the half-sib structure of the data. General linear mixed models with fixed effect of sex and random effects of sire and dam (nested within sire) were fitted. Separate univariate analyses were performed for each study variable, and the heritabilities were estimated as the ratio of double sum of sire and dam variances to total phenotypic variance: $h^2 = 2(\sigma^2_S + \sigma^2_D)/\sigma^2_P$. The analyses were performed with MIXED procedure in SAS 9.4 (SAS Institute Inc., Cary, NC, USA).

Second, to account for all available pedigree information and the phenotypes measured in both generations and on two host plants (*V. myrtillus* and *C. vulgaris*), a multivariate animal model (Wilson *et al.*, 2001) was applied to simultaneously estimate heritabilities as well as genetic and environmental correlations for the four variables, separately for two host plants. With such a model, two quartets of variables – phenotypic values of four traits for individuals reared on *V. myrtillus* and phenotypic values of four traits for individuals reared on *C. vulgaris* – were analysed together, which, due to the shared pedigree information, allows the estimation of cross-environment genetic correlations. The model included fixed effects of generation and sex, random additive genetic effects of individuals and residual environmental effects. Additive genetic effects in the form of vector **a** as well as residual environmental effects in form of vector **e** were assumed to follow multivariate normal distributions: $\mathbf{a} \sim N(\mathbf{0}, \mathbf{A} \otimes \mathbf{G})$ and $\mathbf{e} \sim N(\mathbf{0}, \mathbf{I} \otimes \mathbf{R})$, respectively, where $\otimes$ denotes the Kronecker product, **G** and **R** are 8×8 additive genetic and residual variance–covariance matrices, respectively. The matrix **G** contains four 4×4 blocks with block $\mathbf{G}_{11}$ containing additive genetic (co)variances between variables measured on *V. myrtillus*, block $\mathbf{G}_{22}$ containing additive genetic (co)variances between variables measured on *C. vulgaris* and blocks $\mathbf{G}_{12} = \mathbf{G}_{21}{}^T$ containing cross-environment genetic covariances. Similarly, the matrix **R** contains four 4×4 blocks with blocks $\mathbf{R}_{11}$ and $\mathbf{R}_{22}$ containing residual (co)variances between variables measured on *V. myrtillus* and between variables measured on *C. vulgaris*, respectively, and blocks $\mathbf{R}_{12} = \mathbf{R}_{21}{}^T$ are null matrixes (there are no cross-environment residual covariances). Matrix **A** is the additive genetic relationship matrix considering additive genetic relationship coefficients between all three generations of individuals in the pedigree (2206 individuals in total). The heritabilities were estimated as the ratio of additive genetic variance to total phenotypic variance: $h^2 = \sigma^2_A/\sigma^2_P$, and the genetic and environmental correlations r_G and r_E were calculated as based on corresponding components of variance–covariance matrices **G** and **R**. Animal model was fitted and genetic parameters were estimated using software VCE 6.0 (Groeneveld *et al.*, 2008).

Calculating the heritabilities in two different ways allowed us to estimate the robustness of the results. Moreover, comparing the results of within-generation (half-sib) and cross-generation (animal model) analyses facilitated evaluating the role of more complex inheritance mechanisms (such as sex-specific inheritance and transgenerational environmental effects, which affect the results of the latter but not of the former analysis).

Results

The average cuticular reflectance spectrum of the dark larvae closely resembles that of melanin (Fig. 1); reflectance of incident light was less than 10% over the range of wavelengths 350 to 1000 nm, with a gradual rise in reflectance with increasing wavelength. Melanin is the only natural pigment with these characteristics,

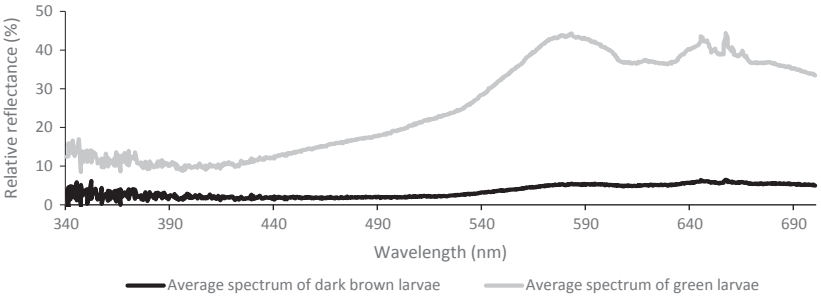


Fig. 1 Reflectance spectra of larval cuticle of dark brown (black line) and green (grey line) larvae of *Ematurga atomaria*, averaged over 6 larvae of both types.

Table 1 Descriptive statistics for the focal variables and estimated heritabilities according to the half-sib model of F2 individuals of *Ematurga atomaria* reared on *Vaccinium myrtillus* ($N = 1380$).

	Mean (SD)	Min, Max	Heritability
Pupal weight (mg)**	Males: 64.1 (6.27) Females: 75.1 (9.17)	39.2, 83.0 26.2, 100.5	0.585
Duration of the 5th instar (days)*	Males 9.72 (1.30) Females 11.03 (1.32)	6, 17 6, 18	0.366
Growth ratio in 5th instar	2.59 (0.301)	0.89, 4.51	0.320
Darkness (MCA score)	−0.037 (0.708)	−1.14, 1.35	0.255

*The among-gender difference in duration of last instar was significant ($F_{1,1379} = 150.5$, $P < 0.001$).
**The among-gender difference in pupal weights was significant ($F_{1,1379} = 313.4$, $P < 0.001$).

namely strong but slightly declining absorbance across the wavelength range discussed here (Riley, 1997). Thus, the spectral properties of these dark caterpillars are most consistent with increased cuticular melanization, similar to a number of other lepidopteran larva for which this has been studied in detail (e.g. Goodwin, 1953; Bear *et al.*, 2010; Futahashi *et al.*, 2010).

The reflectance spectrum of lightest larvae in Fig. 1 exhibits distinct long wavelength peaks, suggesting that the colour of these morphs is the product of non-melanin pigments. The reflectance of the light morph larvae is lowest in the violet (390–410 nm) and increases rapidly in the green range (530–560 nm). This spectral curve is consistent with a mix of biliverdin and carotenoids and/or ommochromes, a combination known from other Lepidopteran larvae (Kayser, 1985). Thus, the most likely driver of differences in larval darkness is differences in overall melanization, potentially accompanied by changes to the concentration of other cuticular pigments such as biliverdin, carotenoids and/or ommochromes.

The basic descriptive statistics of study variables measured on individuals reared on *V. myrtillus* are presented in Table 1. All the growth-related variables had coefficients of variation between 11 and 14%, with pupal masses and development times being sexually dimorphic. The analyses involving also individuals reared on *C. vulgaris* revealed statistically significant among-host differences in all four focal traits (all $P < 0.001$): the individuals reared on *C. vulgaris* had 8.8% lower pupal mass, 20.2% longer duration of the 5th instar, 10.8% lower growth ratio and higher darkness compared with individuals reared on *V. myrtillus* (see Sandre *et al.*, 2013; Vellau *et al.*, 2013, for a discussion of these among-host differences).

The half-sib analysis of F2 data indicated that all four focal traits are moderately heritable: the heritability estimates lay between 0.26 for darkness and 0.59 for pupal mass (Table 1). Including individuals reared on *C. vulgaris* and considering the effect of host plant, we see that heritability estimates for all traits only decrease by 0.04–0.05. An additional analysis (not shown) revealed also the potential effect of F1-parents' rearing plant on their F2-offspring, whereby the effect depended on the trait and parent gender. Nevertheless, the magnitudes of the estimated quantitative-genetic parameters were not affected. The three indices of growth performance correlated positively with each other (to be presented and discussed in another manuscript).

The results from the multivariate animal model as applied to individuals reared on *V. myrtillus* are presented in Fig. 2a. In comparison with the half-sib analysis (Table 1), this approach considers a broader range of relationships between the individuals, and also the phenotypic records of F1 individuals. Heritability estimates delivered by the animal model approach somewhat differed from those derived from the half-sib model (Table 1). Nevertheless, the magnitude of the heritabilities of the growth performance traits remained unchanged: about one-half of the variability in pupal weight, about one-third of the variability in the duration of 5th instar and about one-fourth of the variability in final instar growth ratio were heritable (see Fig. 2a for exact values). However, the heritability estimate of darkness increased more than two times. This difference may indicate that, in contrast to the directly heritable growth performance traits, the determination of coloration is more complex, possibly allowing for genetic interactions and/or epigenetic effects. The nearly symmetrical distributions of additive genetic effects of darkness – despite the bimodal phenotypic distribution of

the variable (Fig. 2, lower panel) – and respective model errors indicate that the trait has an underlying continuous character (liability), and a polygenetic background.

We subsequently used this information to investigate whether there are genetic correlations between darkness and growth performance of the larvae. As a notable result, we found that for the individuals reared on *V. myrtillus*, larval darkness showed almost no correlations, either genetic or environmental, with the indices of growth performance (Fig. 2a, see Table S1 for full results of the analysis). This suggests that the pathways of genetic determination and the responses to environmental conditions associated with larval darkness are independent from those for larval growth performance.

Animal model analysis of individuals reared on another host plant, *C. vulgaris,* returned somewhat higher but nevertheless similar heritability estimates for all three growth performance traits (Fig. 2b, and Table S1 for full results of the analysis). However, in this analysis, the estimates of genetic correlations related to larval darkness changed. There was still no relationship between darkness and duration of the 5th instar, but the genetic correlations of darkness with growth ratio and pupal weight were positive whereas environmental correlations were negative. However, none of these estimates exceeded 0.3 in absolute value (Fig. 2b). In the framework of the applied model, there is no possibility to explicitly test if the values of the quantitative-genetic parameters differ significantly from zero, or whether differences in the estimates for the two host plants are statistically significant. However, statistical significance can be approximately inferred from standard errors of respective estimates (Fig. 2a,b).

Genetic correlation between larval darkness on the two different hosts was estimated as $r_G = 0.63$ (SE = 0.08). This value considerably differs from unity, which indicates that the mechanism of genetic determination of larval darkness may partly differ on different host plants, so that the genetic basis for variation in larval darkness on bilberry may not – from the genetic perspective – precisely match the genetic variation responsible for differences in larval darkness on heather.

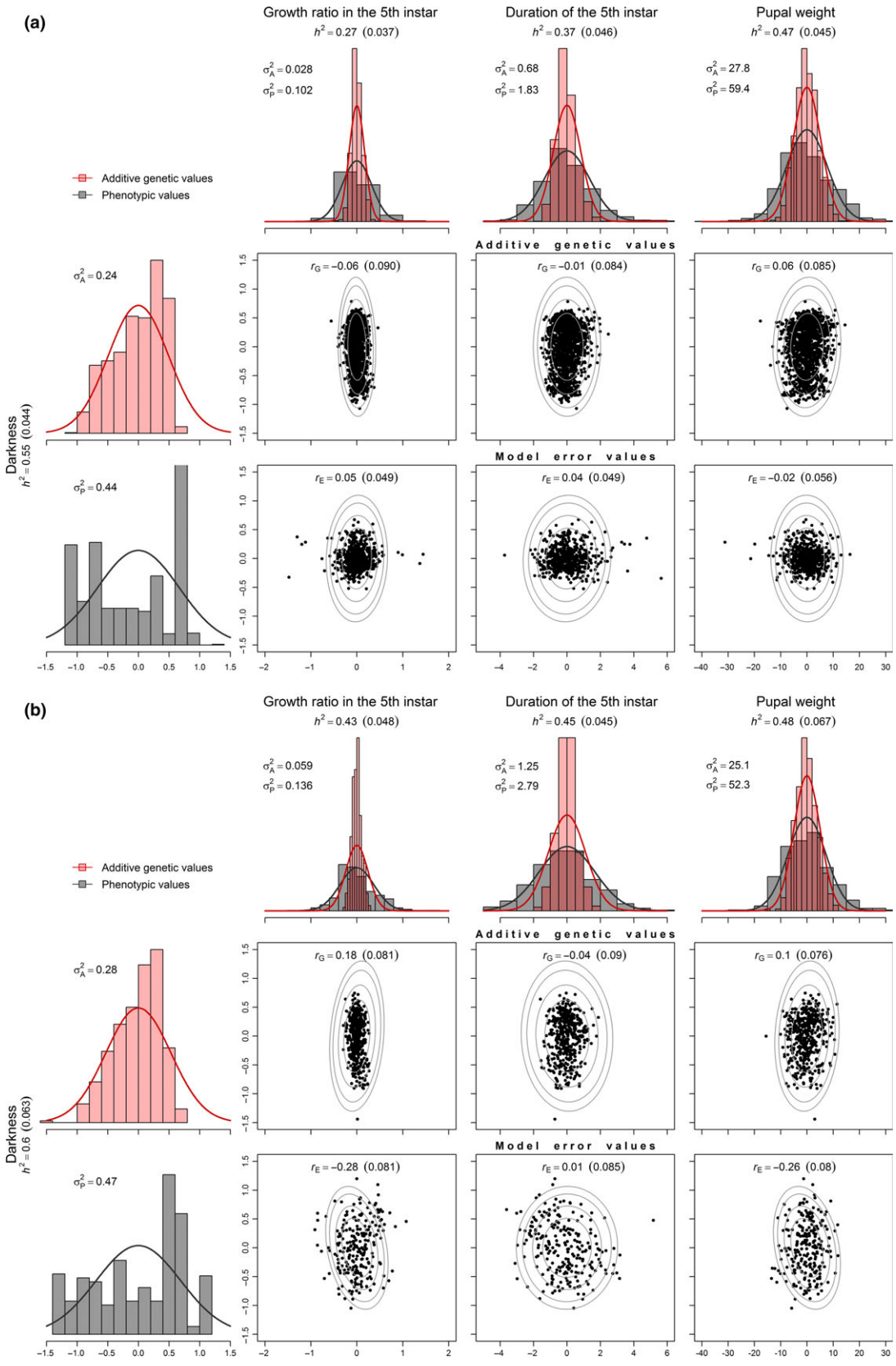
Discussion

The larvae of *E. atomaria* varied considerably in coloration-related traits (for visualization, see Fig. 2; Sandre *et al.*, 2013). About a quarter to a half of the variance in the larval darkness was estimated to be genetic (Table 1, Fig. 2). The indices of growth performance were similarly variable and showed moderately high heritabilities (Table 1). These patterns of high genetic variance in both larval darkness and growth rate facilitated estimation of both genetic and environmental correlations with a reasonable statistical power.

As the central result of the present study, for the larvae reared on *V. myrtillus*, we did not find any – neither environmental nor genetic – correlations between the index of larval darkness and the measures of fitness used. In particular, the dimension of larval appearance associated with darkness did not correlate with either pupal mass, relative mass increment in the last instar or developmental period of the larvae in the last instar. If melanization was traded off against fitness-related growth performance traits, both negative and positive correlations between the degree of melanization and indices of growth performance could arise (see Introduction). Any such correlations, either genetic or environmental, were however absent. This supports a view of microevolutionary independence between larval coloration and fitness-related growth performance in this species. Although growth performance should provide a satisfactory correlate of fitness for insects like *E. atomaria* (see Methods), the possibility nevertheless remains that fitness costs of certain coloration traits would appear in specific conditions, in the case of immune challenge in particular (Krams *et al.*, 2016). Further research on coloration of lepidopteran larvae should address also this possibility.

In the smaller sample reared on the alternative host *C. vulgaris*, we still found moderate but statistically significant correlations of darkness with pupal weight and growth ratio. Genetic correlations between the index of melanization and growth performance were positive, but the respective environmental correlations were negative. Due to the relatively high standard errors associated with these values, we find it premature to make definitive conclusions about possible causes of such a difference among host plants. Nevertheless, given that different host plants induce presumably adaptive plastic changes in the coloration of *E. atomaria* larvae (Sandre *et al.*, 2013), a positive genetic correlation between darkness and growth performance on *C. vulgaris* might indicate the existence of genotypes adapted to this particular host. In other words, rearing

Fig. 2 Heritabilities (h^2) and genetic (r_G) and environmental (r_E) correlations (with standard errors in brackets) for three studied life history traits and darkness for *Ematurga atomaria* larvae reared on (a) bilberry (N = 1380)and (b) heather (N = 416). The darker and lighter histograms present the empirical distributions of phenotypic and additive genetic values (breeding values), respectively; the black and grey fitted curves present the normal distributions with estimated additive genetic and phenotypic variance, respectively, and illustrate in such a way the ratio of variances which defines heritability (in case of $h^2 = 1$, the two distributions should overlap). In the scatterplots, the dots mark the additive genetic values and model error values of single individuals, respectively; the grey ellipsoids denote the 50%, 75%, 90% and 95% regions of bivariate normal distributions.


(a)
Growth ratio in the 5th instar
$h^2 = 0.27$ (0.037)
$\sigma_A^2 = 0.028$
$\sigma_P^2 = 0.102$
Duration of the 5th instar
$h^2 = 0.37$ (0.046)
$\sigma_A^2 = 0.68$
$\sigma_P^2 = 1.83$
Pupal weight
$h^2 = 0.47$ (0.045)
$\sigma_A^2 = 27.8$
$\sigma_P^2 = 59.4$
Additive genetic values
Phenotypic values
Darkness
$h^2 = 0.55$ (0.044)
$\sigma_A^2 = 0.24$
$\sigma_P^2 = 0.44$
Additive genetic values
$r_G = -0.06$ (0.090)
$r_G = -0.01$ (0.084)
$r_G = 0.06$ (0.085)
Model error values
$r_E = 0.05$ (0.049)
$r_E = 0.04$ (0.049)
$r_E = -0.02$ (0.056)
(b)
Growth ratio in the 5th instar
$h^2 = 0.43$ (0.048)
$\sigma_A^2 = 0.059$
$\sigma_P^2 = 0.136$
Duration of the 5th instar
$h^2 = 0.45$ (0.045)
$\sigma_A^2 = 1.25$
$\sigma_P^2 = 2.79$
Pupal weight
$h^2 = 0.48$ (0.067)
$\sigma_A^2 = 25.1$
$\sigma_P^2 = 52.3$
Additive genetic values
Phenotypic values
Darkness
$h^2 = 0.6$ (0.063)
$\sigma_A^2 = 0.28$
$\sigma_P^2 = 0.47$
Additive genetic values
$r_G = 0.18$ (0.081)
$r_G = -0.04$ (0.09)
$r_G = 0.1$ (0.076)
Model error values
$r_E = -0.28$ (0.081)
$r_E = 0.01$ (0.085)
$r_E = -0.26$ (0.08)

E. atomaria on particular host plants may reveal genetic variation that was otherwise hidden when rearing them on other host plants. In the context of the present study, however, the difference of the correlation structure between host plants reinforces the idea that there is no consistently shared genetic architecture connecting coloration and growth performance in this species. The relatively low genetic correlation between larval darkness as expressed on the two host plants provides further evidence that physiological state is unlikely to be a major determinant of larval colour phenotype.

The results of the present study caution against uncritically assuming a trade-off between coloration and physiologically based fitness-related traits. Empirical results confirming condition dependence of coloration in vertebrate animals (e.g. Van Buskirk & Schmidt, 2000; Griffith *et al.*, 2006; Costantini *et al.*, 2007; Svensson & Wong, 2011) have been echoed by examining this relationship in insects (e.g. Talloen *et al.*, 2004). Indeed, in a recent review, Roulin (2016) suggested that the condition-dependent component of melanin-based coloration is stronger in invertebrates than vertebrates. Studies showing no association between coloration and performance indices in insects are, to our knowledge, scarce (see, however, Contreras-Garduño *et al.*, 2007; Sandre *et al.*, 2007; Karl *et al.*, 2010). However, this paucity of evidence may be the result of a publication bias for positive results, rather than a reflection of the 'typical' relationship between melanization and other physiological processes in insects. Moreover, as indicated by this and other (Roulin, 2016) studies, the overall picture may be biased by the dominant practice of studying the correlations in one environment only: lots appears to be gained from examining those across an environmental gradient.

There are a number of reasons to expect differences in the strength and/or direction of correlations between coloration and growth performance both within and among species. For example, we might expect strong correlations when precursors of the pigments are limiting but not when they are in ample supply (Morehouse *et al.*, 2010; Morehouse, 2014). The observation that correlations were stronger on heather than on bilberry is consistent with this possibility as the former plant has lower nitrogen content (Vellau *et al.*, 2013). Moreover, synthesizing different pigments must differ in terms of costliness (e.g. McGraw & Hill, 2000), and pigments may differ in the amount of the concentration of pigment molecules needed to produce a change in the animal's appearance. For example, melanin has a particularly high extinction coefficient, that is, very little of it is needed to achieve the same visual effect, as compared to pterins and carotenoids (Kayser, 1985). Also, distribution of the pigment in the body of the animal should matter: the costs of dark coloration may indeed be higher in such insects as crickets or beetles in which melanization affects massive cuticular structures (Roff & Fairbairn, 2013; Evison *et al.*, 2017), as compared to hairless larvae in which pigmentation is limited just to the body surface.

Bird predation is a major cause of mortality among lepidopteran larvae (Cornell & Hawkins, 1995) and should thus form the primary source of natural selection on coloration and body size in insects (Remmel & Tammaru, 2009; Remmel *et al.*, 2011). We are therefore inclined to see the notably high diversity of colour morphs in *E. atomaria* as an outcome of selection caused by birds as visually guided predators. On the one hand, this is supported by host plant-driven plastic changes in larval coloration (darkness included), which may help to increase camouflage on particular hosts (Sandre *et al.*, 2013). On the other hand, the high variability in coloration and patterning does not require an explanation more complex than negative frequency-dependent selection, a very common epiphenomenon of selection caused by vertebrate predators (Ruxton *et al.*, 2004). The considerable heritability of coloration traits revealed by this study is consistent with this scenario (Maynard Smith, 1982). The results of the present study thus provide no evidence that physiologically mediated trade-offs could interfere with adaptive evolution of larval coloration caused by the need to remain undetected by the predators.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1 Heritabilities (in bold face on main diagonal) and genetic and environmental correlations (above and below the diagonal, respectively) with standard errors in brackets for three studied life history traits and darkness for *E. atomaria* larvae reared on heather *Calluna vulgaris L.* ($N = 416$) or on bilberry *Vaccinium myrtillus* ($N = 1380$).

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