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# The evolution of alternative morphs: density-dependent determination of larval colour dimorphism in a butterfly

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Understanding the ultimate causes for the presence of polymorphisms within populations requires knowledge of how the expression of discrete morphs is regulated. In the present study, we explored the determination mechanism of a colour dimorphism in larvae of the butterfly *Pararge xiphia* (Satyrinae: Nymphalidae) with the ultimate aim of understanding its potential adaptive value. Last-instar larvae of *P. xiphia* develop into either a green or a brown morph, although all individuals are invariably green during the preceding three instars. A series of laboratory experiments reveal that morph development is strongly environmentally dependent and not the result of alternative alleles at one locus. Photoperiod, temperature, and in particular larval density, all influenced morph determination. The strong effect of a high larval density in inducing the brown morph parallels other known cases of density-dependent melanization in Lepidopteran larvae. Because melanization is often correlated with increased immune function, this type of determination mechanism is expected to be adaptive. However, the ecology and behaviour of *P. xiphia* larvae suggests that increased camouflage under high-density conditions may be an additional adaptive explanation. We conclude that the colour dimorphism of *P. xiphia* larvae is determined by a developmental threshold that is influenced both by heredity and by environmental conditions, and that selection for increased immune function and camouflage under high-density conditions may be responsible for maintaining the dimorphism. 

ADDITIONAL KEYWORDS: developmental threshold – melanization – morph – nymphalidae – pararge – plasticity – polymorphism – polyphenism – satyrinae – threshold trait.

## INTRODUCTION

Understanding the factors that cause and maintain variation in natural populations is of central interest in evolutionary biology, ecology, and developmental biology. The presence of discrete alternative phenotypes within populations (i.e. polymorphism or polyphenism) provides very obvious and often fascinating examples of such variation (Ford, 1975; Shapiro, 1976; Roff, 1996; West-Eberhart, 2003; Leimar, Hammerstein & Van Dooren, 2006). It is therefore not surprising that this phenomenon has attracted the attention of researchers for a long time and, indeed, some of the classic examples of evolutionary change and adaptation involve polymorphisms (Kettlewell, 1973; Ford, 1975; Majerus, 1998). Although it is a research area rich in examples and theory, the typical questions asked often fall into two categories: (1) ultimate reasons of variation (i.e. what type of selective regime may favour, or at least allow, more than one 'optimal' phenotype?) and (2)

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proximate reasons of variation (i.e. what determines the expression of the different morphs?). However, to be able to understand the ultimate causes for the presence of polymorphism in a given species, it is necessary to understand the developmental processes that allow the expression of different morphs (Moran, 1992; Roff, 1996; Nijhout, 1999; West-Eberhart, 2003; Hazel, Smock & Lively, 2004; Leimar *et al.*, 2006). This includes answering the question of whether the polymorphism is completely genetically determined or if it is also influenced by the environment and involves plasticity (typically referred to as polyphenism). In cases where morph determination is influenced by the environment, it is interesting to explore if the plasticity may be adaptive, so that alternative morphs are expressed in response to some particular aspect of the environment that provides information about future selective conditions (Lively, 1986; Moran, 1992; West-Eberhart, 2003; Leimar *et al.*, 2006). Therefore, the proximate mechanisms for morph determination may provide information about the potential adaptive value of a given polymorphism/polyphenism.

Discontinuous variation within populations may be determined by Mendelian mechanisms such as alternative alleles at a single locus, or by a developmetal threshold that integrates genetic and environmental influences on the phenotype (Hazel, Smock & Johnson, 1990; Roff, 1996; Nijhout, 1999; Hazel *et al.*, 2004). A Mendelian mode of inheritance (i.e. one locus) is typically expected to be less sensitive to environmental variation than is a developmental threshold (Roff, 1996). Indeed, a particularly interesting aspect of developmental thresholds is that they provide a mechanism whereby natural selection can influence the degree of environmental sensitivity in the development of phenotypes by influencing the genetic basis of the threshold (Hazel *et al.*, 1990; West-Eberhart, 2003; Leimar *et al.*, 2006). This perspective on developmental thresholds suggests that they can be viewed as a mechanism that adaptively integrates genetic and environmental effects on phenotypes and that they are likely to be important for the evolution of adaptive plasticity (Lively, 1986; Moran, 1992; Nijhout, 1999; West-Eberhart, 2003; Leimar *et al.*, 2006; Gotthard, 2008).

Insects provide numerous examples of polymorphisms in morphology, life cycles, and behaviour, where the determination includes both simple Mendelian inheritance as well as environmentally-dependent threshold mechanisms (Roff, 1996; West-Eberhart, 2003). Colour polymorphisms within the Lepidoptera not only include some classic cases of Mendelian inheritance (Ford, 1975), but also fascinating examples of where morph determination is strongly dependent on the environment during development (Wiklund, 1975; Shapiro, 1976; Hazel, 1977; Hazel & West, 1979; Greene, 1989; Greene, 1996). For example, butterfly larvae and adults may develop darker morphs in response to late or early seasonal cues, such as shorter photoperiods and colder temperatures, which allows more efficient thermoregulation under both cold and warm periods of the season (Shapiro, 1976; Fields & McNeil, 1988; Goulson, 1994; Kingsolver, 1995; Hazel, 2002; Nice & Fordyce, 2006). Other examples include lepidopteran larvae that develop into a darker form when grown under high-density conditions (Goulson & Cory, 1995; Wilson *et al.*, 2001; Hagen, Ims & Yoccoz, 2003; Cotter *et al.*, 2004a). In some cases, these more melanized larvae have been shown to be more disease resistant compared to lighter morphs, which is likely beneficial when high-density conditions increase the risk of infection or parasitoid attack (Wilson *et al.*, 2001; Wilson & Cotter, 2008). It has also been speculated that a high larval density may alter the larval environment, so that the high-density morph provides better camouflage in high-density conditions (Hagen *et al.*, 2003). These examples illustrate how the determination mechanism (i.e. seasonal cues and crowding, respectively), provide information about the potential functional significance of the dimorphism.

In the present study, we report on a series of experiments aimed at characterizing the determination mechanism of a colour dimorphism where larvae of the Madeiran speckled wood butterfly, *Pararge xiphia*, develop into either a green or a brown morph in the last instar. The research strategy was based on the idea that the alternative morphs are expressed in response to an environmental cue that provides information about future selective conditions (Lively, 1986; Moran, 1992; Hazel *et al.*, 2004; Leimar *et al.*, 2006) and that the nature of the determination mechanism therefore will provide information about the potential adaptive value of the alternative morphs. Because the dimorphism is only expressed during the last-larval instar, it is likely that the potential adaptive value is the result of some size-dependent selection where the importance of an ecological factor (e.g. ambient temperature, competition, predation, parasitism, starvation risk) varies with the size of individual larvae (Berger, Walters & Gotthard, 2006; Berger & Gotthard, 2008). Moreover, last-instar larvae switch to strictly nocturnal feeding and typically hide during the day (Berger & Gotthard, 2008). Both the nocturnal feeding and the colour dimorphism in the last instar are particular for *P. xiphia* because none of these traits occur in its two closest relatives *Pararge aegeria*, and *Pararge xiphioides* or within the sister genus *Lasiommata* (D. Berger & K. Gotthard, unpublished data). Because larvae of these other species are invariably green, it is clear that the expression of the

brown morph is an apomorphy in *P. xiphia* and that it is the presence of this morph that requires an evolutionary explanation.

In the present study, we first characterized the two colour morphs and the differences in their spectral properties, and then went on to test whether the dimorphism in the last instar is determined by a simple Mendelian mechanism (i.e. alternative alleles at one locus) or, instead, whether is the result of a developmental threshold that is also sensitive to environmental conditions. Because of the aforementioned adaptive hypotheses for larval melanization, we tested to what degree morph development depended on seasonal cues (i.e. photoperiod and temperature), larval density, and larval food stress.

## MATERIAL AND METHODS

The Madeiran speckled wood butterfly, *P. xiphia*, is endemic to the subtropical island of Madeira. It occurs in most of the woodlands of the island, although is particularly common above altitudes of 500 m a.s.l. where it can be observed flying in clearings also on foggy and relatively cold days (Wakeham-Dawson, Salmon and Franquinho Aguiar, 2001). Adults can be seen all year round and there is no recorded diapause in the species. Females oviposit on a number of different grasses on which the larvae feed and go through four instars before pupation. Observations made in the field suggest that large larvae can be either green or brown (Wakeham-Dawson *et al.*, 2001) and laboratory rearings clearly demonstrate that the brown morph is only expressed in the fourth instar.

### General experimental procedures

All experiments were conducted in the laboratory under controlled photoperiod and temperature regimes where *P. xiphia* larvae were given *Dactylis glomerata* as host plant. First-instar larvae were put on host plants that were kept in transparent plastic cups (0.4 litres) where the plants were cultured in fertilized water through a small hole in the bottom of the cup. Unless specifically stated, larvae were always reared individually and host plants were renewed when consumed or showing signs of deterioration. In all experiments, we scored the larval morph by eye a few days after the last larval moult. In most experiments, we also noted larval development time, pupal weight, pupal development time, and sex of each individual (sex was determined by visual inspection of pupal genitals). Adults were allowed to mate and oviposit in cages ($0.5 \times 0.5 \times 0.8$ m) in the laboratory.

### Experiment 1: Spectral characterization of the two morphs

To provide an objective characterization of the two colour morphs, we measured the reflectance spectra of seven live larvae of each morph. Reflectance was measured using a USB2000 spectrometer and a PX-2 light source (Ocean Optics). Reflectance was measured in the range 300–700 nm and relative to a white, diffuse reflectance standard. For each larva, we determined the reflectance spectrum of the side and the reflectance spectrum of the dark dorsal mid stripe (Fig. 1A). These two spectra of each individual were based on the averages of three separate measurements of the side and of the mid stripe. To graphically present the reflectance data, we then calculated the mean and confidence interval for dorsal and side measurements of each morph.

Because passerine birds are potential predators of these larvae, we estimated the chromatic contrast between the colour morphs using a model of bird colour vision (Vorobyev & Osorio, 1998; Vorobyev *et al.*, 1998). This calculation assumes that receptor noise limits discrimination of colours. It reveals whether the colours of two patches are distinguishable to a viewer. The unit of the output is the 'just noticeable difference' (JND). An output of 1 JND or larger indicates that there is a chromatic contrast distinguishable to the viewer, and an increasing value indicates an increasing probability of detecting a chromatic difference under given conditions. In our calculations, we assumed clear skies daylight conditions and used the cone sensitivities of the blue tit (*Cyanistes caeruelus*) as identified by (Hart *et al.*, 2000). The blue tit has a chromatic sensitivity that is representative for Passeriformes (Hart, 2001; Håstad, Victorsson & Ödeen, 2005).

### Experiment 2: Genetic background and the effect of seasonal cues

The main purpose of this experiment was to test whether the colour dimorphism was the result of simple Mendelian inheritance with two alternative alleles at one locus. If so, within-morph matings should produce at least one monomorphic group even if one of the alleles is dominant (i.e. the mating group that is homozygotic for the recessive allele). Moreover, morph determination should be relatively insensitive to environmental variation. The larvae used to start this experiment were the offspring of ten females collected in the field on Madeira and we set up two breeding treatments where we only allowed monomorphic matings for two consecutive generations (i.e. the third generation of larvae in the monomorphic mating groups had parents and grandparents

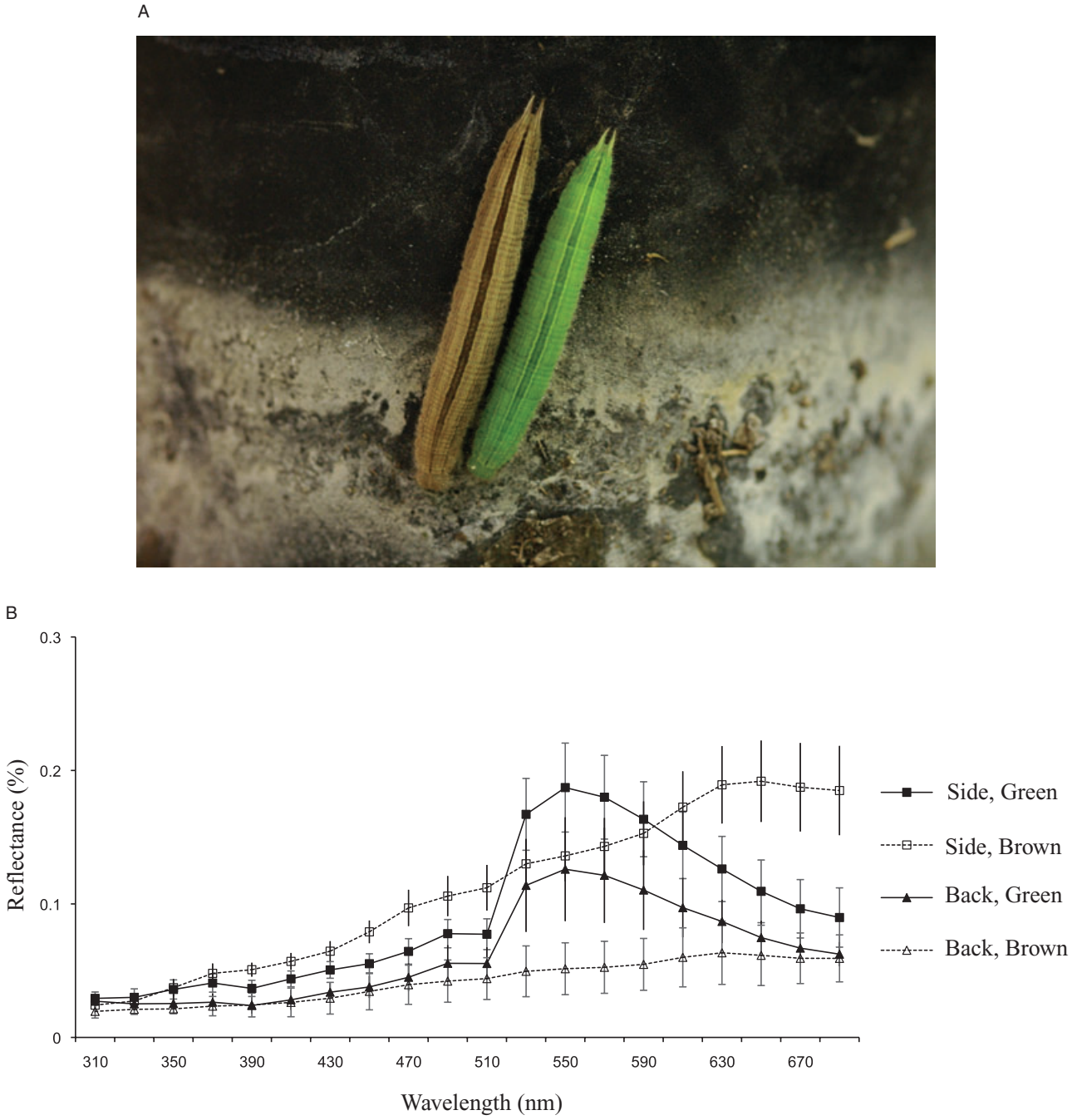


**Figure 1.** Image of the two morphs (A) and the results of Experiment 1 (B) showing the reflectance spectra of the side and the dorsal stripe of the green and the brown morph based on seven larvae of each morph (means ± 95% confidence intervals).

that had expressed the same morphs). In addition, we kept a control group where mating was random with respect to larval morph. The number of mating adults within each group and generation varied in the range 40–12. The rearing conditions for the first laboratory generation (offspring of wild collected females) comprised a of 12 : 12 h light/dark cycle and a temperature of either 17 °C or 22 °C, whereas the second generation of larvae was kept under 12 : 12 h light/dark cycle and a temperature of 17 °C. In the third laboratory generation, we assayed the response by using the three mating types (Brown, Green and Control) in an experiment where we exposed growing larvae to variation in cues of seasonal change (two

different photoperiods and two different temperatures in a two-by-two design). The conditions were chosen to mimic the extreme photoperiods and monthly mean temperatures on Madeira, which amounted to photoperiods of either an 11 : 13 h or 15 : 9 h light/dark cycle and temperatures of 15 °C or 22 °C. If the darker morph would be an adaptation for increasing body temperature during colder parts of the year, a higher incidence of the darker brown morph in shorter photoperiods and lower temperatures would be expected (Hazel, 2002; Nice & Fordyce, 2006).

### Experiment 3: The effect of larval density

In this experiment, we used a new stock of butterflies that were the first generation offspring of eight females collected in the field on Madeira. Because of our own observations in the laboratory and results from other lepidoperans, we wanted to experimentally test to what degree morph expression was dependent on larval density during development (Wilson *et al.*, 2001; Cotter *et al.*, 2004a). The experiment included two main factors (larval density and photoperiod) with two levels each that were crossed in a two-by-two design. Photoperiod was included as a factor in this experiment to allow comparisons of the relative effects of different environmental factors tested in the different experiments. First-instar larvae were either put individually or in groups of five in the type of rearing cups described earlier. They were then randomly assigned to either a 11 : 13 h or 15 : 9 h light/dark cycle. The temperature was kept constant at 17 °C in all treatments. All individuals that reached the last instar were scored for larval morph. As each rearing cup can be considered an independent observation, we performed the analysis on this level of observation. The high-density cups were scored as brown when 50% or more of surviving larvae within a rearing cup developed the brown morph. In the statistical analysis, we only included rearing cups of the high-density treatment where two or more larvae survived until they could be scored for larval morph in the last instar. Because there were more than one individual per rearing container in this experiment, it was not possible to track the development of each individual pupa. We therefore could not analyse life-history traits with respect to morph in this dataset.

### Experiment 4: The effect of starvation prior to morph development

In this experiment, we again used a new stock of larvae that were the third generation of 12 females collected on Madeira. We aimed to assess to what degree starvation just prior to the last moult and subsequent morph development may influence morph determination. The reason for this was to evaluate whether the effect of larval density on morph development could be explained by a suspected lower food level and quality in high-density conditions. Again, we performed a two-factor experiment that included the main effects photoperiod and starvation treatment where photoperiod was included to allow comparisons across experiments. The same two photoperiods used in the earlier experiments were also used here, and, in addition, we randomly assigned one-half of the individuals to a period of starvation during the penultimate third-larval instar. During this third-larval instar, larvae in the starvation treatment were put in a plastic cup without any host plant for 5 days in total. These 5 days, however, were split in one 2-day and one 3-day period between which larvae were allowed to feed on low quality food for 1 day to avoid death by starvation. Host plants were categorized as being of low quality when they had already been almost completely consumed by larvae and only contained one or two green grass leaves. Larvae in the control treatment had access to good quality host plants throughout the third instar.

### Statistical analysis

In each experiment, we analysed the effects of experimental treatments on the propensity to develop the brown morph by generalized linear models (GLM with binomial data and a logit link function). After fitting a full model with all main effects and interactions, we subsequently removed all interaction effects where $P > 0.1$. In Experiment 2, where one of the factors had three levels (mating types), we also tested the main effects and interactions by pairwise contrasts between groups.

All life-history traits were analysed using analysis of variance (ANOVA) that included experimental treatments, mating type (Experiment 2), sex, and morph (except for Experiment 3, see above) as fixed factors in the analysis. It is typically the case that factors such as sex, temperature, photoperiod, larval density, and starvation have strong effects on life-history traits. However, because the purpose of these analyses was to investigate potential difference in life-history strategies between morphs, we only report the results obtained when morphs showed significant differences (either through main effects or interactions). We also report the potential effects of experimental treatments on larval survival, which were analysed by GLM with binomial data and a logit link function. All statistical analyses were performed with STATA, version 9.2 (StataCorp, 2005).

## RESULTS

### Spectral characterization of the morphs

Visual scrutiny of Figure 1 confirms that the two morphs had distinctly different reflectance spectra. The green larvae showed a reflectance peak at around 550 nm, whereas the reflectance of the brown larvae increased with longer wavelengths. Furthermore, the general shape of the reflectance curve appears similar between the dorsal and the lateral colours within a morph, although the curves appear more elevated for the lateral measurements than for the dorsal measurements. This indicates that the lateral and dorsal measurements were similar in colours, but differed in lightness (the dorsal measurements being darker in both morphs). Thus, the reflectance measurements correspond to those differences between colour morphs and between dorsal and lateral areas that are detectable by the human eye. Furthermore, the measurements reveal that both morphs showed very low reflectance within the ultraviolet range (300–400 nm). Thus, there appears to be no differences between the morphs that would be invisible for humans but detectable by some potential predators, such as passerine birds with colour sensitivity stretching into the ultraviolet range.

Modelling of avian colour vision suggests that the two larval coloration phenotypes are distinctive to birds. The mean ± SD colour contrast between the lateral measurements of the green and the brown larvae was 14.4 ± 4.5 JND. There was also noticeable variation within each phenotype; the average contrast among the green larvae was 10.0 JND and it was 4.5 JND among the brown larvae. Similarly, for the measurements of the dorsal stripe, the contrast between the two morphs was 15.4 ± 7.3 JND. The mean contrast was 11.0 JND among the green larvae and 6.2 JND among the brown larvae.

### Genetic background and the effect of seasonal cues

All three breeding treatments expressed both larval morphs after two generations of controlled mating (Fig. 2). Nevertheless, there were still significant effects of mating type, photoperiod and temperature on morph development (GLM: residual d.f. = 175, $Z_{\text{mating}} = -2.42$, $P_{\text{mating}} = 0.016$, $Z_{\text{photoperiod}} = -2.71$, $P_{\text{photoperiod}} = 0.007$, $Z_{\text{temperature}} = -3.11$, $P_{\text{temperature}} = 0.002$). The Brown mating type did express the brown morph significantly more compared to the Green type (GLM: residual d.f. = 174, $Z_{\text{B versus G}} = -2.46$, $P_{\text{B versus G}} = 0.014$), whereas there were no significant differences between Brown and Control (GLM, residual d.f. = 174, $Z_{\text{B versus C}} = -1.60$, $P_{\text{B versus G}} = 0.11$) or between Green and Control (GLM, residual d.f. = 174 $Z_{\text{G versus C}} = -1.01$, $P_{\text{G versus C}} = 0.34$). The short photoperiod (11 : 13 h light/dark cycle) and warm temperature (22 °C) induced a higher incidence of the brown morph (Fig. 2). The difference in the response of the Brown and the Green groups to photoperiod was close to significant (mating × photoperiod interaction for the contrast Green versus Brown: residual d.f. = 173,

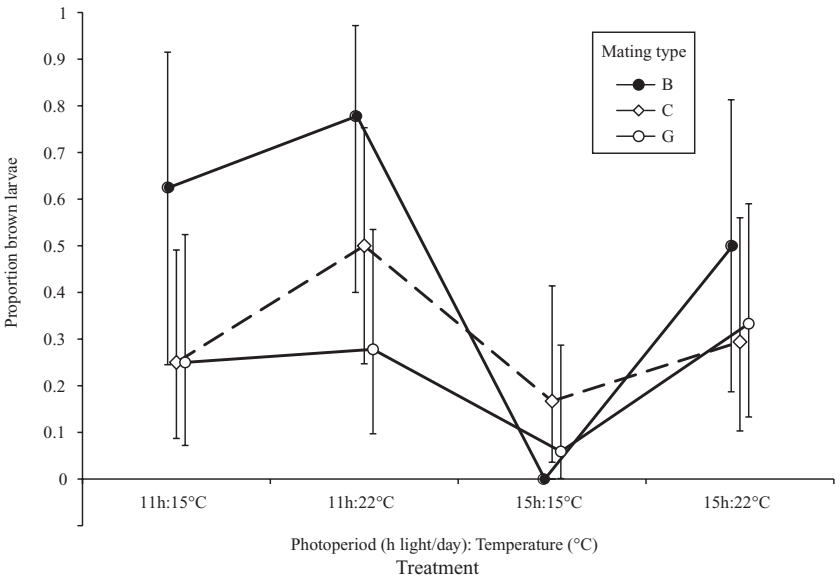


**Figure 2.** Results obtained in Experiment 2 showing the proportion of brown larvae produced by the three genetic lines in different combinations of photoperiod and temperature (means ± 95% confidence intervals). Sample sizes were between 18 and nine individuals within each category.

$Z = 1.88$, $P = 0.06$). There were no significant differences between mating types in any other responses to photoperiod or temperature (for all other interaction terms $P > 0.1$).

Larval survival was significantly lower in the Brown type compared to the Green and Control types, suggesting that inbreeding may have been stronger in the Brown treatment (GLM: residual d.f. = 236, $Z_{mating} = 3.96$, $P_{mating} < 0.0001$; mean ± SE survival for the Brown = 0.38 ± 0.05, Green = 0.68 ± 0.05, Control = 0.71 ± 0.05).

Larval development time was the only life history trait that differed significantly among mating treatments (ANOVA: $F_{2,137} = 5.83$, $P = 0.0037$), and this was the result of a shorter larval development time in the Brown type compared to the Green and Control types (mean ± SE in days in 15 °C: B = 64.7 ± 2.9, G = 71.1 ± 2.2, C = 73.7 ± 2.5; in 22 °C: B 41.7 ± 1.6, G = 43.6 ± 1.0, C = 48.9 ± 2.0). However, there were no significant differences among morphs in any of the measured traits.

## Effects of larval density

Both larval density (one or five larvae per container at start) and photoperiod (11 h or 15 h of light) significantly affected morph development (GLM, residual d.f. = 70, $Z_{density} = 4.03$, $P_{density} < 0.0001$, $Z_{photoperiod} = -2.12$, $P_{photoperiod} = 0.034$). Larvae that were reared in high density and a short day length were more likely to develop the brown morph (Fig. 3). Because mortality in the high-density treatment reduced the number of individual larvae in some of the containers, we performed an additional analysis where we use the actual number of larvae surviving to the fourth instar as our estimate of density (rather than five in all containers as in the previous analysis). The result of this analysis was nevertheless very similar to the first analysis (GLM, residual d.f. = 70, $Z_{density} = 4.10$, $P_{density} < 0.0001$, $Z_{photoperiod} = -2.46$, $P_{photoperiod} = 0.014$).

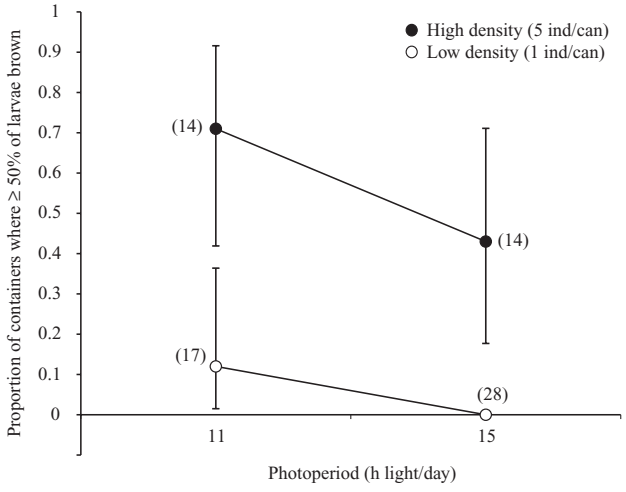


**Figure 3.** Results obtained in Experiment 3 showing the effect of rearing density (one or five larvae per container) and photoperiod on morph development. The result are the means ± 95% confidence intervals calculated with rearing container as an independent observation. Numbers within parentheses show sample sizes.

There was a significant effect of photoperiod but not of density on larval survival (GLM: residual d.f. = 192, $Z_{density} = -0.36$, $P_{density} = 0.72$, $Z_{photoperiod} = 2.66$, $P_{photoperiod} = 0.008$). Larvae growing under an 11 : 13 h light/dark cycle had a lower survival compared to larvae growing under an 15 : 9 h light/dark cycle (mean ± SE survival: 11 h = 0.73 ± 0.05, 15 h = 0.89 ± 0.03).

Pupal weight was significantly affected by photoperiod but not of larval density (ANOVA, photoperiod: $F_{1,112} = 9.51$, $P = 0.0026$; density: $F_{1,112} = 1.65$. $P = 0.20$) with larvae reared under a 15-h day length being larger on average. Larval development time on the other hand was significantly affected by density but not by photoperiod (ANOVA, photoperiod: $F_{1,126} = 0.27$, $P = 0.60$; density: $F_{1,126} = 55.6$. $P < 0.0001$), which, as expected, was because larvae reared in a higher density had longer development times.

## The effect of starvation

A 5-day period of starvation during the penultimate third instar did not have a significant effect on morph development in the last instar (GLM, residual d.f. = 101, $Z_{starvation} = -1.69$, $P_{starvation} = 0.091$). In line with the two earlier experiments, there was a significant effect of photoperiod on morph determination where the brown morph was more frequently expressed in a shorter photoperiod (GLM residual d.f. = 101, $Z_{photoperiod} = -2.44$, $P_{photoperiod} = 0.015$; Fig. 4).

There was no significant effect of the starvation treatment or photoperiod on larval survival to pupation (GLM, residual d.f. = 115, $Z < 0.5$ and $P > 0.5$ in both cases).

In this experiment, larvae of the green morph pupated on average at smaller sizes than the brown larvae (ANOVA: $F_{1,88} = 6.0$, $P = 0.017$, Mean ± SE pupal weight (mg): Green morph = 287.1 ± 5.2, Brown morph = 313.8 ± 10.0). In the case of development time, the brown larvae had on average shorter larval development time compared to the green larvae (ANOVA, main effect morph: $F_{1,87} = 5.9$; $P = 0.017$, mean ± SE developmental time in days: Green morph = 54.4 ± 1.1, Brown morph = 49.2 ± 1.3). There was also a significant effect of the interaction between starvation treatment and morph as starved larvae of the green morph prolonged development to greater

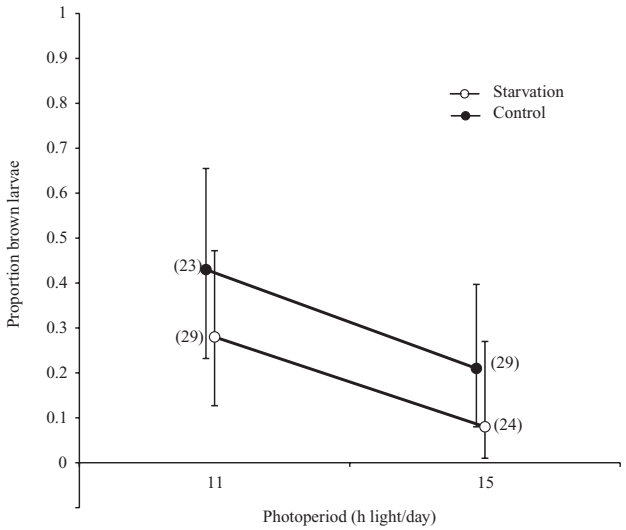


**Figure 4.** Results obtained in Experiment 4 showing the effect of starvation treatment and photoperiod on the propensity to develop the brown morph (means ± 95% confidence intervals). Numbers within parentheses show sample sizes.

extent than did starved larvae of the brown morph (ANOVA, starvation × morph interaction: $F_{1,87} = 7.8$, $P = 0.007$).

## DISCUSSION

In our experiments with *P. xiphia*, two successive generations of monomorphic matings did not produce offspring that were monomorphic with respect to larval coloration (Fig. 2). Instead, we found strong effects of larval environment in all three rearing experiments. Accordingly, we conclude that larval morph in *P. xiphia* is not determined by simple Mendelian inheritance with two alternative alleles at one locus, but more likely by an environmentally-dependent developmental threshold. The difference between Brown and Green mating types suggests that there is variation in the underlying genetic basis for the threshold and that natural selection could alter the pattern of morph determination.

Even though there were no consistent differences between morphs in life history, the particular restriction of morph expression to the last instar and the lack of anything similar in the closest relatives of *P. xiphia* still suggest that the dimorphism is unlikely to be selectively neutral in nature. If the variation in larval colour is an adaptation to seasonally varying thermal conditions for development, we would expect the darker brown morph to be most prevalent in the combination of photoperiod and temperature that is most similar to winter conditions on Madeira (i.e. short day and low temperature). This pattern was, for example, found by (Hazel, 2002) in a study of larval coloration in the eastern black swallowtail butterfly, *Papilio polyxenes*, where the amount of black pigment in the cuticle varied substantially in the two last-larval instars. The results obtained in the present study were, however, not as clear-cut as in *P. polyxenes*. The effect of photoperiod was in line with the prediction (shorter day: higher incidence of brown larvae) but the effect of temperature was opposite of the expected (higher temperature: more brown larvae). Indeed, it appears that the significant results largely originated from the peculiar effect of the treatment with long day and low temperature producing almost no brown larvae in any of the lines (Fig. 2).

Other instances of colour polyphenisms in lepidopteran larvae where selection for efficient thermoregulation has been suggested typically report strong effects of low temperature in inducing the darker morph (Goulson, 1994; Hazel, 2002; Nice & Fordyce, 2006). Moreover, in some cases, the polymorphism is not restricted to just one larval instar (Goulson, 1994; Nice & Fordyce, 2006) and the colour change may even be reversible between instars if the temperature is changed (Nice & Fordyce, 2006). It also appears that the amount of dark pigments in the cuticle of larvae of these other examples often varies in a more continuous fashion compared to that observed in *P. xiphia* (Goulson, 1994; Hazel, 2002; Nice & Fordyce, 2006). Finally, in contrast to these other cases (Hazel, 2002; Nice & Fordyce, 2006), larvae of *P. xiphia* become strictly night active during the dimorphic fourth instar, which clearly reduces the exposure to direct sunlight. During the light hours, larvae typically hide at the bottom of the grass tuft or under a leaf and we have not observed any type of basking behaviour. Hence, even though photoperiod, which is a very reliable cue of seasonal change, did have an effect in the expected direction, it is unlikely that the brown larval morph in *P. xiphia* is the result of selection for an increased capacity to gain heat by basking when temperatures are low.

By contrast to the effects of temperature and photoperiod, the effect of larval crowding was relatively strong and straightforward (Fig. 3). This result is also confirmed in mass rearings of *P. xiphia*, where up to 85% of larvae typically develop the brown morph (K. Gotthard, unpublished data). The lack of any effect on morph development of the starvation treatment in Experiment 4 suggests that the effect of larval density is not an indirect consequence of food stress during the period just preceding morph development (Fig. 3).

Examples of density-dependent polyphenims in the degree of cuticular melanization are known from several insect orders (Cotter, Kruuk & Wilson, 2004b; Wilson & Cotter, 2008) and at least five families

within the Lepidoptera (Noctuidae, Saturnidae, Sphingidae, Geometridae, Tortricidae) (Goulson & Cory, 1995; Wilson *et al.*, 2001). However, *P. xiphia* appears to be the first reported example from the true butterflies (Papilionoidea) showing this pattern. In other lepidopteran species, the darker larvae often are more disease-resistant than lighter larvae, which fits well with the knowledge that melanin and its precursors are important components of the insect immune system (Wilson *et al.*, 2001; Wilson & Cotter, 2008). Because pathogen transmittance is often positively density-dependent, it has frequently been argued that density-dependent larval melanization is likely to be adaptive. Many of the lepidopteran species that show density-dependent larval polymorphism are agricultural pests that regularly occur in high densities in nature (Goulson & Cory, 1995) and consequently experience natural selection for surviving under high-density conditions at relatively frequent intervals. By contrast, there are no indications that *P. xiphia* shows this type of extremely high abundance in the field, even if several larvae may be feeding on the same host plant individual. It is clearly possible that the density-dependent colour dimorphism in *P. xiphia* is related to variation in immune function, although it appears as if the ecological situation is quite different compared to the other known cases of Lepidoptera. Direct experimental tests of the correlation between larval morph and immune function are required to resolve this issue.

Selection for crypsis is obviously one of the main adaptive explanations for variation in colour patterns and for larval polymorphisms/polyphenisms (Kettlewell, 1973; Greene, 1989; Greene, 1996; Majerus, 1998). In *P. xiphia*, the two morphs have reflectance spectra that are distinguishable by the human eye and by potential bird predators, which are generally better able to discriminate between colours than are humans (Vorobyev *et al.*, 1998). Therefore, it is reasonable to expect that colour morph is not a selectively neutral trait, but will influence the detection of the larvae by some of their predators. Furthermore, if the adaptive function of the coloration is concealment, it is reasonable to expect that the colour variation would be connected to variation in the visual habitat of fourth-instar larvae (Merilaita, Tuomi & Jormalainen, 1999), and that larval crowding should provide information of this variation. It is, for example, possible that more crowded conditions typically arise when there is a lack of good host plants because many potential hosts have wilted, creating a less green feeding environment.

The development of the two morphs during the last-larval instar in *P. xiphia* coincides with a change in larval feeding from day and night activity to strict nocturnal feeding (Berger & Gotthard, 2008). This ontogenetic shift in behaviour is likely to be caused by a size-dependent change in the cost–benefit ratio for growth, partly because of an increase in the risk of predation from day-active and visually-hunting predators (Berger *et al.*, 2006; Berger & Gotthard, 2008). Direct effects of colour morph on the risk of detection will mainly prove to be important during daylight conditions when larvae are resting and hiding. A group of larvae will produce considerably more leaf damage compared to a single larva, and this is likely to attract the attention of visually-hunting predators (e.g. wasps, birds, and lizards). In such circumstances, it may be beneficial to leave the green host plant during day-time and find a resting place in dry and wilted vegetation where the brown morph is well camouflaged. Interestingly, this type of correlation between larval behaviour and colour morph is reported from the hawkmoth *Erinnyis ello* (Sphingidae) that express different colour morphs in the fifth- and last-larval instar (Curio, 1970a, b). All non-brown larvae feed and rest on the leaves of their host plant, whereas the brown larval morph move away from the green leaves and rests on the brown trunk of the host plant during a part of the day. This behaviour appears to be adaptive because the risk of predation from wasps that hunt among the leaves during the day is significantly lower for the brown larvae resting on the trunk than the other morphs that constantly stay among the green leaves (Curio, 1970b). Unfortunately, there no information is available about what determines morph development in *E. ello*.

It is important to note that these two adaptive hypotheses for density-dependent colour dimorphisms (i.e. increased immune function and camouflage during day-time resting) are not mutually exclusive. High-density conditions are likely to create selection pressures that differ from low-density conditions in many aspects and the development of the brown morph under high density may be maintained by selection for more than one function. Even though it is likely that a polyphenism typically originate in response to particular selection pressure, it is probable that the alterative morphs subsequently will experience quite different selection. If so, selection may favour developmental mechanisms that lead to phenotypic divergence between morphs in several traits downstream of the original developmental threshold (West-Eberhart, 2003). As long as a cue, such as larval crowding, is reasonably accurate for predicting future selective conditions, the evolution of polyphenism will often be favoured by natural selection (Lively, 1986; Moran, 1992; Hazel *et al.*, 2004; Leimar *et al.*, 2006).

In conclusion, the experiments reported in the present study strongly suggest that the larval dimor-

phism of *P. xiphia* is determined by a developmental threshold that is influenced both by heredity and by environmental conditions. Because larval density, rather than photoperiod or temperature has the by far strongest effect on morph development, we argue that it is unlikely that selection for improved thermoregulation is responsible for the maintenance of the two morphs. Given other examples of density-dependent larval colour polymorphisms and the ecology of *P. xiphia*, we find that increased immune function in the darker morph in response to high density, and concealment on different visual backgrounds during day-time resting are adaptive hypotheses that warrant future study.

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