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THE EVOLUTION OF WING COLOR IN *COLIAS* BUTTERFLIES: HERITABILITY, SEX LINKAGE, AND POPULATION DIVERGENCE

JACINTHA ELLERS[1,2,3] AND CAROL L. BOGGS[1,2]
[1]*Center for Conservation Biology, Department of Biological Sciences, 371 Serra Mall, Stanford University, Stanford, California 94305-5020*
[2]*Rocky Mountain Biological Laboratory, Crested Butte, Colorado 81224*

Abstract.—We investigated the genetic background of intraspecific variation in wing color across an elevational gradient in the butterfly *Colias philodice eriphyle*. The degree of wing melanization was an accelerating function of elevation, and differences in wing melanization persisted in a common environment. Full-sibling analysis and parent-offspring regression yielded consistent, moderate to high heritabilities for the degree of wing melanization. The breeding experiments also demonstrated that wing melanization is strongly sex linked. Because traits that differentiate sister species also tend to be sex linked, our results suggest that the genetic mechanisms underlying intraspecific differences in wing melanization are not fundamentally different from those that have been shown to differentiate sister species.

Key words.—Ecological gradient, genetic adaptation, heritability, sex linkage, speciation, wing melanization.

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The evolution of wing coloration in butterflies is strongly affected by its multiple functions, such as thermoregulation, mate choice, defense against predators, and mimicry (Vane-Wright and Boppré 1993; Van Dyck et al. 1997; Kapan 2001; J. Ellers and C. L. Boggs, unpubl. ms.). Intraspecific variation in wing color is often a local adaptation to ecological conditions (Guppy 1986; Brakefield and Reitsma 1991). Simultaneously, wing color plays a role in mate choice and species recognition, which can drive interspecific divergence in wing color (Silberglied and Taylor 1973, 1978; Wiernasz and Kingsolver 1992; Mallet et al. 1998). The parallel importance of wing color at the intra- and interspecific level implies that local selection pressures may have consequences for reproductive isolation between species. However, for such variation to contribute to future evolutionary pathways of populations, there must be a genetic basis underlying phenotypic divergence.

Wing coloration in Lepidoptera is determined by genetic and environmental components. In several species, wing color or wing pattern depends on rearing temperature (Hoffmann 1978; Brakefield and Mazzotta 1995) or photoperiod (Douglas and Grula 1978; Jacobs and Watt 1994; Kingsolver 1995) during the larval stage, although genetic variation is also present for the shape and size of color patches such as eye spots and mimetic pattern coloration (Smith 1976; Holloway et al. 1993; Windig 1998; Gilbert 2002). However, differences in background wing color often involve variation in the degree of wing melanization, the genetics of which are rarely studied (Van Dyck et al. 1998).

Here we report on population divergence in the degree of wing melanization and its genetic basis in the butterfly *Colias philodice eriphyle* Edwards across an elevational gradient. In *Colias*, melanization is essential in thermoregulation because darker wings absorb more sunlight and enable lateral basking butterflies to raise their body temperature sufficiently to allow flight. Flight is necessary for fitness-related activities such as oviposition and nectaring in females and patrolling, mating, and nectaring in males (Watt 1968; Kingsolver 1983a,b). Variation in the degree of wing melanization exists both within and between species of *Colias* (Watt 1968; Roland 1982; Kingsolver 1983a). Genetic analysis has shown that interspecific differences in wing color between *C. p. eriphyle* and *C. eurytheme*, such as ultraviolet reflectance, yellow wing pigmentation, and the width of the black band on the forewing, are heritable and sex linked (Silberglied and Taylor 1973, 1978; Grula and Taylor 1980). Intraspecific differences in wing melanization in these two species are known to be affected by larval rearing conditions (Hoffmann 1978; Jacobs and Watt 1994). However, the genetic component of wing melanization has not been studied, and it is unknown whether intraspecific differences in wing melanization are caused by the same genetic structure as traits that have been shown to differentiate sister species.

In this paper we explore genetic factors affecting wing melanization at the intraspecific level in *C. p. eriphyle*. We measure phenotypic variation in melanization in the field and use common-garden and breeding experiments to quantify the genetic basis of differences in wing melanization. Furthermore, comparison of sex-specific heritabilities reveals whether sex linkage plays a role in within-species differences in wing color.

MATERIALS AND METHODS

Species Description and Study Site

In the Colorado Rocky Mountains, *C. p. eriphyle* lives in alfalfa fields and on native legumes in meadow habitat at elevations from 1000 to 3000 m. 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),

[3] Present address: Vrije Universiteit, Institute of Ecological Science, Faculty of Earth and Life Sciences, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands; E-mail: ellers@bio.vu.nl.

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 and 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. The age of each individual was estimated based on wing wear using a scale from one (no scale loss) to five (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 sealed 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 at 25 ± 1°C, with 16:8 L:D. Because we used wild clover plants collected near Rocky Mountain Biological Laboratory (Gunnison County), 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 wing melanization was tested with a breeding experiment. Sixty females were collected at Hl, and brought into the greenhouse at Stanford University 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 sealed 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 and Watt 1981), which means that the offspring of one female are full-siblings. The degree of wing melanization was measured for all offspring.

Heritability of wing melanization using a full-sibling analysis was calculated using the components of variance from one-way ANOVAs (Falconer 1989). In a full-sibling analysis, heritability is estimated as $(V_A + 0.5V_D)/V_P$, 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-sibling 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. To avoid an overestimation of the heritability, we also did a parent-offspring regression, which omits most of the nonadditive variance (although maternal effects and additive × additive epistatic genetic variance could still play a role; Lynch and Walsh 1998). Obviously, any heritability estimate is dependent on the conditions in which it is measured, and should be interpreted accordingly. The full-sibling analysis consisted of 24 families and a total of 116 male and 69 female offspring. The parent-offspring analysis included 25 mother–mean offspring values. Both analyses were performed separately for male and female offspring, because phenotypic values differed significantly by sex. 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; Grula and Taylor 1980) were eliminated from the analysis.

Wing Melanization Measurement

Butterfly wings contain 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 in cell Cu2, below the discal cell at the crossing with vein Cu2. The wing was photographed on a standard gray background using a black-and-white digital camera mounted on a microscope and connected to a computer with the software Morphosys (Meacham and Duncan 1991). In Adobe Photoshop, the image was standardized for contrast through a percentage saturation stretch of the tonal histogram of the image (Wilkie and 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 pure white. Subsequently, the degree of melanization was determined as the percentage of the standardized image that was melanized using a threshold value of 70 (on a scale of 0–255). Because 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 for an effect of wing wear on the degree of melanization in our field-collected individuals.

RESULTS

The degree of wing melanization differed among elevations and between sexes (Fig. 1). At all elevations, females were significantly darker than males (ANOVA with elevation and sex: $F = 38.3$, df = 1,196, $P < 0.001$). Wing melanization was an accelerating function of elevation, indicating that there was a greater rate of change in the degree of melani-

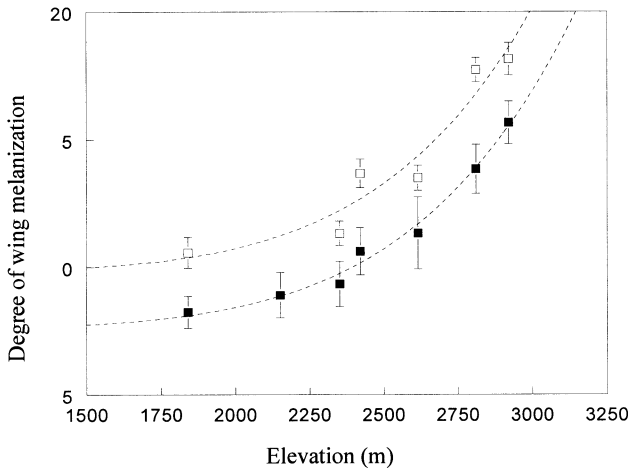

FIG. 1. The relationship between elevation and the degree of wing melanization (± SE) in *Colias philodice eriphyle* males (solid squares; $y = 7.61 + [1.28 \times 10^{-20}]x^6$) and females (open squares; $y = 9.84 + [1.41 \times 10^{-20}]x^6$).

zation at higher elevations (males: $r^2 = 0.99$, $F_{1,5} = 405.5$, $P < 0.001$; females: $r^2 = 0.93$, $F_{1,4} = 51.12$, $P = 0.002$; Fig. 1). We also tested the effect of wing wear on the measured degree of melanization, but in none of the populations was wear significant (for all elevations $r^2 < 0.12$ and $P > 0.27$).

Population differences in wing melanization persisted when offspring were reared in a common environment. Individuals from the high-elevation population (Wc) were significantly darker than their conspecifics from low elevation (Pa; $F_{1,54} = 12.0$, $P = 0.001$), and females were significantly darker than males ($F_{1,54} = 29.6$, $P < 0.001$) under common conditions. The interaction between sex and elevation was not significant. This reveals a genetic component to wing melanization.

Heritability and Sex Linkage

The full-sibling ANOVA and heritabilities for the degree of wing melanization are given in Tables 1 and 2. The full-sibling analysis showed significant heritability for both males and females, yet heritability for females was much lower. The mother-offspring analysis showed a significant heritability for males but not for females (Table 2, Fig. 2). The low female heritabilities along with high male heritabilities calculated from mother-offspring regressions do not fit an autosomal model for the genetic inheritance of wing melanization. Because the variances for males and females were of the same magnitude, the low heritability for females cannot be due to a reduced variance in the degree of wing melanization in females.

The most parsimonious explanation for the observed heritabilities is that hind wing melanization is at least in part a sex-linked trait. In Lepidoptera, females are the heterogametic sex, resulting in a higher relatedness between full-sibling males for traits located on the X-chromosome and a low heritability between mother and daughter for sex-linked traits. When all the genes affecting a trait are sex linked, the coefficient of coancestry for males is 0.75 instead of 0.5, while it remains 0.5 for females (Trivers 1985; Lynch and Walsh 1998). Assuming a fully sex-linked model with all genes located on the X-chromosome and no dominance or epistatic interactions, the heritability estimate between male full-siblings becomes 0.43 for ventral wing melanization (Table 2). This estimate is roughly consistent with our estimated heritability for females.

TABLE 1. ANOVA of full-sibling design. Males and females are analyzed separately because phenotypic values differed significantly for sex.

Source	Mean square	df	*F*-value	*P*-value
Males ventral				
Family	11.45	22	3.47	<0.001**
Error	3.30	91		
Females ventral				
Family	8.51	23	1.89	0.022*
Error	4.52	72		

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

DISCUSSION

The accelerating function for wing melanization indicates that populations are more strongly diverged at high elevations. Females were consistently darker than males over the full elevational range in the field, as well as in our common-garden and breeding experiments. This raises the question of whether the selection pressures for acting on wing melanization differ between the sexes. All else being equal, the higher degree of melanization of females should result in a more efficient body heating for females. This may be adaptive: Watt (1968) found in other North American *Colias* species that females initiated flight at a higher body temperature than males. The reason for this difference in thermal behavior is not clear. It may be that the larger body mass of females requires a higher body temperature for effective flight. Also, there may be an adaptive effect of body warming on egg maturation rates in females (J. Ellers and C. L. Boggs, unpubl. data).

The common-garden and breeding experiments showed that the degree of wing melanization is genetically based. Both the full-sibling analyses and the parent-offspring regression yielded roughly similar estimates for heritability, indicating that there was little dominance variance. Both

TABLE 2. Heritability estimates ($h^2 \pm$ SE) of wing melanization in *Colias philodice eriphyle.*

		Autosomal model	Sex-linked model
Full-sibling analysis (ventral)	Males	0.64 ± 0.21	0.43 ± 0.21
	Females	0.36 ± 0.22	0.36 ± 0.22
Mother-offspring analysis (ventral)	Son	0.47 ± 0.20	
	Daughter	0.07 ± 0.14	

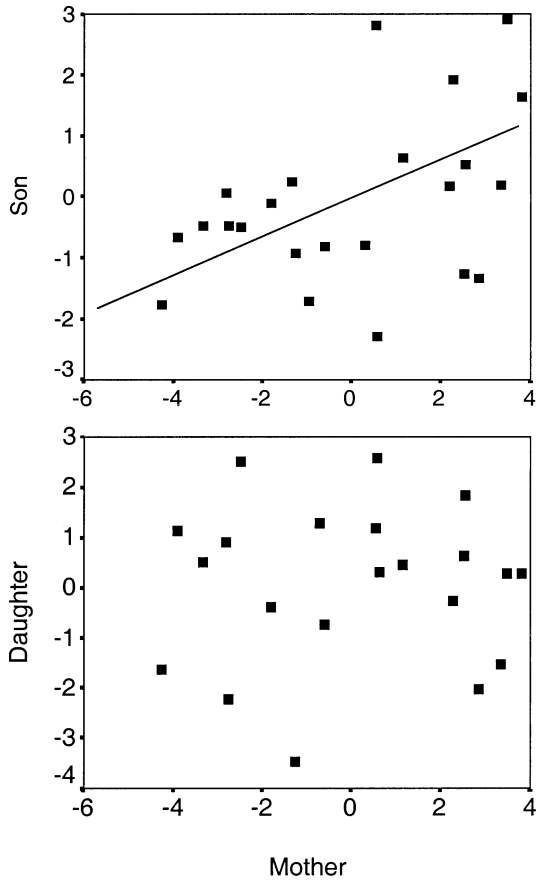

FIG. 2. Regression of mean offspring on mother for the degree of ventral wing melanization in males (upper graph) and females (lower graph) of *Colias philodice eriphyle.*

methods may still include maternal effects in the estimate of heritability. However, because wing melanization is sex linked, the mother-daughter regression does not contain additive variance and should provide an upper estimate for maternal effects. Almost no heritability was detected between mother and daughter, indicating that the maternal effect is negligible and our heritability estimates are based primarily on additive genetic variance.

Moderate to high heritabilities are not uncommon for morphological traits in butterflies. Other studies also found significant heritable variation in wing coloration (Holloway et al. 1993; Van Dyck et al. 1998; Windig 1998), but in none of these cases was variation in wing coloration associated with ecological differences. Some researchers have argued that traits with high heritabilities are not important for fitness, because selection is expected to quickly reduce genetic variation in traits closely linked to fitness (Mousseau and Roff 1987). However, the relation between wing melanization and fitness has been firmly established through its effect on thermoregulation and flight ability (Watt 1968; Kingsolver 1983a,b; Kingsolver and Watt 1984). Therefore, it is more likely that the genetic variance for wing melanization is maintained by other contrasting selection pressures or temporal variation in selection pressures.

Our experiments demonstrate that wing melanization is controlled by sex-linked genes. The method for determining sex linkage has been described for between-species comparisons using reciprocal hybrid crosses (Sperling 2002), and this method has been applied to intraspecific comparisons by Janz (1998). Alternatively, on theoretical grounds sex linkage is expected to increase the relatedness for traits located on the X-chromosome between males in a full-sibling design and strongly reduce relatedness for such traits between mother and daughter (Trivers 1985; Falconer 1989; Lynch and Walsh 1998). Our findings for the heritability of wing melanization are consistent with the theoretical predictions of a sex-linked model in which all genes affecting the trait are located on the X-chromosome. Thus, we conclude that the genetic component of wing melanization is primarily regulated by sex-linked genes.

In Lepidoptera, genes located on the sex chromosome tend to be important in prezygotic isolation and species differences (Sperling 1994, 2002; Prowell 1998; Ritchie and Phillips 1998). Sex linkage of characters is thought to facilitate the formation of adaptive gene complexes because of reduced recombination rates on the sex chromosome (Sperling 2002) and thus could promote major differentiation across an ecological gradient. Earlier reviews showed that a disproportionate fraction of the traits differentiating sister species are sex linked, and these include several examples of wing color (Prowell 1998). Differences between *C. philodice* and its close sister species *C. eurytheme* conform to this pattern, as the majority of species differences investigated to date are sex linked (Silberglied and Taylor 1973, 1978; Grula and Taylor 1980). Previous studies concentrated on interspecific differences in species pairs or well-defined races. However, if sex linkage has an important role in prezygotic isolation and speciation, as suggested by many authors (e.g., Charlesworth et al. 1987; Coyne and Orr 1989; Ritchie and Phillips 1998), sex-linked traits that differentiate species pairs after speciation should also differ intraspecifically, for example, among diverged populations of one species. Therefore, the finding of sex linkage for intraspecific differences in wing melanization in *C. p. eriphyle* supports the notion that genetic mechanisms that underlie intraspecific adaptation are not fundamentally different from those involved in speciation.

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