

Evolutionary genetics of dorsal wing colour in *Colias* butterflies

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Abstract

The evolution of butterfly wing colouration is strongly affected by its multiple functions and by the correlated evolution of wing colour elements. Both factors may prevent local adaptation to ecological conditions. We investigated one aspect of wing colouration, the degree of dorsal wing melanization, in the butterfly *Colias philodice eriphyle* across an elevational gradient and its correlation with another aspect of wing colouration, ventral wing melanization. Dorsal wing melanization increased with elevation and these differences persisted in a common environment. Full-sibling analysis revealed high heritability for males but only intermediate heritability for females. The correlation between ventral and dorsal melanization showed significant elevational and sex-specific differences. In males the two traits were highly correlated, whereas in females the strength of the correlation decreased with increasing elevation. We conclude that uncoupling of ventral and dorsal melanization has evolved in females but not in males and discuss possible mechanisms underlying uncoupling.

Introduction

Butterfly wing colouration is important in multiple functions such as thermoregulation, aposematic signaling and mate recognition (Vane-Wright & Boppre, 1993; Jiggins *et al.*, 2001; Kapan, 2001; Ellers & Boggs, 2003). Intraspecific variation in wing colour in butterflies often has adaptive significance and is under natural selection in the field. For example, morphs with darker wings can achieve higher flight activity and faster egg maturation under cold conditions (Kingsolver, 1983b; Van Dyck & Matthyssen, 1998; Ellers & Boggs, 2002, 2004), and differences in the size and number of wing spots can affect mating success (Fordyce *et al.*, 2002). However, the evolution of traits with multiple functions, including wing colour, depends on the cumulative effect of both direction and strength of selection associated with each individual function. Hence, the evolution of the optimal phenotype for each function may be constrained if

opposing selection pressures act on different functions (Ellers & Boggs, 2003). Therefore, separate functions of a complex trait may not be adapted to local conditions.

Simultaneously, the evolutionary response of wing colouration may be limited by developmental mechanisms. For example, the size and shape of wing pattern elements such as eyespots show strong genetic and phenotypic correlations, indicating developmental coupling (Kingsolver & Wiernasz, 1991; Paulsen, 1994; Brakefield & French, 1999; Beldade & Brakefield, 2002; but see Beldade *et al.*, 2002). Less attention has been given to more subtle wing colouration, such as the evolution of the tone of the wing, including variation in the degree of wing melanization. In many species variation in wing melanization is seen on both dorsal and ventral wing surfaces (Kemp & Jones, 2001). High heritabilities for dorsal or ventral wing melanization have been found (Van Dyck *et al.*, 1998; Ellers & Boggs, 2002) but the question of whether the two traits are correlated to the same extent as conspicuous wing pattern elements are, has remained unaddressed. Yet this is an important question because a strong correlation constrains the potential for independent changes in melanization and may prevent local adaptation.

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In the butterfly, *Colias philodice eriphyle* Edwards hind wing melanization varies extensively on both the ventral and dorsal side. Wing melanization is caused by a diffuse spread of black scales from the base of the wing towards the outer edges, giving the yellow wings a green-grey appearance. Melanization on the ventral wing surface is functional in thermoregulation during basking, and its evolution has been extensively studied (Watt, 1968; Kingsolver, 1983a,b; Kingsolver & Watt, 1984; Ellers & Boggs, 2002). Ellers & Boggs (2002) showed significant genetic variation for the degree of ventral wing melanization and moderate to high heritabilities. In addition, female ventral wing melanization plays an important role in mating success of females because *Colias* males prefer more yellow (i.e. less melanized) females (Ellers & Boggs, 2003). There is no sexual selection on male ventral wing melanization. Much less attention has been directed to the function and genetics of the dorsal wing colour. This side is exposed to sunlight only during flight and may play a role in the regulation of body temperature during flight (Tsuji *et al.*, 1986). No sexual selection on female or male dorsal wing melanization has been shown so far. Therefore, the two wing sides typically have different functions and are exposed to different selection pressures, thus creating the potential for independent changes. However, if the correlation between the degree of melanization on the two wing surfaces is strong, the evolution of wing melanization will reflect a compromise between the optimal phenotypes of both sides.

The aim of this work is to answer the following questions: (1) Is there variation in dorsal wing melanization for male and female *C. p. eriphyle* across an elevational gradient? (2) What is the heritability of dorsal wing melanization? and (3) Is there a correlation between ventral and dorsal melanization, and are there elevational and sex-specific differences in this correlation?

Methods

Species description and study site

In the Colorado Rocky mountains, *C. p. eriphyle* occurs in alfalfa fields and on native legumes in meadow habitat at elevations from 1000 to 3000 m. It has two generations per year at the higher elevations and up to four at the lower elevations. Seven sites located in Gunnison and Delta counties were used for our field work: native meadow habitat in Paonia (Pa, 1840 m), Cimarron (Ci, 2150 m), lower Gold Basin (Gb, 2350 m), Elk Meadows (Em, 2420 m), Jack's Cabin (Jc, 2615 m), Brush Creek (Bc, 2810 m) and Willow Creek (Wc, 2920 m). An additional site at Honey Lake (Hl, 1225 m), near Susanville in the Sierra Nevada of California, is a large alfalfa field.

Population divergence

Intraspecific divergence in the degree of wing melanization in *C. p. eriphyle* was assessed in seven field populations across an elevational gradient from 1840 to 2920 m: Pa, Ci (only males), Gb, Em, Jc, Bc and Wc. All populations were sampled in August/September 1999 and 2000, except Pa, which was sampled in June 2000. Where density allowed, 15 males and 25 females were caught in each population and stored in the freezer until further measurements were taken in the laboratory. For each individual wing wear was estimated using a scale from 1 (no scale loss) to 5 (more than 50% of the scales lost).

We also collected six females from each of Pa and Wc and raised their offspring in a common environment to assess whether differences in wing melanization were genetically based. The females were allowed to oviposit on clover (*Trifolium hybridum*), which is a natural host plant. Any eggs laid were collected daily and stored at 5 °C in a plastic bag until the experiment started. At the start of the experiment, 25 eggs per female were put on clover in a cage and reared in an incubator with 16 : 8 (L : D), which is comparable with light conditions in the field. The incubator temperature was 25 ± 1 °C, which is close to the average low elevation temperature. We used wild clover plants collected near Rocky Mountain Biological Laboratory and therefore host plant quality may have varied. When two-thirds of the clover was eaten, the larvae were transferred to a new plant. This was repeated until all the larvae had pupated. The cages were checked daily for emerging butterflies. After emergence, the butterflies were stored in the freezer for further analysis. For the analyses of wing melanization, 15 males and 15 females were chosen at random for each of Pa and Wc from the pooled offspring of all females from each population.

Breeding experiment

The heritability of the degree of dorsal wing melanization was estimated with a breeding experiment. Sixty females were collected at Hl, and brought into the greenhouse at Stanford at a temperature cycle of 24 °C day and 15 °C night and 16 : 8 (L : D). Females were allowed to oviposit on vetch (*Vicia americana*), which was grown hydroponically in the greenhouse. Every day, any eggs laid were collected and stored at 5 °C in a plastic bag until the next generation was started. For the next generation, 25 eggs per female were placed on vetch, with eggs of each female spread across four plants in the same cage. Whenever two-thirds of the vetch was eaten, the larvae were transferred to new plants. This was repeated until all the larvae had pupated. The use of several plants per family and changing the plants multiple times over the rearing period minimized the confounding effects of common environmental differences. *Colias* show complete sperm precedence (Boggs & Watt, 1981), which

means that the offspring of one female are full sibs. The degree of wing melanization was measured for all offspring.

Heritability of wing melanization using a full-sib analysis was calculated using the components of variance from one-way ANOVAs (Falconer, 1989), as well as using Restricted Maximum Likelihood (REML) analysis which has the advantage that it gives unbiased estimates even if family sizes are unbalanced. In a full-sib analysis, heritability is estimated as $(V_A + \frac{1}{2}V_D)/V_P$, so that one cannot distinguish the contributions to the total phenotypic variation (V_P) of the additive variance component, V_A and a nonadditive variance component, dominance variance V_D . Thus, a full-sib analysis estimates broad-sense heritability and can only set an upper limit to heritability, whereas heritability in the strict sense is V_A/V_P (Lynch & Walsh, 1998). Obviously, any heritability estimate is dependent on the conditions under which it is measured, and should be interpreted accordingly. Any families with females of the *alba* phenotype [missing the yellow pigment (Gerould, 1911; Watt, 1973)], or which showed signs of introgression with the close sister species *C. eurytheme* [orange pigment in wing (Taylor, 1972; Gula & Taylor, 1980)], were eliminated from the analysis. The full-sib analysis consisted of 24 families and a total of 110 male and 91 female offspring, with each of the 24 families more or less evenly represented. The corrected family size under an unbalanced design (k_3 , Lynch & Walsh, 1998, p. 574) was 3.5 for males and 3.2 for females. The analysis was performed separately for male and female offspring, because phenotypic values differed significantly by sex. A power analysis showed that our experimental design has 73% resp. 67% probability to detect heritability for males resp. females of 0.5 using the components of variance from one-way ANOVAs.

Genetic correlations between dorsal and ventral melanization were calculated using the mean squares and cross-products of analyses of (co)variance (Lynch & Walsh, 1998). The genetic correlation is estimated by $\rho_A = \text{Cov}(s_1, s_2) / [\text{Var}(s_1) \times \text{Var}(s_2)]^{0.5}$, where $\text{Var}(s_1)$, $\text{Var}(s_2)$ and $\text{Cov}(s_1, s_2)$ denote estimates of the sire components of variance and covariance. Phenotypic correlations between dorsal and ventral melanization were calculated using a standard least-squares regression.

Wing melanization measurement

Butterfly wings consist of a fine mosaic of tiny scales, which in *Colias* can be yellow, or black (melanized). Melanization of the hind wing was measured in an area of fixed size, below the discal cell at the crossing with the cubital vein (Ellers & Boggs, 2003). The wing was photographed on a standard grey background using a black and white camera mounted on a microscope and connected to a computer with the software MORPHOSYS. In Adobe Photoshop, the image was standardized for contrast through a percentage saturation stretch of the

tonal histogram of the image (Wilkie & Finn, 1996). This is a commonly used remote sensing technique, which ensures that the full range of black tones for each image is used by setting the bottom and top first percentile of the black tonal range to pure black and white. Subsequently, the percentage of the standardized image that was melanized was measured using a threshold value of 70 (on a scale of 0–255). The repeatability of the wing melanization measurements was high. We took a random set of 30 individuals from the breeding experiment and photographed the same wing twice. The correlation between the wing melanization measurements on the two photographs was $r = 0.94$, $n = 30$, $P < 0.001$. Since no significant consistent difference was found between left and right wing melanization, the degree of melanization used in the analyses was averaged over both wings. The scales on butterfly wings overlap to a great degree so that some loss of scales should not affect melanization measurements. To justify this assumption, we tested the effect of wing wear on the measured degree of melanization our field-collected individuals, but in none of the populations was wear significant (for all elevations: $r^2 < 0.12$, ns). Measurements of ventral wing melanization were taken from Ellers & Boggs (2002).

Results

Elevational gradient in dorsal wing melanization

The degree of dorsal wing melanization differed among elevations and between sexes (Fig. 1). At all elevations,

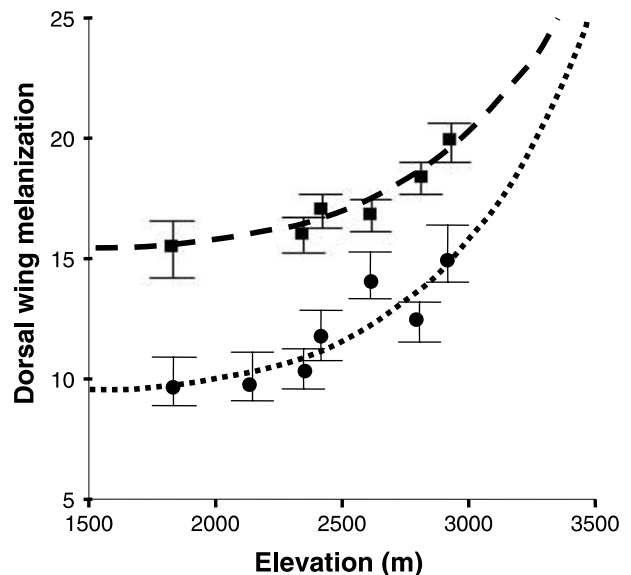


Fig. 1 The relationship between elevation and the degree of dorsal wing melanization (\pm SE) in *Colias philodice eriphyle* males (dotted line, $y = 9.51 + 9.21 \times 10^{-21} \times x^6$) and females (dashed line, $y = 15.39 + 7.30 \times 10^{-21} \times x^6$).

females were significantly darker than males (ANOVA with elevation and sex: elevation: $F_{[5,170]} = 5.61$, $P < 0.001$; sex: $F_{[1,170]} = 67.8$, $P < 0.001$). Wing melanization was an accelerating function of elevation, indicating that there was a greater rate of change in the degree of melanization at higher elevations (males: $r^2 = 0.80$, $F_{[1,5]} = 20.27$, $P < 0.01$; females: $r^2 = 0.94$, $F_{[1,4]} = 60.22$, $P < 0.001$) (Fig. 1).

Population differences in wing melanization persisted when offspring were reared in a common environment (Fig. 2). Individuals from the high elevation population (Wc) were significantly darker than their conspecifics from low elevation (Pa) ($F_{[1,110]} = 27.0$, $P < 0.001$), and females were significantly darker than males ($F_{[1,110]} = 59.1$, $P < 0.001$) under common conditions. The interaction between sex and elevation was not

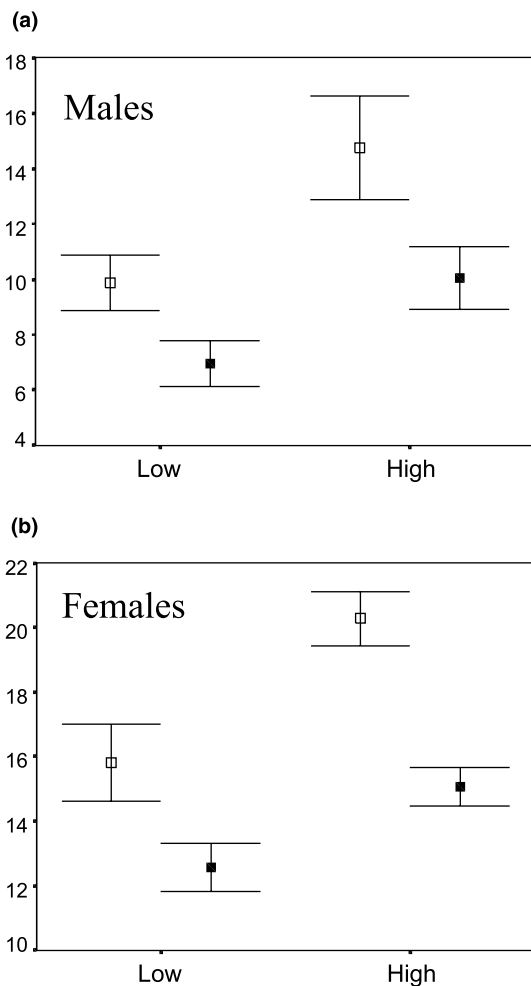


Fig. 2 The degree of dorsal wing melanization (\pm SE) in low (Pa) and high (Wc) elevation males and females of *Colias philodice eriphyle* reared in a common garden experiment (filled squares) compared with wild-caught individuals (open squares).

significant. This reveals a genetic component to wing melanization. Furthermore, common garden individuals from both populations were less melanized than wild-caught individuals ($F_{[1,110]} = 31.4$, $P < 0.001$), showing that there is an environmental component for wing melanization as well. The data suggest a greater environmental effect for the high elevation population, but this is most likely due to our choice of temperature in the common garden regime.

Genetics of dorsal wing melanization

The full-sib ANOVA for the degree of dorsal wing melanization is given in Table 1. The full-sib analysis showed significant heritability for both males and females. The upper estimate of heritability for males was $h^2 = 0.90$ (± 0.21 SE) and for females $h^2 = 0.35$ (± 0.25 SE). REML analysis yielded very similar estimates ($h^2 = 0.82$ for males and $h^2 = 0.32$ for females). Given these estimates, broad sense heritability for females seemed lower. As the variances for males and females were of the same magnitude, the low heritability for females cannot be due to reduced variance in the degree of wing melanization in females.

Genetic and phenotypic correlations between dorsal and ventral melanization

In the breeding experiment, there is a positive phenotypic correlation between the degree of ventral and dorsal melanization in both males ($r = 0.68$, $n = 110$, $P < 0.001$) and females ($r = 0.48$, $n = 91$, $P < 0.001$). However, a positive genetic correlation was only found for males ($r = 0.82$, $n = 110$, $P < 0.001$), not females ($r = 0.004$, $n = 91$, ns).

In the field, the overall phenotypic correlation between ventral and dorsal melanization was also positive for both males ($r = 0.57$, $n = 122$, $P < 0.001$) and females ($r = 0.53$, $n = 130$, $P < 0.001$), but there were large differences among elevations and sexes in the strength of the correlations. In males, ventral and dorsal melanization were positively correlated at all elevations (Fig. 3), although marginally significant at the highest

Table 1 ANOVA of full-sib design for dorsal wing melanization in *Colias philodice eriphyle*. Males and females are analysed separately because phenotypic values differed significantly by sex.

Source	Mean square	d.f.	F-value	P-value
Males dorsal				
Family	46.91	22	4.86	<0.001
Error	9.65	88		
Females dorsal				
Family	19.35	23	1.69	0.05
Error	11.47	68		

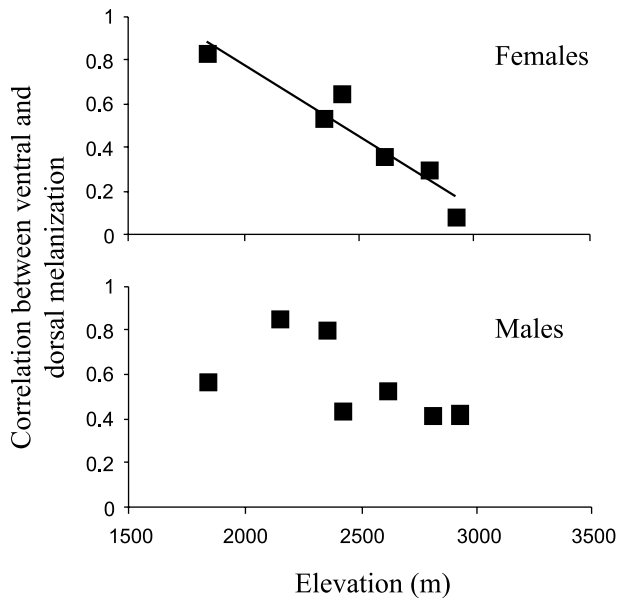


Fig. 3 The relationship between elevation and the strength of the phenotypic correlation between dorsal and ventral melanization for males and females.

Table 2 Phenotypic correlations of the degree of ventral and dorsal wing melanization in *Colias philodice eriphyle* males and females.

Site	Elevation	Males (<i>r</i>)	<i>P</i> -value	Females (<i>r</i>)	<i>P</i> -value
Pa	1840	0.56	0.037**	0.83	<0.001****
Ci	2150	0.85	<0.001****		
Gb	2350	0.80	<0.001****	0.53	0.008***
Em	2420	0.43	0.053*	0.65	<0.001****
Jc	2615	0.53	0.014**	0.36	0.070*
Bc	2810	0.41	0.066*	0.30	0.147 NS
Wc	2920	0.42	0.061*	0.08	0.761 NS

* $P < 0.1$; ** $P < 0.05$; *** $P < 0.01$; **** $P < 0.001$.

elevations (Table 2). There was no relationship between elevation and the strength of the correlation between ventral and dorsal melanization ($r = -0.57$, $n = 7$, $P = 0.186$). In contrast, in females the strength of the correlation decreased significantly with increasing elevation ($r = -0.95$, $n = 6$, $P < 0.01$). Female ventral and dorsal melanization were only positively correlated in low elevation populations, whereas in high-elevation populations ventral and dorsal melanization were uncoupled (Fig. 3, Table 2). We used Fisher's Z-transformation to compare the correlation coefficients for males and females. Despite the fact that the strength of the correlation between ventral and dorsal melanization decreased with increasing elevation in females but not in males, there was no evidence that this relationship differed significantly between sexes ($Z = 1.55$, ns).

Discussion

Dorsal wing melanization increased with elevation in *C. p. eriphyle*, as has been found in earlier studies for ventral wing melanization (Kingsolver, 1983a; Ellers & Boggs, 2002). The function of ventral melanization has been extensively studied (e.g. Watt, 1968; Kingsolver, 1983a,b; Kingsolver & Watt, 1984); however, little attention has been directed to the function of dorsal wing melanization (Tsuji *et al.*, 1986). Data in Tsuji *et al.* (1986) suggest that dorsal basking increases thorax temperature substantially, and thus dorsal melanization may serve to help regulate body temperature during flight. Flying results in net cooling as a consequence of the greater convective heat loss (Tsuji *et al.*, 1986). Therefore, dorsal melanization may slow down the heat loss by increasing solar heat absorption during flight. Since ambient temperature decreases with altitude, the increase in dorsal wing melanization with altitude may be under natural selection.

Our results show that the observed population divergence in dorsal wing melanization has a genetic basis. Compared with the genetic differences in ventral wing melanization found earlier (Ellers & Boggs, 2002), the common garden environment revealed genetic differences between populations to be larger for dorsal wing melanization. Therefore, we argue that dorsal wing melanization is under stronger genetic control than ventral melanization. An explanation for this may lie in *Colias* biology. Because *Colias* is a lateral basker, the dorsal wing surface is exposed solely when flying. Flying takes place only under a very restricted set of climatic conditions, i.e. when solar radiation and air temperature are sufficiently high to allow body temperatures between 28 and 39 °C (Watt, 1968). As a consequence, the dorsal wing surface is exposed to relatively little variance in weather conditions. On the contrary, the ventral wing surface is exposed to a much larger range of climatic conditions as it is exposed during basking as well as resting. Therefore, the selective pressures on ventral melanization may vary over a broader range, depending on experienced climatic conditions. A higher degree of phenotypic plasticity in ventral wing melanization may allow *Colias* to adjust to such temporal differences.

The breeding experiment showed significant heritabilities for dorsal wing melanization, comparable with estimates for other species (Van Dyck *et al.*, 1998; Windig & Lammar, 1999; Windig, 1999). The heritability estimate for dorsal wing melanization was very high in males, whereas only weak to moderate in females. One explanation for this sex-specific difference in heritability could be that the traits are sex-linked. In Lepidoptera, females are the heterogametic sex, and as a consequence, in a full-sib design the estimate of male heritability would be biased upward if dorsal melanization is located on the X-chromosome (Trivers, 1985; Lynch & Walsh, 1998).

Ventral wing melanization is sex-linked in *C. p. eriphyle* (Ellers & Boggs, 2002), and several examples of sex-linked wing colour traits are found in other species (Prowell, 1998).

The breeding experiment also showed a significant genetic correlation between dorsal and ventral melanization for males but not for females. In the field, we could only estimate phenotypic correlations, which reflect the joint effect of environment and genetic correlations. Therefore, the value of a phenotypic correlation may not be equal to that of a genetic correlation (Mousseau & Roff, 1987; Willis *et al.*, 1991). However, extensive comparative studies have shown that phenotypic and genetic correlations are generally of the same sign and magnitude in the majority of cases, especially if morphological traits are involved (Cheverud, 1988; Roff, 1995, 1996). With appropriate caution, the field data on the phenotypic correlation in *C. p. eriphyle* may be interpreted as showing that uncoupling of ventral and dorsal melanization has evolved in females but not in males.

Although we have no evidence of the evolutionary history of the coupling between ventral and dorsal melanization, two lines of evidence suggest that it is more likely that uncoupling has evolved in females than vice versa, i.e. that coupling has evolved in males. First, coupling between dorsal and ventral characters seems to be the rule in *Colias*, because it is found in males of all populations and in females of some populations. Secondly, coupling of dorsal and ventral melanization has been observed in other species such as *Hypolimnas bolina* (Kemp & Jones, 2001) and the melanic form of *Papilio glaucus* (Koch *et al.*, 2000a,b).

One likely explanation for the sex-specific differences in *Colias* lies in the multiple functions of wing melanization in the two sexes. In females, both dorsal and ventral melanization contribute to thermoregulation, which causes an increase in melanization at higher elevations. In addition, the degree of female ventral melanization is also important in male mate choice, with males preferring less melanized females throughout their range (Ellers & Boggs, 2003). The cumulative effect of both selection pressures on female wing melanization results in a reduced melanization on the ventral wing surface compared with the dorsal wing surface. Males, on the contrary, are not subject to female sexual selection for wing colour (Silberglied & Taylor, 1978), and therefore experience only selection pressures for thermoregulation on both ventral and dorsal melanization at all elevations. Consequently, only in females has an uncoupling of the degree of melanization on the two wing surfaces evolved.

Alternatively, the uncoupling between female ventral and dorsal wing melanization at high elevations may have evolved because of the cost of melanin production. Melanin is a nitrogen-rich pigment that is deposited in the wing scales late in the pupal development. Within

the developing pupa the total supply of nitrogen is limited, so that trade-offs in nitrogen allocation may arise between melanin synthesis and other nitrogen-consuming functions such as growth or egg production. Talloen *et al.* (2003) showed that experimentally induced nitrogen limitation causes a significant reduction in wing melanization in *Pararge aegeria*. The high nitrogen costs of egg production in females, together with the cumulative nitrogen cost of high ventral and dorsal melanization at high elevation may exceed their pupal nitrogen supply. Females may therefore have evolved to reduce wing melanization in favour of nitrogen allocation to eggs.

What are the developmental processes that underlie the uncoupling of ventral and dorsal wing melanization? The various pigments that make up the colour pattern in the wing are deposited in a fixed order, with the black pigment melanin being deposited last. Pigments are only deposited in fully developed scales so that the colour of individual scales is determined by timing of scale development (Koch *et al.*, 2000a,b). Through delaying or accelerating scale development of specific wing areas, the colour or position of a wing pattern element can be changed. Uncoupling of colour patterns is achieved through compartmentalization so that each specific wing area acts as an autonomously developing element (Nijhout, 2001). This mechanism has been shown for various colour patterns on the same wing surface (Koch *et al.*, 2000b), but could equally well apply to wing tone on the ventral and dorsal surface. Uncoupling of dorsal and ventral wing melanization in females would thus require heterochrony of scale development on the two wing surfaces. Heterochrony provides a testable hypothesis of a possible mechanism underlying the independent changes in wing colouration.

In conclusion, more subtle wing colouration such as the tone of the wing is at least partly genetically determined, as is found for more conspicuous wing patterns. Also, correlations between ventral and dorsal wing colour elements can be equally strong as correlations between wing pattern elements on the same wing surface. Yet, no evidence was found for developmental constraints shaping the evolution of traits even when they are as highly correlated as are ventral and dorsal wing melanization in males. The uncoupling of wing elements found in females shows that selection is the dominating factor in the evolution of wing colour.

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