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Evolutionary genetics of seasonal polyphenism in the map butterfly *Araschnia levana* (Nymphalidae: Lepidoptera)

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ABSTRACT

Araschnia levana shows two spectacularly different seasonal forms: a predominantly orange spring form with black dots and a predominantly black summer form with a white band. The forms are induced by length of day. We quantified differences in wing pattern between the forms and sexes with the help of image analysis. We used a split family design to analyse the genetic background. Each wing pattern element responded in its own way to length of day. Heritabilities within forms were generally high. Genetic correlations across lengths of day, between forms, ranged from around 0 to around 1. Wing pattern may thus rapidly respond to natural selection, but this response is for some traits not independent from selection in the other form. The overall heritability for producing a spring or summer form in an environment where both forms were produced was very high. There was a tight relationship between the length of the 5th larval instar and the adult form produced. We discuss a physiological model which can explain this relationship, and which has some interesting implications for the debate on whether genes for plasticity exist. The overall results are discussed in the light of West-Eberhard's theory of polyphenism as a first step towards speciation.

Keywords: morphometrics, phenotypic plasticity, quantitative genetics, speciation.

INTRODUCTION

Phenotypic plasticity, or environmentally induced variation in phenotypes, has attracted considerable attention from evolutionary biologists and ecologists over the last 15 years (for reviews, see Schlichting, 1986; Sultan, 1987, 1995; Stearns, 1989; Van Noordwijk, 1989; West-Eberhard, 1989; Thompson, 1991; Moran, 1992; Scheiner, 1993; Gotthard and Nylin, 1995; Via *et al*., 1995; Brakefield, 1996). This followed a period of neglect (Schlichting, 1986) during which phenotypic plasticity was seen mainly as instability, which was better avoided. Many animal and plant breeders focused on stable genotypes that gave a constant high yield across a range of environments (e.g. Becker, 1964). At the end of the last century,

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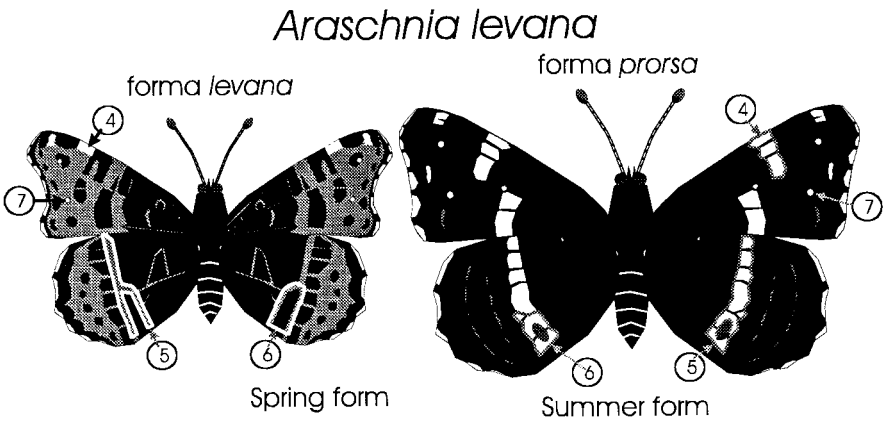
however, environmentally induced variation received much attention, because of its possible role in speciation (Shapiro, 1976). Bradshaw (1965) wrote the first modern review on phenotypic plasticity that recognized its importance for evolutionary biology. Many of the concepts developed in that review still influence contemporary evolutionary research on plasticity.

A common opinion is that plasticity can reduce the impact of natural selection on populations by concealing genetic variation within environments, and by producing phenotypes with high fitness across a range of environments (Bradshaw, 1965; Thompson, 1991; Redbo-Torstensson, 1994; Sultan, 1995). Evolution, however, may still proceed despite the presence of high amounts of plasticity. Bradshaw (1965) and Schlichting (1986) proposed that plasticity may be seen as 'a trait in and of itself', on which natural selection may act. This has led to considerable controversy. Via (1993a) considers this view misleading and sees plasticity as a by-product of selection in different environments. Much of the debate that followed concentrated on whether genes exist that influence the degree of phenotypic change across environments regardless of the values of the traits within environments (genes for plasticity) (Schlichting and Pigliucci, 1993; Via, 1993b; Via *et al*., 1995). This debate suffered and still suffers, however, from a lack of understanding of the physiological basis of plasticity and the uncertainty as to which genes, apart from regulatory genes, are involved (Windig *et al*., in press).

A different angle on the evolutionary importance of plasticity comes from West-Eberhard (1989). She proposed that evolution is not reduced by the presence of plasticity, but that plasticity may accelerate evolution. Whenever alternative phenotypes (either in the form of genetic polymorphism or phenotypic plasticity) evolve, there are more phenotypes that can be influenced by natural selection. In the case of polyphenism, this can lead to a change in one of the forms without loss of the other form in the same genotype, the latter possibly having a high fitness in at least some environments. In evolutionary quantitative genetic terms, two forms may differentiate by natural selection if the heritability within forms is high and if genetic correlations between the forms are low.

One of the more striking examples of phenotypic plasticity is that of the European Map butterfly (*Araschnia levana* L.). It has a spring and a summer form that differ dramatically in wing pattern (Fig. 1). It is thus an example of seasonal polyphenism, in which environmentally induced discrete forms are produced in different seasons (Shapiro, 1976). This phenomenon is especially well studied in butterflies (Brakefield, 1987; Braby, 1994; Windig *et al*., 1994; Kingsolver, 1995). *A. levana*, however, has received considerably little attention recently from evolutionary biologists, although Shapiro (1976) considered it one of the most promising organisms for elucidating relationships between physiology, environment, genetics and natural selection.

Linneus first described *A. levana* as two species. Later breeding showed that one form gave rise to the other form (Hübner, 1816). At the end of the nineteenth century, Weismann (1875) and others were interested in the species because they thought the dimorphism might be related to speciation. They performed extensive temperature experiments in an attempt to induce the different forms, but had limited success. Danilevski (1948) was the first to establish that photoperiod was the dominant factor determining the seasonal form. Reinhardt (1969, 1984) showed that photoperiod in the last larval instar was the prime factor determining the seasonal form and that temperature in the pupal stage could modify the wing pattern to a lesser extent. He was able to obtain a continuous range from extreme spring forms via intermediate forms to extreme summer forms.



Traits measured	
on whole wing	as indicated
① Wing Area ② Orange Area ③ Black Area	④ Fore Band ⑤ Hind Band ⑥ Band Color ⑦ White spot

Fig. 1. Wing pattern traits measured to quantify variation within and between seasonal forms and sexes. Colour of band was measured on a grey scale from 0 (= black) to 255 (= white). All other traits were areas measured in mm^2.

A. levana is one of the few examples of plasticity where information on its physiological basis is relatively extensive. The physiological basis of wing pattern determination in *A. levana* has been studied extensively (Koch and Bückmann, 1987; Koch, 1992, 1995, 1996). This makes the species attractive for the study of the evolutionary biology of plasticity. There are, however, considerable gaps in our knowledge of its evolutionary biology. The variation in colour pattern between and within the forms has not been adequately described. Previously, the amount of red as estimated by eye (Reinhardt, 1969, 1984) or by planimetry (Koch and Bückmann, 1987) has been used to describe the seasonal forms. The forms differ in more traits, however, such as wing size. Furthermore, no information exists on the genetic basis of variation in wing pattern. Nothing is known about the heritability of the wing pattern within or across forms.

We try to fill these two gaps in this paper. First, we describe the wing pattern in detail by answering the following questions: What characters best describe the differences between the forms? Do these characters covary within and across forms? Is the variation in wing pattern similar in both sexes? Is there a gradient within a form in the direction to the other form, or is the variation more discrete? Second, we evaluated the genetic basis of this variation. Bundles of reaction norms are used to visualize the interaction between genotype and environment. Heritability within forms is presented as a measure of whether selection may change the average of traits within forms. Genetic correlations within traits across forms (Via and Lande, 1985; Windig, 1997) are used to determine whether selection can

influence one of the forms independently of the others. Or, in other words, whether evolution of divergent forms is constrained by high genetic correlations across forms.

MATERIAL AND METHODS

Breeding of butterflies

Butterflies were raised in the laboratory following the methods of Pullin (1986) for the related Peacock butterfly *Inachis io*. Females of *A. levana* of the summer generation were caught in the field and allowed to lay egg batches in small cages placed over naturally growing foodplants in a garden, where the batches were allowed to hatch. DNA-fingerprinting was later used to determine if members of a family had the father as well as the mother in common. After removal of two individuals in one family, all families comprised full sibs (data not reported here).

As soon as the larvae reached the second stage and were robust enough to withstand handling, they were brought to the laboratory. Families were split between a long-day (16 h light/8 h dark) and a short-day (12 h light/12 h dark) climate room. The temperature in both climate rooms varied between 19°C and 21°C, the short-day climate room tending to be some tenths of degrees cooler. Sixteen families of *A. levana* were raised in July and August 1995. Breeding space was limited, and a maximum of 30 larvae were used for each family in each environment. Two families with about 30 larvae were raised only in the long-day environment.

Families were placed in a petri dish with a fresh nettle leaf. At the start of the 3rd stage they were split in groups of five and at the 4th stage in groups of two. Fresh leaves were provided every other day. The petri dishes were stacked in piles of five. All families were split equally over all five levels and over piles placed at the sides or in the middle. Each petri dish was placed one level higher when fresh food was provided, except the top dishes which were placed at the bottom. When a larva had pupated, the pupa was placed in a small peat pot with the top of the petri dish as the lid. Pupae from the short-day environment, destined to produce spring forms, went into diapause to overwinter, as did some of the pupae in the long-day environment. Pupae that did not hatch within 4 weeks after pupation were stored in a cold room (0–5°C) for 4 months, after which they were returned to 20°C. One day after emergence, adult butterflies were placed in the freezer. They were later stored in small envelopes (of the type stamp collectors use) at −70°C.

Measurement of the seasonal forms

To take account of the full variation between forms and between the sexes, we used image analysis (Windig, 1991). Altogether, 112 wing pattern traits were examined on 23 male and 23 female butterflies. The 46 butterflies were chosen from the collection in the Natural History Museum in Leiden such that the full range of variation in that collection was represented in the subset. Discriminant analysis was used to distinguish the seasonal forms and sexes. The 34 traits with the highest scores on the first two axes were measured a second time. On the basis of ease and accuracy of measurements, the set of traits was reduced to nine. To speed up measurements further, the two traits not on the dorsal side (amount of purple on the ventral side and colour of the ventral side of the abdomen) were also left out. This resulted in a set of seven traits (Fig. 1). Scores for the first two axes in

the discriminant analysis for the set of 112 traits as a whole were nearly identical to the subset of seven traits ($r = 0.989$, $P < 0.0001$). Within forms and sexes, scores for the first two axes in a principal component analysis were also highly correlated (all $r > 0.962$, $P < 0.0001$).

Measurement of penis length

Reinhardt (1984) noted that the form of the penis was different between the two seasonal forms (Fig. 1). As differences in the morphology of genitalia are often associated with species differences, an accurate description of the form of the penis in both seasonal forms and the overlap between them is interesting from an evolutionary viewpoint. The penis was dissected from the male abdomen and examined under a microscope connected to a computer at a magnification of 250×. The image was analysed using AXIODOC software. The length, width, perimeter and area of the top of the penis were measured twice on a small subset. Of these four measures, length was the most accurate measurement and showed the largest differences between the seasonal forms; consequently, length was measured for all penises.

Larval development time

Larval development time plays an important role in another Nymphalid butterfly, *Bicyclus anynana*, in determining the seasonal form (Brakefield and Reitsma, 1991; Windig, 1992; Kooi *et al*., 1996), and it is also an important life-history trait. The correlation between the development time in the final instar and the preceding four instars is low, probably because of different coloration influencing thermoregulation (Windig, 1999). We therefore analysed larval development time separately for the first four instars (larval development I–IV) as the number of days from egg hatch until start of the 5th instar, and the 5th instar (larval development V). To obtain a normal distribution, the data were log-transformed before analysis.

Statistical analysis

All traits except band colour were log normally distributed. Band colour, which was not an area measurement, was normally distributed. The area measurements were log-transformed before further analysis. All pattern elements showed allometric effects within forms and sexes. Slopes were similar across forms and sexes, but intercepts differed (Fig. 2). To correct for the allometric effects, 'regression' lines were calculated with slopes and intercepts equal to the average of the slopes and intercepts of the separate regression lines within forms and sexes (Fig. 2). Unless otherwise stated, traits refer to the residuals of the trait on the calculated regression lines.

Discriminant analysis was used to separate spring and summer forms and males and females, and to determine the relative importance of the different traits for distinguishing the different categories. Traits were not corrected for size effects before discriminant analysis. The scores of the first axis were also used as an index of seasonal form. Analyses of variance were used for the individual traits (corrected for allometric effects where necessary) and the first two axes of the discriminant analysis to analyse the effects of seasonal form/daylength, sex and their interaction. Since spring forms were formed both under long and

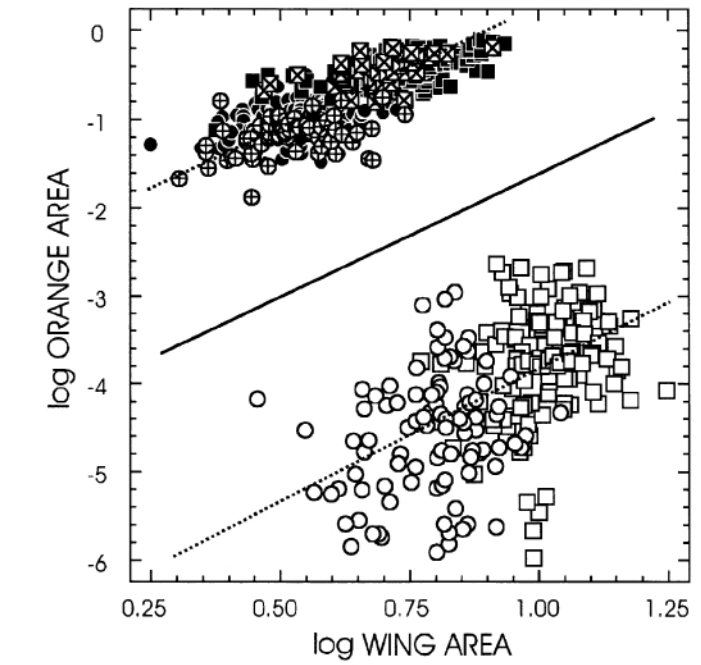


Fig. 2. Relationship between trait area and wing area for 'orange area'. Dashed lines indicate regression lines within forms; solid line is the average of the two. Residuals with solid lines were used to eliminate allometric effects.

short daylengths, but summer forms only under a long daylength, seasonal form/daylength had three levels: summer form, long-day spring form and short-day spring form.

Genetic analysis

Reaction norms were used as a visual description of the differences between forms and of the genetic variation within and across forms. Reaction norms were compiled by connecting family means for the different forms by lines. The width of such bundles of reaction norms is an indication of the genetic variation within forms, and the amount of crossing of the lines an indication of the genetic variation for plasticity (Van Noordwijk, 1989). The variance of family means, however, also contains part of the non-genetic residual variance (Windig, 1997), so this variation is also reflected in the bundles.

The heritability (h^2), additive variance (V_A) and residual variance (V_R) components of traits within forms were calculated using restricted error maximum likelihood (REML) methods to obtain a more precise description of the genetic variance. Since the families consisted of full sibs, the estimated V_A also contains half of the non-additive variance component (V_D; Falconer, 1989). The estimated V_A may be further inflated by cage effects, although this inflation was limited here, since each family was restricted to one dish in the first two larval instars only, split over as many as 15 dishes in each environment in the last larval instars, and raised individually in the pupal stage.

In a REML analysis, variance components are fitted to the data in an iterative procedure, where the fit is judged by a criterion called the log-likelihood (Shaw, 1987; Roff, 1997). The procedure stops when there is no further improvement in the log-likelihood, in other words when the maximum likelihood is reached. The REML procedure has the advantage that it gives unbiased estimates, even if family sizes are unbalanced, that can be tested directly for a difference with 0. To test for a difference with 0, the procedure is run for a second time, but this time with the component to be tested restricted to 0. The difference between the two maximum likelihoods is χ^2-distributed with one degree of freedom (Shaw and Platenkamp, 1993). However, given the large number ($n = 157$) of tests we performed on heritabilities and genetic correlations, we cannot focus upon the significance level of any single parameter. We were unable to apply Bonferonni correction because of non-independence, so the calculated probabilities must be viewed as upper limits. The REML procedure was carried out using the nf3-program of Shaw and Shaw (1992) adapted for use on a personal computer.

Genetic correlations (r_G) within forms were also estimated using REML procedures. The r_G across forms within traits was estimated using the following formula:

$$r_G = \frac{\mathrm{COV}_{\mathrm{meanFd1,meanFd2}}}{\sqrt{V_{\mathrm{A,Fd1}}, V_{\mathrm{A,Fd2}}}}$$

where $\mathrm{COV}_{\mathrm{meanFd1,meanFd2}}$ is the covariance between the family means of one form/daylength and another, and $V_{\mathrm{A,Fd1}}$ and $V_{\mathrm{A,Fd2}}$ are the additive variances for the two forms as estimated in the REML procedures. The 95% confidence intervals were calculated using a jack-knife procedure. In this way, r_G could be tested for a difference both with 0, indicating independence of forms, and with 1, indicating absence of genetic variation for plasticity (Windig, 1997).

RESULTS

Altogether, 742 larvae (76.5%) survived to the pupal stage. Significantly more larvae in the long-day environment survived ($n = 396$, 85.4%) than in the short-day environment ($n = 346$, 72.8%; $\chi^2 = 17.11$, $P < 0.001$). Survival in the pupal stage was around 80% in both environments and not significantly different (long day, $n = 338$, 80.6%; short day, $n = 279$, 78.5%; $\chi^2 = 0.50$, $P = 0.479$). More males ($n = 311$, 52.7%) than females ($n = 279$, 47.3%) were raised, but this was not different from a 1:1 sex ratio ($\chi^2 = 1.73$, $P = 0.187$). The percentage of males was especially high in the short-day environment (56.7 *vs* 49.7%), but this difference was not significant ($\chi^2 = 2.87$, $P = 0.090$). In the short-day environment, all butterflies were of the spring form; in the long-day environment, 116 (34.3%) spring forms and 222 (65.6%) summer forms emerged (Table 1). Not all families produced spring forms in the long-day environment and, consequently, the average numbers of individuals per family were the lowest of all the daylength forms, especially for the females (Table 1).

Table 1. Numbers of individuals of *Araschnia levana* and numbers of families of both seasonal forms raised in long- and short-day environments

	Spring form				Summer form	
	Short day		Long day		Long day	
	Individuals/ families	Mean	Individuals/ families	Mean	Individuals/ families	Mean
Males	143/14	10.21	73/13	5.62	94/15	6.27
Females	109/14	7.79	42/10	4.20	128/16	8.00
Total	252/14	18.00	115/13	9.82	222/16	14.27

Wing pattern

Plasticity

Responses of individual traits are visualized with reaction norms in Fig. 4. All traits responded to daylength but there is no general pattern common to all traits. They all differed significantly between the forms (Table 2). The discriminant analysis (DA; Fig. 4) indicated band colour, orange area and wing size as the three most important traits distinguishing the forms (standardized DA scores: 0.779, –0.532 and 0.333 respectively). Summer forms had whiter bands, larger areas of orange and larger wings (Fig. 3). Hind band and fore band had intermediate DA scores (–0.207 and 0.211 respectively) and black area and white spot the lowest DA scores (–0.110 and –0.075 respectively). The discriminant analysis clearly separated spring and summer forms, and no intermediate forms were present.

The differences between the spring forms from the long-day and short-day environments were small compared with the differences between the spring and summer forms, although significant differences did exist. The long-day spring form did not look consistently more like the summer form than the short-day spring form. For example, the orange area that was small to absent in summer forms was smaller in long-day spring forms, while band colour, which was paler in summer forms, was paler in short-day spring forms, and wing area did not differ between the spring forms. Overall, the DA scores indicated that for males long-day spring forms looked more like summer forms, while for females short-day spring forms looked more like summer forms (Fig. 3), resulting in a significant daylength × sex interaction (Table 2).

In both summer and spring forms, females had clearly larger wing areas with relatively larger orange areas (Fig. 2) and smaller black areas, especially in the spring forms. This resulted in a significant overall difference between the sexes, and significant sex × form interactions, especially significant for the black area (Table 2). The white spot, the hind band and the band colour differed between the sexes, especially in the summer form, resulting in a significant overall difference and relatively large sex × form interactions. The fore band showed no overall difference between the sexes. It was, however, larger for females in the summer form and smaller in the spring form, hence the significant sex × form interaction (Table 2).

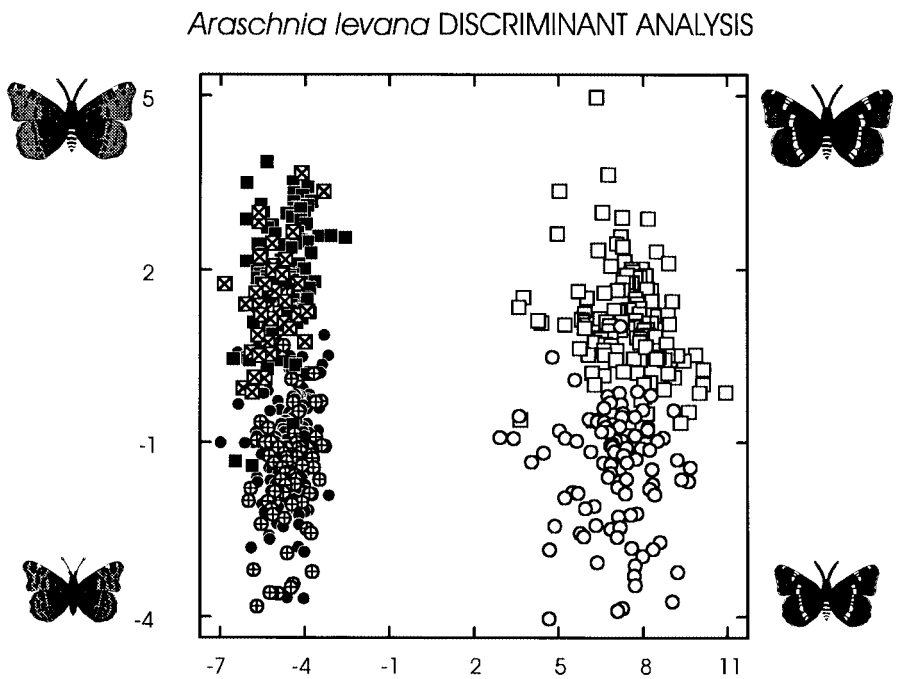


Fig. 3. Discriminant analysis of wing pattern for seasonal forms and sexes. For key to symbols, see Fig. 2.

Table 2. Multi-factor analysis of variance of traits for seasonal form/daylength, sex and their interaction

	Form		Sex		Form × sex	
	F	*P*	*F*	*P*	*F*	*P*
Wing area	612.71	****	568.17	****	3.98	*
Orange area	8073.76	****	52.22	****	3.72	*
Black area	367.43	****	208.17	****	14.53	****
Hind band	169.59	****	110.40	****	49.29	****
Fore band	4637.17	****	0.22	N.S.	564.35	****
Colour band	6159.93	****	25.03	****	20.89	****
White spot	32.65	****	1.29	N.S.	20.17	****
DSC 1	9782.56	****	1.52	N.S.	11.36	****
DSC 2	1092.19	****	20.05	****	3.16	*
Penis length	30.91	****	—	—	—	—
Larval development I–IV	162.57	****	3.54	+	0.32	N.S.
Larval development V	436.70	****	32.81	****	1.80	N.S.

Note: Seasonal form had three levels: summer form, long-day spring form and short-day spring form. DSC = axis of discriminant analysis (see Fig. 3).
+ $P < 0.10$, * $P < 0.05$, **** $P < 0.0001$.

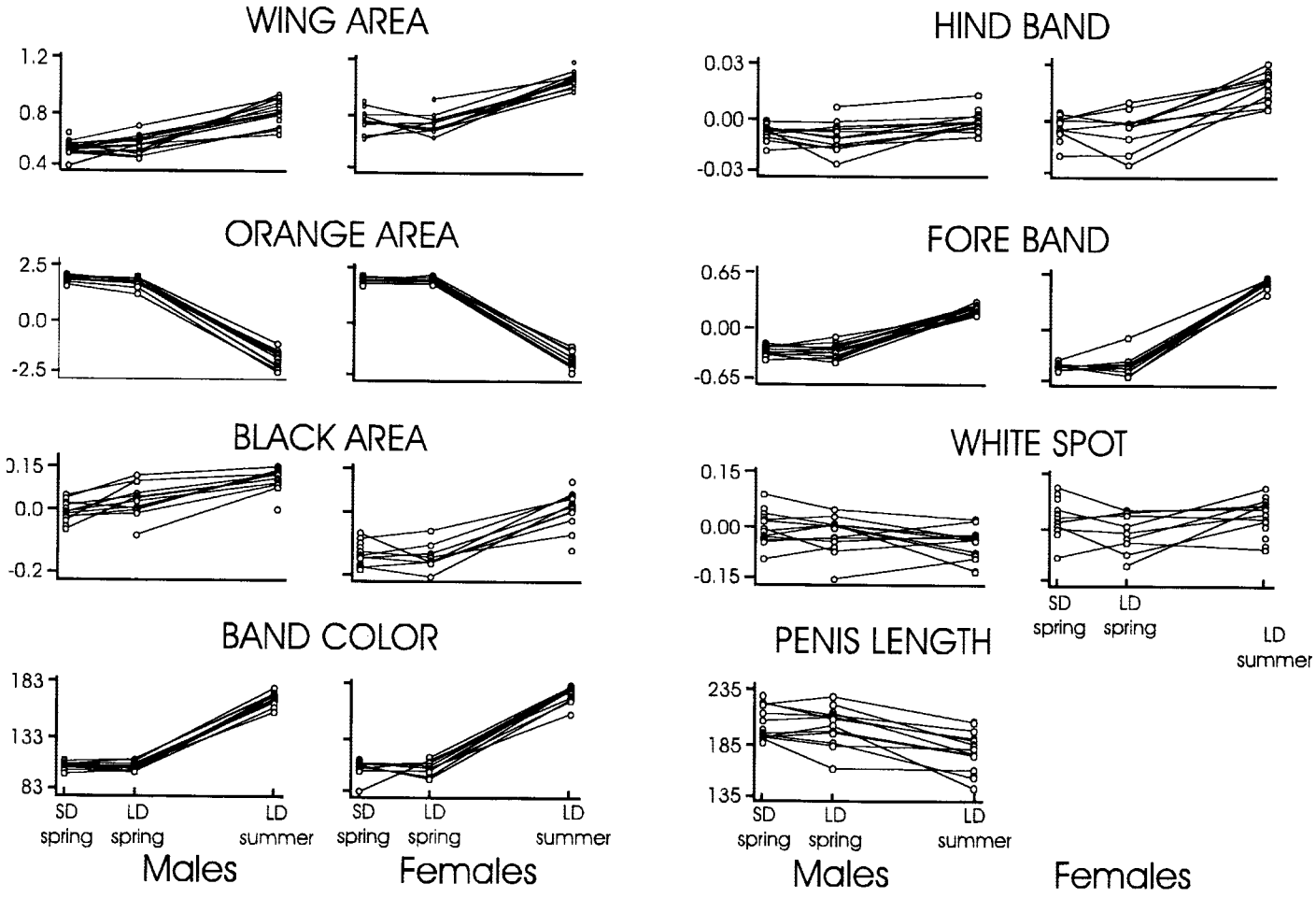


Fig. 4. Bundles of reaction norms. Reaction norms are compiled by connecting family means across environments.

Genetics

Differences between families are visualized by bundles of reaction norms in Fig. 3. They varied over traits. Family differences were relatively small compared with environmental differences in terms of, for example, orange area, but large in terms of, for example, white spot. Reaction norms crossed for all traits, but for some traits less (e.g. hind band in males) than for others (e.g. white spot), suggesting variation in the strength of genetic correlations across environments. There were no environments where the width of the bundles was clearly less, although some variation in the width was present in some traits (e.g. orange area in females). This suggests no extensive variation in heritability across environments.

For only six of the 42 calculated h^2-values, the range $\pm$ 2 standard errors did overlap with 0 (Table 3). These h^2-values were mostly for traits within females of the long-day spring form. They could reflect the small sample size rather than the absence of non-zero h^2-values, since the long-day spring-females had both the lowest number of individuals and the lowest number of families. The average h^2 (0.56) over all traits and both sexes is close to the average of 0.51 reported for morphological traits in general (Mousseau and Roff, 1987). There was no consistent pattern over forms and sexes. For example, h^2 is especially high for the orange area in male summer forms, for the hind band in female long-day spring forms and for band colour in female short-day spring forms.

Genetic correlations within traits across forms/daylengths ranged from –0.191 to 0.859 (Table 4). The r_G-values between the two spring forms were all positive and, except for wing area in males and band colour in both sexes, clearly different from 0. For three traits – orange area, fore band and white spot – $r_G \pm 2$ standard errors overlapped with 1. The r_G-values between the summer form and the two spring forms were generally similar. The exceptions were three r_G-values between the summer form and the long-day spring form in females, which were clearly lower than those between the summer form and the short-day spring form. These three traits all had a low h^2 in the long-day spring form, possibly because of the small sample size. Also, because of the direct relationship between h^2 and r_G (Falconer, 1989), r_G could not be high. For most summer–spring r_G-values, the estimates $\pm$ 2 standard errors did not include 0; they did, however, for band colour, white spot and the fore band. The r_G-values between summer and spring forms $\pm$ 2 standard deviations included 1 for only two estimates. These were r_G in males with the short-day spring form for orange area and the hind band.

Penis length

Penis length was significantly less in summer forms (Table 2, Fig. 3). This was not an allometric effect because the summer forms were larger. The penis of the long-day spring form was for all but one family intermediate between the summer and short-day spring form (Fig. 2). The h^2 of penis length was for all daylengths/forms high (Table 2). Reaction norms did not cross much and r_G across environments was high and close to 1.

Development times

Larval development was significantly faster in the long-day environment, both in the 5th instar and in the preceding instars. Females took longer to develop in the 5th instar, but not in the preceding instars. Phenotypic and genetic correlations between larval

Table 3. Heritability (h^2), additive variance component (V_A) and residual variance component (V_R) as estimated by REML analysis (full-sib estimates)

	Spring form								Summer form			
	Short day				Long day				Long day			
	h^2	V_A	V_R	P	h^2	V_A	V_R	P	h^2	V_A	V_R	P
Males												
Wing area	0.496	0.33	0.33	****	0.199	0.16	0.64	N.S.	0.694	0.79	0.35	****
Orange area	0.358	2.24	1.83	***	0.709	3.70	1.63	***	1.004	22.3	−0.77	****
Black area	0.551	0.45	0.81	****	0.323	0.28	0.58	N.S.	0.753	0.54	0.18	****
Hind band	0.341	3.35	6.48	***	0.558	9.30	7.38	**	0.797	3.47	0.55	****
Fore band	0.485	0.53	0.56	****	0.608	0.67	0.43	**	0.306	0.24	18.3	**
Colour band	0.432	13.3	17.5	****	0.590	18.0	12.5	***	0.716	46.3	0.190	****
White spot	0.681	0.13	0.06	****	0.488	0.087	0.091	*	0.190	0.124	0.190	*
Penis length	0.637	260	171	****	0.915	411	38.0	****	1.024	435	−10	****
Larval development I–IV	0.237	1.72	5.54	**	pooled with summer form				0.924	1.01	0.18	****
Larval development V	0.404	0.96	1.43	****	pooled with summer form				0.926	7.16	0.57	****
Females												
Wing area	0.591	0.71	0.49	****	0.248	0.17	0.57	N.S.	0.307	0.20	0.47	***
Orange area	0.673	2.41	1.17	****	0.220	6.77	2.40	N.S.	0.674	16.4	7.87	****
Black area	0.562	0.85	0.66	****	−0.023	−0.003	0.14	N.S.	0.651	1.26	0.70	****
Hind band	0.486	4.43	4.86	****	1.201	14.23	−2.42	****	0.542	1.77	1.52	****
Fore band	0.331	0.24	0.48	***	0.356	0.28	0.50	*	0.514	0.31	0.29	****
Colour band	1.101	50.4	−4.6	****	0.924	35.0	2.86	***	0.538	45.0	38.6	****
White spot	0.695	0.085	0.037	****	0.219	0.133	0.219	N.S.	0.509	0.048	0.047	****
Larval development I–IV	0.874	1.00	0.14	****	pooled with summer form				1.013	1.48	−0.02	****
Larval development V	0.179	0.44	2.03	N.S.	pooled with summer form				0.771	3.40	1.01	****

Note: All traits except wing area, band colour, penis length and larval development time were corrected for allometric effects. Spring and summer forms were formed in the pupal stage; therefore, the h^2 of larval development time for the long-day environment is not split over spring and summer forms.
* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Table 4. Genetic correlations across environments within traits

	Spring SD–Sping LD						Spring SD–Summer						Spring LD–Summer					
	Male			Female			Male			Female			Male			Female		
	r_G	≠0	<1	r_G	≠0	<1	r_G	≠0	<1	r_G	≠0	<1	r_G	≠0	<1	r_G	≠0	<1
Wing area	0.280		**	0.459	*	*	0.533	***	***	0.633	***	*	0.326		**	0.153		***
Orange area	0.686	****		0.538	***		0.767	****		0.322	*	**	0.621	**	*	0.327		*
Black area	0.378	**	***	0.390	*	*	0.546	**	***	0.515	***	*	0.649	***	*	0.007		***
Hind band	0.340	**	****	0.513	***	*	0.843	****		0.365	*	**	0.662	***	*	0.275		**
Fore band	0.584	****		0.663	***		–0.191		****	–0.054		****	0.162		****	–0.188		****
Colour band	0.256		***	0.024		****	–0.067		****	0.063		****	0.254		***	0.154		***
White spot	0.859	****		0.628	***		0.261		***	0.367		*	0.226		****	0.205		*
Penis length	0.600	****	*	—	—	—	0.852	****		—	—	—	0.792	****		—	—	—
Larval development I–IV	Spring LD pooled with Summer						0.791	****		0.690	****		Spring LD pooled with Summer					
Larval development V	Spring LD pooled with Summer						0.725	****		0.326	**	***	Spring LD pooled with Summer					

Note: Difference with 0 and 1 tested with jack-knife procedures. SD = short day, LD = long day.
* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

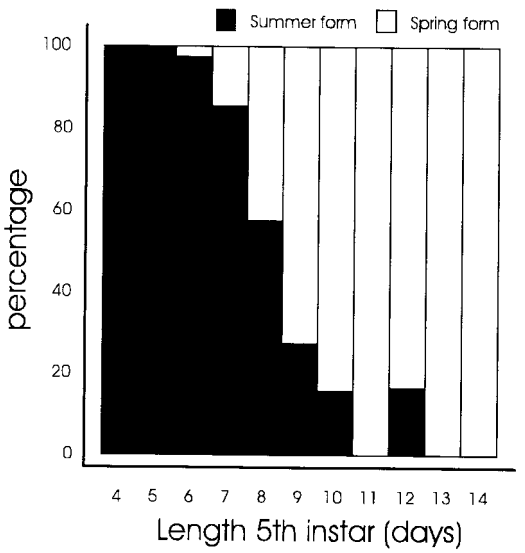


Fig. 5. Percentage of seasonal forms formed in the long-day environment for different lengths of 5th larval instar.

development I–IV and larval development V were weak (<0.25). These weak correlations were caused by differences in coloration between 5th instar larvae and the preceding instars for a part of the larvae. Probably because of differences in thermoregulation, these differences in coloration generate variation in development times (Windig, 1999). There was a clear relationship between larval development V and the production of spring forms in the long-day environment, but no such relationship with larval development I–IV.

In the long-day environment, larvae with longer development times in the 5th instar more often produced spring forms than faster growing larvae (Fig. 5). The average length of the 5th stage for the long-day spring forms (males 9.20 days; females 9.71 days) was closer to that of the short-day spring forms (males 10.04 days; females 10.90 days) than that of the summer forms (males 6.29 days; females 7.06 days). Spring forms in the long-day environment experienced similar development times in the first four instars (males 15.12 days; females 15.52 days) as the summer forms (males 15.05 days; females 15.43 days), whereas short-day spring forms experienced considerably longer development times (males 18.05 days; females 18.18 days).

Heritability for larval development time in the long-day environment was high (>0.77, Table 2). The heritability for seasonal form in the long-day environment, analysed as a threshold trait, was high for males (1.398) but even higher for females (1.633). Dominance variance, maternal effects or cage effects may have inflated both the estimates for the threshold trait and one of the underlying traits, larval development time. It was clear that h^2 for seasonal form was higher than for development time, suggesting that there is also some other underlying trait generating genetic variation for the production of the seasonal form.

Within daylengths/forms, correlations of larval development I–IV with wing pattern traits were weak (<|0.156|) and not significantly different from 0. Correlations in the summer forms with larval development V were also weak (<|0.166|) and not significantly different

from 0, except for wing area in the females (–0.185, $P = 0.036$). In the spring forms, correlations of wing area with larval development V were also significantly negative (short-day males –0.359; long-day males –0.509; short-day females –0.452; long-day females –0.530; all $P < 0.0001$). Orange area was positively correlated (short-day males 0.191; long-day males 0.344; short-day females 0.290; long-day females 0.301; all $P < 0.001$), whereas band colour was negatively correlated (males –0.337; females –0.244, $P < 0.001$). All the other correlations were smaller (<|0.137|) and not significantly different from 0.

DISCUSSION

The wing pattern of *A. levana* does not form an integrated suite of traits, of which all react similarly to environmental variation. Differences between seasonal forms, between spring forms produced under different lengths of day and between the sexes are trait-specific, as are the heritabilities and genetic correlations across environments. The heritability of the wing pattern though, was often high, and strong genetic correlations across environments were seen for some traits. Wing pattern may thus change rapidly under the influence of natural selection, but the two forms cannot evolve independently. Some traits have somewhat different patterns. Band colour, for example, showed very weak genetic correlations across environments.

Form of plasticity is trait-specific

The form of the plasticity depends on the trait. For most traits, the two spring forms are different but for some they are not (e.g. for band colour). Also, the difference is not consistently in the same direction. The difference between the forms was for some traits clearly larger in the females (e.g. for fore band), whereas for others the response was quite similar across the sexes (e.g. orange area). There was also no consistent pattern in heritability. The white spot, for example, had the highest heritability in the short-day spring form, whereas that for black area was highest in the summer form. In this respect, *A. levana* is different from other butterflies for which the quantitative genetics of plasticity in wing pattern have been analysed.

In *Bicyclus anynana*, the responses of different wing pattern traits to temperature are quite similar across traits and sexes (Windig, 1994). A common physiological basis for the different traits seems likely. In *Pontia occidentalis*, wing pattern traits vary as groups in accordance with predictions made on developmental and functional grounds (Kingsolver, 1995). There are two reasons for the apparent weaker integration of wing pattern traits in *A. levana*. First, the two forms of *A. levana* are completely discrete forms, whereas in the two other species there is overlap across environments. In *A. levana*, some traits are so different across environments (e.g. the hind band) that a common functional significance seems highly unlikely. Other traits, however, remain more or less the same, such as wing area and white spot. Second, another factor in addition to length of day may influence the traits. Reinhardt (1969, 1984) reported that temperature modifies the wing pattern within forms. It may be that the influence of temperature is stronger for some traits than for others. The significant correlation of some traits with larval development time, but the absence of it in other traits, may also be caused by different influences of temperature on different traits. Experiments to determine the influence of temperature within and across daylengths are needed.

Genetic basis and mechanics of plasticity

Our results do not provide information on the physiological basis of plasticity, but they do indicate that the development time of the 5th larval instar plays a role. In this way, it is quite similar to *Bicyclus anynana* (Windig, 1992). In this butterfly, differences in temperature seem to induce different wing patterns, but a closer inspection reveals that wing pattern is better explained by differences in development time than by temperature differences. The following model was developed to explain the linear relationship between the log of the development time and the wing pattern. At the start of the larval stage, a hormone is present in a certain amount. Each day a fraction of the hormone is synthesized. At the end of the larval stage, the amount of this hormone determines the wing pattern. A similar model may apply to *A. levana*, except that here a threshold is involved: above a certain level of the hormone the spring form is induced, whereas below this threshold a summer form is produced. Furthermore, length of day has to be involved. This can be done by modifying the model so that the hormone is only produced in the dark (or only in the light) as Roff (1986) proposed in a model on induction of wing forms by daylength. Similar models have been developed for photoperiodic clocks (Vaz Nunes *et al.*, 1991a,b).

If the above model is correct, it has some interesting implications for the debate on whether genes for plasticity exist. Genes that influence the initial amount of the hormone will influence the overall level of the reaction norm, but genes that influence the fraction that is synthesized daily will influence the slope of the reaction norm. There is some information regarding the physiological basis of polyphenism in *A. levana.* The wing pattern is determined at the start of the pupal stage. In the prepupa of the summer morph, a peak in juvenile hormones occurs, and ecdysteroids are released early in pupation (Koch, 1996). In the spring form, the juvenile hormone peak is absent, and ecdysteroids are released late in the pupal stage. How these different hormone levels are induced by different lengths of day is, however, unclear.

Polyphenism as a step towards speciation

West-Eberhard (1989) suggested that polyphenism may play an important role in speciation. She proposed that, once alternative phenotypes have evolved, polyphenism may lead to rapid speciation via the following steps:

1. *Phenotype fixation*. The phenotype can be considered fixed in some populations if only one of the alternative phenotypes is expressed. Interestingly, this is the case in *A. levana* in the northernmost populations. Recently, the species has extended its range considerably, and now occurs also in Finland. The growing season there, however, allows for only one generation and, consequently, only the spring form is expressed. The species also extended its range to the south into northern Spain, but how many generations occur there is unknown. The high h^2 for the production of seasonal form in long days suggests that the induction of seasonal forms may change rapidly when the relationship between length of day and climate changes.
2. *Increased divergence due to phenotype fixation*. Once different forms have developed, natural selection may increase their divergence. Low heritabilities within forms and strong genetic correlations across forms can constrain this divergence. The strong genetic correlations across forms will, however, play no role if phenotypes are fixed in some populations.

In *A. levana*, h^2 is generally rather high and thus natural selection can potentially change both forms. For some traits, however, there are strong genetic correlations across forms, which may slow down their divergence. Some correlations in the males, such as the length of the penis, were close to or equal to 1, which means that further divergence in these traits might be impossible. It would be interesting to compare selection regimes on wing patterns in Finland with areas where both seasonal forms occur, and if the wing pattern in Finland changes more in the absence of (genetic correlations with) the summer form. Nothing is known about the adaptive significance of the two forms.

3. *Acceleration of reproductive isolation due to divergent specialization*. An increase in divergence may lead to further specialization. This, in turn, may lead to reproductive isolation. It is interesting that the male genitalia in *A. levana* are already morphologically different, and have high heritabilities. Differences in genitalia are widespread among animals with internal fertilization (Eberhard, 1985), even for closely related species. There are several evolutionary theories that can account for this phenomenon (Arnqvist *et al.*, 1997). All theories have in common that reproductive success of individuals with divergent genitalia is reduced, either directly (lock and key hypothesis) or indirectly through sexual selection or pleiotropic effects. A small pilot experiment was performed to determine if the two forms might be reproductively isolated. Both forms were raised in the laboratory and split over four cages: only spring forms, only summer forms, male spring forms and female summer forms, or female spring forms and male summer forms. Only one mating (within summer forms) was observed, so no conclusions could be drawn on that basis. Interactions between males and females (Table 5) were significantly more frequent in the summer–summer cage than in the other cages, especially the male spring–female summer cage ($\chi^2_3 = 87.5,\ P < 0.0001$). These observations support reproductive isolation between the forms, but may also be explained by unfit spring forms. Thus although the presence of reproductive isolation between the two forms other than in time is uncertain, evolution of reproductive isolation between the two forms when in allopatry is a possibility.

4. *Rapid attainment of compatibility in sympatry*. Because both forms originated in sympatry, the ability to persist in sympatry after secondary overlap may be relatively easily attained. It is interesting to note that other monomorphic species of the Nymphalidae with spring form like and summer form like wing patterns co-exist. The so-called 'fritillaries'

Table 5. Number of matings and male–female interactions in four cages with sexes of the same or of different forms

Forms present (males–females)	Matings (*n*)	Observed interactions (*n*)	Expected on basis of observation time (*n*)	Expected on basis of observation time and number of butterflies (*n*)
Spring–spring	0	27	38.4	41.6
Spring–summer	0	0	36.0	26.0
Summer–spring	0	21	31.2	26.3
Summer–summer	1	84	26.4	38.1
Total	1	132	132.0	132.0

Note: Expected number of interactions are based on the number of 15 min observation sessions for each cage (fourth column) or the number of observation sessions multiplied with the number of butterflies in each cage.

have predominantly orange wings with black spots as in the spring form, whereas members of the Limenitinae (White Admiral, *Limenitis populi* and relatives) have predominantly black wings with a large white band.

In conclusion, there are quite a number of aspects in the *A. levana* plasticity system that are in line with West-Eberhard's (1989) hypothesis. Much remains to be done but *A. levana* provides interesting opportunities to test aspects of West-Eberhard's (1989) hypothesis.

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