

Concerted evolution and developmental integration in modular butterfly wing patterns

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SUMMARY Developing organisms are thought to be modular in organization so that traits in different modules evolve independently whereas traits within a module change in a concerted manner. The eyespot pattern in *Bicyclus anynana* butterflies provides an ideal system where morphological modularity can be dissected and different levels of genetic integration analyzed. Several lines of evidence show that all eyespots in an individual butterfly are genetically integrated, suggesting that the whole pattern, rather than the separate eyespots, should be considered as a single character. However, despite the strong genetic correlations between the two eyespots on the dorsal forewing of *B. anynana*, there is great

potential for independent changes. Here we use laboratory lines selected in different directions for the size of those eyespots to study correlated responses in the whole eyespot pattern. We show clear changes in eyespot size across all wing surfaces, which depend on eyespot position along the anterior–posterior axis. There are also changes in the number of extra eyespots and in eyespot color composition but no changes in eyespot position relative to wing margin. Our analysis of eyespot pattern modularity is discussed in the light of what is known about the cellular and genetic mechanisms of eyespot formation and the great potential for evolutionary diversification in butterfly wing patterns.

INTRODUCTION

The concept of modularity has been recurrently used in evolutionary developmental biology both to describe patterns of and understand processes in morphological evolution (e.g., von Dassow and Munro 1999; Bolker 2000; Winther 2001). Developing organisms are thought to be modular in organization; traits representing separate modules (Raff 1996; Wagner and Altenberg 1996) or quasi-independent units (Lewontin 1978) are free to follow their own evolutionary paths, whereas features within one module evolve in a concerted manner (i.e., they are coupled). The developmental coupling between traits might impose biases in the production of phenotypic variants and thus constrain or channel morphological evolution in particular directions (Cheverud 1984; Maynard-Smith et al. 1985). Examples of modular traits made up of serial repeats that have received recent attention are the segments of arthropod bodies (Arthur 1999; Williams and Nagy 2001), the dentition of vertebrates (Stock 2001), and serially homologous pattern elements on butterfly wings (Brakefield 2001; Nijhout 2001). The latter are good models with which to study and decompose the modular organization of morphological traits (e.g., Kingsolver and Wiernasz 1987; Paulsen and Nijhout 1993; Paulsen 1994; Brakefield 1998, 2001) and to examine it in the light of a well-studied developmental mechanism (Beldade and Brakefield 2002).

The concept of modularity provides an ideal framework

for examining the evolutionary processes that have led to the spectacular diversity found in butterfly wing patterns. The classic model describing wing pattern morphology recognizes different types of pattern elements repeated in homologous series. This idealized “nymphalid ground plan” (Nijhout 1991) represents the maximum number of pattern elements with little differentiation among the individual homologues within each series. Existing patterns can be seen as derivations from this ground plan, which have proceeded via more or less profound changes in the different pattern elements independently from each other (Nijhout 1991). Experimental data from several butterfly species suggest that indeed there is high independence between different types of pattern elements, but also that there are correlations across homologous pattern elements (Brakefield 1984; Paulsen and Nijhout 1993; Monteiro et al. 1994; Paulsen 1994; Nijhout 2001).

The tropical nymphalid *Bicyclus anynana* has a series of eyespots on both the dorsal and ventral wing surfaces. All eyespots are circular in shape and have a similar color composition but differ in size. Laboratory studies of *B. anynana* have revealed much genetic variation for different aspects of eyespot morphology, including their size and color composition (Beldade and Brakefield 2002). However, all eyespots characteristically respond together to artificial selection (Monteiro et al. 1994, 1997) and are typically affected in concert in wing pattern mutants (see examples in Brakefield 1998, 2001). The coupling be-

tween individual eyespots is, presumably due to the fact that they all share the same developmental basis. All eyespots are formed through the action of central organizers, the foci (French and Brakefield 1995), and all show a characteristic expression of a number of developmental genes in preadult wing primordia (Brakefield et al. 1996; Keys et al. 1999; Brunetti et al. 2001). The evidence for the genetic coupling between eyespots has led to the suggestion that the whole pattern of eyespots should be viewed as a single module that is developmentally and evolutionarily independent from those of other pattern elements (Brakefield 2001). To more thoroughly examine this hypothesis and to be able to make predictions about evolutionary outcomes, we need to understand how the eyespot pattern can be decomposed into units (or modules) with independent development and, consequently, realize the potential for evolutionary change in different directions. The butterfly system lends itself to such an analysis because the genetic integration revealed by the correlated responses to artificial selection can be examined in the light of the knowledge of different aspects of the mechanisms underlying eyespot formation (Beldade and Brakefield 2002).

Although *B. anynana* eyespots show a conserved pattern of relative size and respond to selection on size in a concerted manner (Monteiro et al. 1994), this pattern can readily be broken by artificial selection (Beldade et al. 2002b, 2002c). Despite the genetic correlations between eyespots, it has been possible to select for localized changes in the size of the two dorsal forewing eyespots (Beldade et al. 2002b). Here we use such selection lines to explore the hierarchical organization of the eyespot module as a whole, monitoring correlated responses in eyespot size across different wing surfaces and changes in other features of eyespot pattern morphology (eyespot number, color composition, and position).

MATERIALS AND METHODS

Experimental animals

Bicyclus anynana butterflies have several marginal eyespots on their fore- and hindwings, each centered within a wing region bordered by veins (called a wing cell). A series of marginal wing cells can be recognized on each wing (wing cell 1 being the most anterior one). Typically, these butterflies have eyespots in wing cells 2 and 5 on the forewing (both dorsal and ventral surfaces) and in wing cells 1–7 on the ventral hindwing (Fig. 1a).

Here we use butterflies from groups of 2-fold replicated lines derived by selecting on different combinations of the size of the dorsal forewing eyespots, the anterior eyespot (A) and the posterior (P). Five groups of selection lines were analyzed (Fig. 1b): AP (both eyespots selected for increased size), Ap (selected for a larger anterior and a smaller posterior eyespot), aP (small anterior and large posterior eyespot), ap (both eyespots smaller), and UC (unselected controls). Directional selection was applied on female butterflies for a total of 17 generations as described in Beldade et al. (2002b). From the last generation of selection, adult butterflies from each line were randomly collected at eclosion, frozen soon after, and measured for a series of features on their right fore- and hindwings using a binocular microscope coupled to a digitizing tablet through a camera lucida. All butterflies were reared at 27°C.

Response to selection: eyespot sizes

Response in eyespot size across different wing surfaces was evaluated in 50 female and 50 male butterflies from each replicate selection line. The diameter of the two target eyespots (A and P) and all eyespots characteristically present on the ventral surface of both forewing (two eyespots, vA and vP) and hindwing (seven eyespots, h1–h7) were measured, as were linear indices of fore- (W) and hindwing (hW) sizes (Fig. 1a). All eyespots in *B. anynana* have a central white pupil (the focus), a middle black disk, and an outer gold ring; ventral eyespots usually have additional, less conspicuous, outer rings. Total eyespot size was measured along the midline of each eyespot-bearing wing cell as the diameter of the white pupil plus the black and gold rings (Fig. 1a). Changes in eyespot

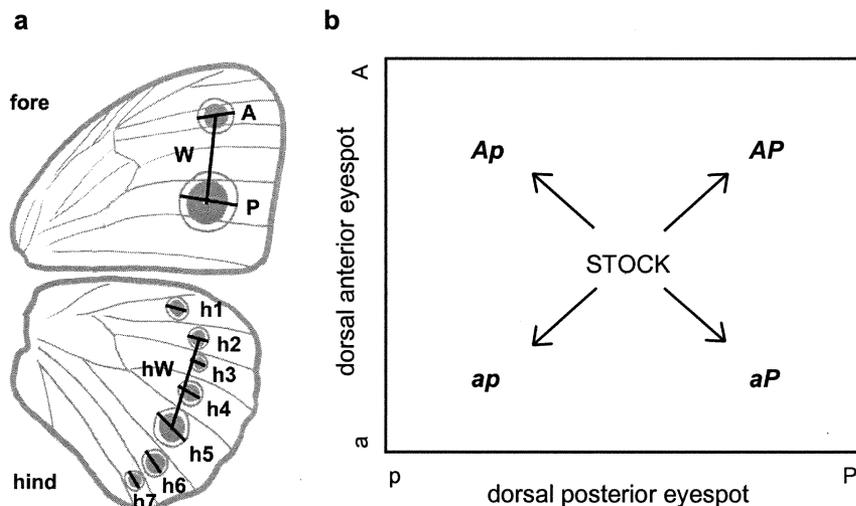


Fig. 1. *Bicyclus anynana* eyespot sizes. (a) Drawings of fore- and hindwing showing all eyespots characteristically present and all size measurements taken (see Materials and methods). Both dorsal and ventral surfaces of the forewing have an anterior and a posterior eyespots (wing cells 2 and 5, respectively). On the hindwing, typically only the ventral surface has eyespots. Note that the vein separating wing cells 6 and 7 on the hindwing is present in veined preadult wing primordia but not in adult butterflies. (b) Diagram illustrating the different directions of artificial selection on the dorsal forewing eyespots (A and P). The selection experiment (Beldade et al. 2002b) included four directional selection groups (arrows) and also unselected control lines (not in the diagram).

size were monitored as the ratio between eyespot diameter and wing size (W or hW for the fore- and hindwings, respectively) to correct for differences in overall wing size and because artificial selection had targeted those ratios (Beldade et al. 2002b).

Response to selection: other features of the eyespot pattern

Correlated responses to selection in other eyespot features were examined in 50 females from each replicate selection line. We monitored eyespot *number* by counting the extra eyespots, that is, those present in wing cells that do not characteristically bear an eyespot in this species (wing cells 1, 3, and 4 on the dorsal and ventral surfaces of the forewing and 1–7 on the dorsal surface of the hindwing; see Fig. 1a). We monitored *color composition* of the two eyespots directly targeted by selection (the A and P dorsal forewing eyespots) using two measures: (a) the proportion of white to total eyespot size, measured as the ratio between the diameter of the white pupil and total eyespot diameter (“Afocus” and “Pfocus” for the anterior and posterior eyespots, respectively), and (b) the proportion of gold to black, measured as the ratio between total diameter minus the diameter to the outer limits of the black disk and the black disk diameter minus white pupil diameter (“Ag/b” and “Pg/b” for the anterior and posterior eyespots, respectively). We monitored the *position* of the forewing eyespots along the proximal–distal axis of the wing by measuring the distance between eyespot centers and wing margin (note that the centers of the dorsal and ventral eyespots overlie each other). These measurements were corrected for differences in overall wing size by using the ratios between measured distance and forewing size (“A-m” and “P-m” for the anterior and posterior eyespots, respectively).

Statistical analysis

To compare phenotypes among the five selection groups (AP, Ap, aP, ap, and UC), we performed analyses of variance (ANOVAs)

using the mean phenotypic values of the two replicate lines for each group. This was done for fore- and hindwing sizes, forewing eyespot diameter/wing size, and eyespot position and color composition. After the ANOVAs testing for differences between all groups, Tukey comparisons between pairs of groups were performed.

We used principal component analysis (PCA) techniques to explore and describe the patterns of variation for the size of the seven hindwing eyespots, using the values of eyespot diameter/wing size for all females or all males from the different selection lines. Principal components (PCs) were calculated based on the correlation matrix described by our data. All PCs explaining more than 5% of the variation in the data were characterized and the scores for these components stored for all individual butterflies. ANOVAs were performed to compare the mean scores for each PC across groups ($n = 2$ mean scores, one for each replicate line, for each of the five selection groups).

All analyses were done using the MINITAB statistics program (MINITAB, Inc., State College, PA, USA). Males and females were analyzed separately not only because of the sexual dimorphism in *B. anynana* wing size (males are, on average, smaller), but also because the artificial selection that derived the different phenotypic groups targeted female butterflies only (Beldade et al. 2002b).

RESULTS

Response in eyespot size

After 17 generations of directional selection, both males and females from the different selection groups showed highly distinct eyespot/wing size phenotypes and no significant differences ($df = 4,5, P > 0.05$; ANOVAs) in forewing (females: $F = 3.81$; males: $F = 3.03$) or hindwing size (females:

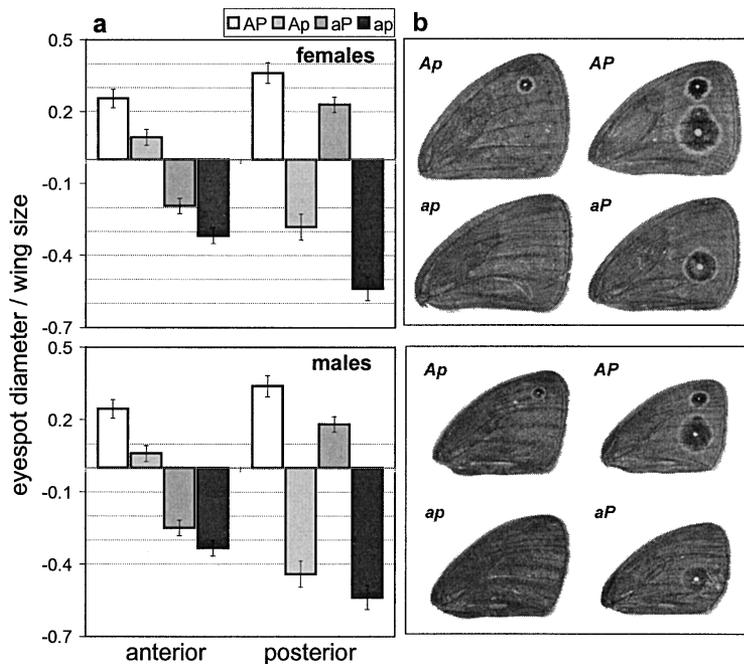


Fig. 2. Response to 17 generations of artificial selection on the size of the eyespots on the dorsal forewing. (a) Mean eyespot/wing size across replicate lines for the different selection groups (inset with labels) are given relative to mean control values (\pm standard error) separately for female (top) and male (bottom) butterflies. (b) Photos of representative phenotypes of the final selected groups.

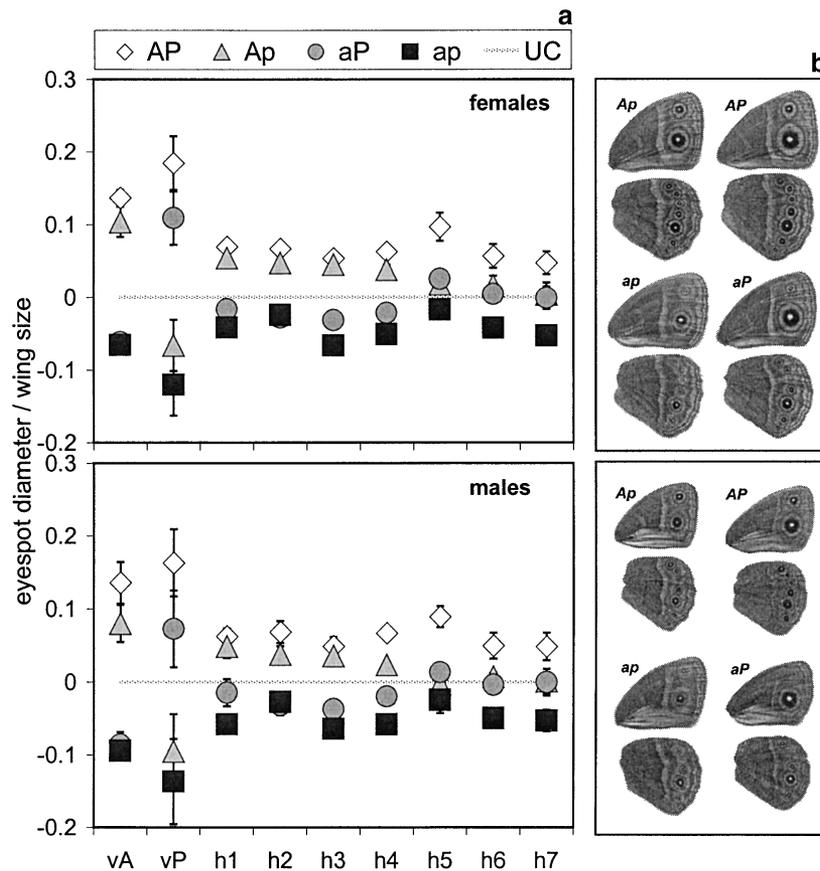


Fig. 3. Correlated changes in ventral eyespot size. (a) Mean eyespot/wing size for the two replicate lines of each selection group (inset with labels) are given relative to mean control values (\pm standard error). (b) Photos of representative fore- and hindwing ventral phenotypes of female (top) and male (bottom) butterflies from the directional selection groups.

F = 2.97; males: F = 3.01). Differences in eyespot size across groups were extreme for the two target eyespots (Fig. 2) but were also clear for most of the others (Fig. 3). Significant differences were found for the size of all eyespots char-

acteristically present on the forewing of both female and male butterflies (Table 1).

The PCA describing the patterns of variation in ventral hindwing eyespot size in the different selection groups has

Table 1. Size phenotypes for the forewing eyespots in butterflies from different selection groups

Sex	Group	Dorsal		Ventral	
		A/W	P/W	vA/W	vP/W
Females	AP	0.589 \pm 0.031	0.997 \pm 0.042 ^b	0.451 \pm 0.004 ^c	0.911 \pm 0.025 ^c
	Ap	0.427 \pm 0.013 ^a	0.354 \pm 0.062	0.418 \pm 0.025 ^c	0.660 \pm 0.018 ^f
	aP	0.141 \pm 0.006	0.864 \pm 0.001 ^b	0.253 \pm 0.005 ^d	0.835 \pm 0.022 ^{e,g}
	ap	0.016 \pm 0.009	0.096 \pm 0.053	0.249 \pm 0.011 ^d	0.606 \pm 0.038 ^f
	UC	0.334 \pm 0.045 ^a	0.635 \pm 0.046	0.315 \pm 0.014	0.727 \pm 0.046 ^{e,g}
	ANOVA F	158.43***	129.98***	87.55***	31.16**
Males	AP	0.578 \pm 0.024	0.877 \pm 0.034 ^j	0.473 \pm 0.036 ⁿ	0.841 \pm 0.003 ^q
	Ap	0.393 \pm 0.039 ^b	0.098 \pm 0.095 ^k	0.417 \pm 0.031 ^{n,p}	0.583 \pm 0.032 ^{r,s}
	aP	0.084 \pm 0.003 ⁱ	0.719 \pm 0.021 ^{j,l}	0.252 \pm 0.017 ^o	0.751 \pm 0.035 ^{q,s}
	ap	0.000 \pm 0.000 ^j	0.000 \pm 0.000 ^k	0.243 \pm 0.008 ^o	0.542 \pm 0.051 ^r
	UC	0.333 \pm 0.041 ^h	0.538 \pm 0.047 ^l	0.337 \pm 0.017 ^{o,p}	0.679 \pm 0.065 ^{q,r}
	ANOVA F	147.06***	114.01***	34.72**	16.26*

Values are mean \pm SD for female and male eyespot diameter/wing size phenotypes in the five selection groups ($n = 2$ replicate lines per group). All eyespots characteristically present on the forewing were measured (i.e., the anterior [A] and posterior [P] eyespots on the dorsal and ventral surfaces) as well as the index of forewing size [W] (see Fig. 1a). The ANOVA test statistic for the comparisons of phenotypes across groups (within sex) shows significant differences for both forewing eyespots on each wing surface: * $P = 0.005$, ** $P = 0.001$, *** $P < 0.0005$ ($df = 4,5$). Superscript letters indicate values that are not significantly different upon Tukey's pairwise comparisons at a 5% family error rate (MINITAB statistical package).

Table 2. Results of a principal component (PC) analysis on ventral hindwing eyespot size

Variable ¹	Females			Males		
	PC1	PC2	PC3	PC1	PC2	PC3
h1/hW	-0.392	-0.255	0.050	-0.375	-0.324	-0.160
h2/hW	-0.383	-0.393	0.043	-0.382	-0.419	0.192
h3/hW	-0.384	-0.351	-0.318	-0.390	-0.366	-0.270
h4/hW	-0.394	-0.249	0.160	-0.402	-0.123	0.172
h5/hW	-0.351	0.371	0.776	-0.349	0.348	0.777
h6/hW	-0.377	0.463	-0.212	-0.379	0.451	-0.276
h7/hW	-0.361	0.494	-0.470	-0.365	0.496	-0.394
Eigenvalue	5.547	0.647	0.357	5.315	0.677	0.423
% variation	79.2	9.2	5.1	75.9	9.7	6.0

¹h1–h7 are the diameters of the seven eyespots on the ventral hindwing, and hW is a linear measure of hindwing size (as in Fig. 1a). For each PC explaining more than 5% of the variation in female and male hindwing eyespot sizes, the table displays the Eigenvalues, the proportion of the variation explained, and the contribution of each eyespot/wing size variable (+/- signs contrast variables, whereas values close to zero indicate that a particular variable does not contribute to the definition of the PC MINITAB Statistical Package).

enabled us to reduce this variation to three main PCs, together accounting for over 90% of the total variance (females, 93.6%; males, 91.6%; Table 2). Examination of the PCA coefficients for the different eyespots shows the same patterns for males and females (Table 2). PC1 describes overall eyespot size, with all eyespots contributing equally (all coefficients have the same sign and same approximate value). PC2 contrasts variation in the more anterior eyespots (h1–h4 with a negative coefficient) to that in the more posterior ones (h5–h7 with a positive coefficient), with eyespots h6 and h7 showing the highest contribution and eyespot h4 (together with h1 in females) the lowest. PC3 is defined mostly by eyespot h5 and, to a lesser extent, eyespots h3, h6,

and h7. ANOVAs comparing mean PC scores across groups (five groups and two values per group; $df = 4,5$) showed significant differences for PC1 (females: $F = 32.45, P = 0.001$; males: $F = 59.90, P < 0.0005$) and marginally significant or nonsignificant differences for PC2 (females: $F = 5.73, P = 0.041$; males: $F = 3.65, P = 0.094$) and PC3 (females: $F = 4.70, P = 0.060$; males: $F = 6.33, P = 0.034$). PC1 and PC2 together clearly separate the groups with different histories of selection on the dorsal forewing eyespots (Fig. 4).

Response in other features of eyespot pattern morphology

Selection on dorsal forewing eyespot size produced correlated responses in other features of the eyespot pattern. We found differences between females from the selection groups in the number of extra eyespots and in dorsal forewing eyespot color composition. However, we found no differences in forewing eyespot position.

The selection groups showed clear differences in the number of extra eyespots. Lines selected for larger dorsal forewing eyespots had more extra eyespots, whereas lines ap (selected for smaller eyespots) had none, and UCs were intermediate (Fig. 5). There were also clear differences in the distribution of these extra eyespots over wing regions; lines selected for a larger dorsal forewing anterior eyespot tended to have extra eyespots in more anterior positions, whereas those selected for a larger posterior eyespot had extra eyespots in more posterior positions (especially clear for group *ap*; Fig. 5b). We also found differences across groups for the two measures of target eyespot color composition. Eyespots from group *ap*, in which both dorsal forewing eyespots are small or absent, have proportionally less white than all other selection groups, and eyespots selected for increased size appear to have proportionally more black relative to gold than

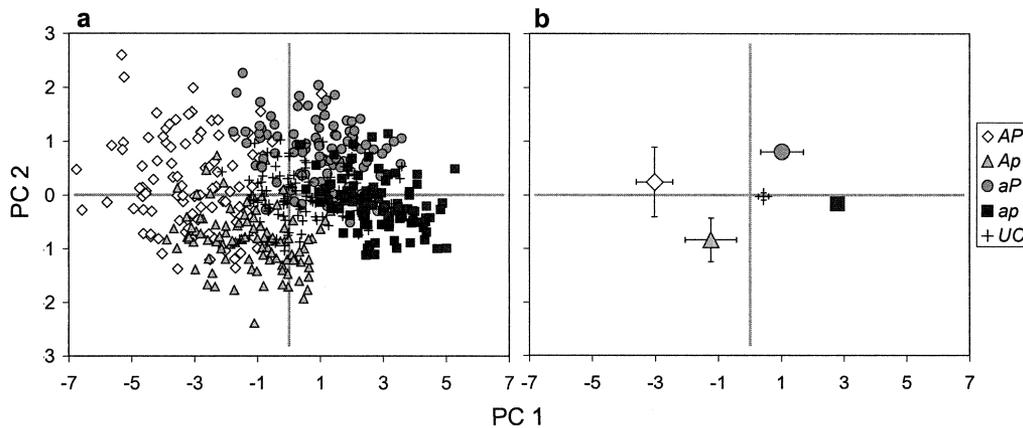


Fig. 4. Variation along the principle component (PC) axes. The distribution of females according to their scores for the PC1 and PC2 is shown for the different selection groups (inset with labels). (a) All individuals from each group ($n = 100, 50$ from each replicate line). (b) Mean group values (\pm standard error) between the two replicate lines. Similar patterns of distribution were found for males.

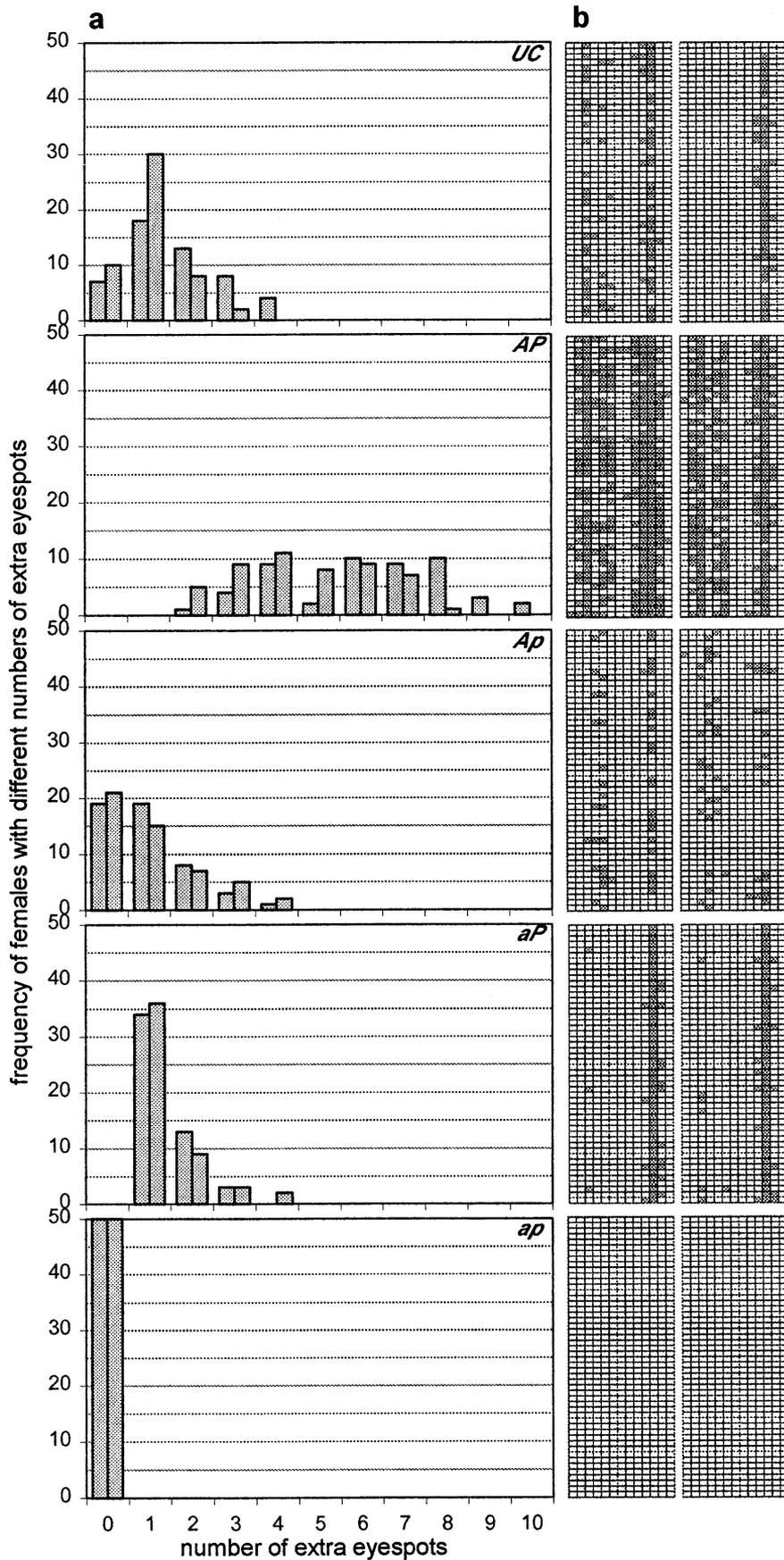


Fig. 5. Correlated changes in eyespot number. (a) Number of extra eyespots found on 50 females from the different selection groups (see Materials and Methods). The absolute frequency of females within each number class is given for the two replicate lines (side by side columns) in each selection group (different histograms). (b) Each matrix illustrates the number and position of extra eyespots on the 50 females (each individual is one line) for each selection line in a particular group (the two replicate lines correspond to side by side matrices). Extra eyespots were monitored on 13 different wing cells (see Materials and Methods), each corresponding to a column on the matrices; from left to right wing cells 1, 3, and 4 on the dorsal surface of the forewing, wing cells 1, 3, and 4 on the ventral forewing, and wing cells 1–7 on the dorsal hindwing (see Fig. 1a). Filled cells indicate presence of eyespot, and empty ones absence.

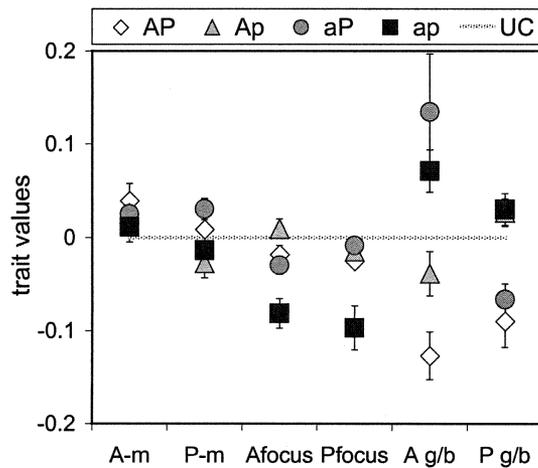


Fig. 6. Correlated changes in other features of forewing eyespot morphology; eyespot position (A-m and P-m) and color composition of dorsal eyespots (Afocus, Pfocus, Ag/b, and Pg/b) (see Materials and methods). Mean trait values across replicate lines for each selection group (inset with labels) are given relative to control mean values (\pm standard error) for female butterflies.

eyespot selected for reduced size (Fig. 6, Table 3). Finally, there were no correlated responses in eyespot position (Fig. 6); selection groups showed no significant differences ($df = 4,5$, $P > 0.05$; ANOVAs) for the distance between eyespot center and wing margin, either for the anterior ($F = 2.21$) or the posterior ($F = 2.92$) eyespots.

DISCUSSION

Butterfly wing patterns provide an ideal opportunity to decompose the modular organization underlying the development and evolution of morphological traits. Here we studied

a series of eyespot traits in *B. anynana* lines derived by artificial selection on the size of the two eyespots on the dorsal forewing. We found changes in size not only of the two target eyespots but also of all eyespots across wing surfaces. We also found differences in other features of eyespot pattern morphology, namely eyespot number and color composition.

Correlated responses in eyespot size across wing surfaces

Artificial selection on the size of the anterior and posterior eyespots on the dorsal forewing produced changes in eyespot size across all wing surfaces. Less extreme changes were observed for the lines where the two target eyespots were selected in opposite directions (groups *Ap* and *aP*), which produced phenotypes that were intermediate between those of the *AP* and *ap* groups. The response was very clear for all eyespots on the forewing but was more extreme for the dorsal wing surface in females (the direct targets of selection). Changes in the anterior and posterior eyespots on the ventral forewing followed closely the changes in the corresponding eyespots on the dorsal surface, revealing a genetic coupling between the two wing surfaces.

There were also correlated responses to selection for the eyespots on the ventral hindwing. The selection groups showed significant differences for the mean scores of PC1 defined by total eyespot/wing size. The response of hindwing eyespot size seemed to follow an anterior/posterior division, the more anterior eyespots (h1–h4) following the changes in the forewing anterior eyespot and the more posterior eyespots (h5–h7) following those in the forewing posterior eyespot. The latter, however, did not change in groups *Ap* and *aP*, where the two target eyespots were selected in opposite directions, suggesting that the response of the posterior eyespots on the hindwing also depends on the selection applied to the target anterior eyespot. The contrast between hindwing anterior and posterior eyespots accounts

Table 3. Color composition for the dorsal forewing eyespots in female butterflies from the different selection groups

Group	White to total		Gold to black	
	Afocus	Pfocus	Ag/b	Pg/b
<i>AP</i>	0.111 \pm 0.001 ^a	0.132 \pm 0.005 ^b	0.426 \pm 0.023 ^c	0.269 \pm 0.039 ^f
<i>Ap</i>	0.139 \pm 0.006 ^a	0.143 \pm 0.007 ^b	0.514 \pm 0.018 ^{c,d}	0.385 \pm 0.021 ^g
<i>aP</i>	0.100 \pm 0.001 ^a	0.149 \pm 0.005 ^b	0.687 \pm 0.084 ^e	0.292 \pm 0.024 ^{f,g}
<i>ap</i> ¹	0.048 \pm 0.018	0.061 \pm 0.033	0.624 \pm 0.015 ^{d,e}	0.389 \pm 0.024 ^g
<i>UC</i>	0.130 \pm 0.014 ^a	0.158 \pm 0.001 ^b	0.553 \pm 0.028 ^{c,d,e}	0.359 \pm 0.003 ^{f,g}
ANOVA F	23.53	12.58	11.32	9.78
<i>P</i> ($df = 4,5$)	0.002	0.008	0.010	0.014

Values are mean \pm SD for the dorsal anterior (A) and posterior (P) eyespot color proportion indices (see Materials and methods) of the different selection groups ($n = 2$ replicate lines per group). The values of the ANOVA test statistic comparing phenotypes across groups are given together with the respective *P* values. Superscript letters indicate values that are not significantly different upon Tukey's pairwise comparisons at a 5% family error rate (MINITAB statistical package manual).

¹Only a small proportion of the 50 females measured in each of the two *ap* replicate lines had any dorsal eyespots; for replicate lines 1 and 2, respectively, 3 and 9 individuals had an anterior eyespot and 10 and 33 had a posterior eyespot.

for 9–10% of the eyespot/wing variation on the hindwing (PC2) and seems to clearly separate groups *AP* and *aP* from each other and the other groups. Less extreme correlated responses were observed for eyespot h5 for which only *AP* has a phenotype that is clearly different from the other selection groups. This eyespot seems to have particular properties relative to all other hindwing eyespots, as reflected by the fact that PC3 is mostly defined by variation in h5. Furthermore, in a mutagenesis screen for mutant wing patterns in *B. anynana*, mutants were found affecting the size and presence/absence of all hindwing eyespots except h5 (Monteiro et al. 2003).

Results of other selection experiments targeting the dorsal forewing posterior eyespot alone had shown correlated responses in all eyespots but especially those on the same wing surface (Monteiro et al. 1994). Our results show that not only are independent changes of eyespots within the same wing surface possible (Beldade et al. 2002b, 2002c), but also that there are genetic correlations between fore- and hindwings and between dorsal and ventral wing surfaces that depend on eyespot position along the anterior–posterior axis. Future work will further dissect this anterior–posterior compartmentalization and attempt to understand its mechanistic basis. In particular, it should be very interesting to compare our results for selection on the A (forewing eyespot 2) and P (forewing 5) eyespots with selection on two eyespots firmly within a tighter cluster, either in the anterior or posterior compartment (e.g., neighbor hindwing eyespots 6 and 7 or eyespots 2–4). Our results also show that although selection was only applied directly to females in each generation, clearly divergent phenotypes evolved in males that corresponded closely to those observed in females. This suggests that the same genes contribute to eyespot variation in the two sexes, which is unsurprising given the absence of clear sexual dimorphism in eyespot patterns in *B. anynana*.

Correlated responses in other eyespot features

Different eyespot features show different patterns of correlated responses to selection on dorsal forewing eyespot size. We found clear differences in the number of extra eyespots across selection groups, some differences in target eyespot color composition, and no differences in forewing eyespot position.

Selection on dorsal eyespot size produced significant changes in eyespot number. This type of correlated response has been described for other lines selected for increased eyespot size (Holloway et al. 1993; Monteiro et al. 1994). It seems likely that all marginal wing cells have the potential to produce eyespots, as implied in the basic nymphalid ground plan (Nijhout 1991). Empirical evidence for this includes the formation of ectopic eyespots in response to different types of surgical manipulations on wing cells that characteristically do not bear eyespots (e.g., Brakefield and French 1995; French and Brakefield 1995) and the existence of mutant

stocks with extra eyespots in some of these wing cells (such as *Spotty*; Brakefield 1998). The distribution of the extra eyespots on the different selection groups again reveals an important anterior–posterior coupling component underlying such correlated responses.

Our selection groups also showed differences in target eyespot color composition. There was a significantly smaller proportion of white in the extremely small eyespots of *ap* lines than in all other groups. Despite the fact that the color composition estimates in *ap* lines were based on a very small number of individuals carrying any eyespots, observations of many more *ap* butterflies from previous generations of selection give us confidence that the effect detected is real. When present, the small *ap* eyespots very often have no discernible white pupil. The other selection groups showed no significant differences in the proportion of white, but there were across group differences in the proportion of gold to black. Eyespots selected for larger size appear to respond through proportionally larger changes in the diameter of the black disk. These results, though, are not very strong, yielding low significance levels and few instances of significant differences upon pairwise comparisons between selection groups. Nonetheless, they are consistent across groups with identical selection directions on individual eyespots (e.g., groups *AP* and *Ap* show a similar type of response for the anterior eyespot, whereas *AP* and *aP* are comparable for the posterior eyespot). These differences in the proportions of gold to black detected in response to selection on eyespot size contrast with previous results of selection experiments directly targeting the proportion of black in the dorsal forewing posterior eyespot which showed no correlated changes in size (Monteiro et al. 1997).

We found no differences between selection groups in eyespot position along the wing proximal–distal axis. *A priori* evidence suggesting we might find such differences included the reported genetic correlations between eyespot diameter and distance from wing margin for *Precis coenia* butterflies (Paulsen 1994). Furthermore, in *B. anynana*, more marginal wing regions can produce larger eyespots in response to surgical manipulation of pupae (Brakefield and French 1995; Monteiro et al. 1997), and there is genetic variation for eyespot position along this axis (Brakefield 1998).

Correlated responses and the mechanisms of eyespot development

A better understanding of the correlations among different features of eyespot morphology needs to take into account what is known about the cellular and molecular mechanisms of eyespot development (Beldade and Brakefield 2002). Surgical manipulations of pupal wings have been traditionally used to characterize the cellular interactions taking place during eyespot induction in butterfly pupae. This process has been dissected into a two-component signal/response sys-

tem: A central group of focal cells produce a signal that induces surrounding epidermal cells to respond with pigment synthesis (Nijhout 1980, 1991; French and Brakefield 1995). This signal is likely to be a morphogen produced (or degraded; French and Brakefield 1992) in the focus and whose concentration determines the fate of the surrounding epidermal cells to produce particular color pigments (Nijhout 1991; French and Brakefield 1995). All eyespots (within and across species) appear to be formed by this same mechanism. Phenotypic changes in eyespot morphology have been traced to changes in the properties of the focal signal and epidermal response components (Monteiro et al. 1994, 1997). Differences in eyespot size due to eyespot identity (French and Brakefield 1995) or artificial selection (Monteiro et al. 1994) are mostly determined by the properties of the focal cells and, to a lesser extent, by epidermal response sensitivities (Monteiro et al. 1994). Correlated responses in eyespot size and eyespot number across wing surfaces suggest that changes in the strength of the focal signal is not restricted to the target wing cells but rather affects other wing cells, wing surfaces, and wings. The correlated changes in proportion of gold to black, for which variation has been mostly attributed to changes in response sensitivity thresholds (Monteiro et al. 1997), suggests that this component might have also been changed in response to our selection on eyespot size. The changes in the proportion of white in ap lines can also be related to the cellular mechanism of eyespot formation. The white center in adult eyespots presumably matches the signal-producing focus of larval and pupal eyespots (e.g., see adult phenotype and gene expression patterns of *cyclops* mutant in Brakefield et al. 1996). A smaller area of signal-producing cells might determine a smaller amount of signal and, consequently, induce the production of a smaller eyespot.

Correlated responses can also be interpreted in the light of the properties of the developmental genetic pathways underlying eyespot formation. In recent years the study of gene expression patterns in butterfly wings has greatly advanced our knowledge on the molecular basis of eyespot formation. A number of genes known to be involved in *Drosophila melanogaster* wing patterning have been implicated in the formation of butterfly eyespots (Brakefield et al. 1996; Keys et al. 1999; Brunetti et al. 2001). These genes are involved in the formation of each single eyespot and in different phases of eyespot development. The expression of the same sets of genes in association with the eyespots in different wing cells might explain correlated responses in eyespot size and number across wing surfaces. Correlated responses in color composition, on the other hand, might be a consequence of the fact that the same genes are deployed in different phases of eyespot formation, possibly determining different properties of eyespot morphology. For example, the genes *Distal-less*, *spalt*, and *engrailed* are expressed together in the foci of larval and pupal wings and separately in the pupae in associ-

ation with different rings of color (Brunetti et al. 2001). At least one of these genes (*Distal-less*) has been directly implicated as contributing to interindividual variation in dorsal forewing eyespot size (Beldade et al. 2002a). Correlated responses in the proportion of gold to black might be due to this gene, as well as others.

Flexibility in eyespot pattern evolution

It has been suggested that the whole of the eyespot pattern in *B. anynana* butterflies behaves as a single module or character (Brakefield 2001) with different levels in a hierarchical organization (Beldade et al. 2002c). Genetic correlations between eyespots have been reported for different aspects of eyespot morphology and shown to be particularly strong for eyespots on the same wing surface (e.g., Monteiro et al. 1994, 1997). Here we show that the genetic correlations among eyespots determine the response in eyespot size to selection across the whole pattern, including in different wing surfaces and, to some degree, other features of eyespot morphology. However, despite the strong genetic correlations between eyespots within the same wing surface, great potential has been found for independent variation of eyespot size (Beldade et al. 2002b, 2002c). This argues against a prevalent role of developmental constraints derived from the coupling between individual eyespots in shaping evolution in eyespot size (Beldade et al. 2002b). The relative ease with which the coupling between eyespots within a wing surface appears to have been overridden (Beldade et al. 2002b) suggests that the (weaker) genetic correlations reported here are also not a major factor constraining wing pattern evolution. For example, despite the obvious genetic correlations we found, it is known that dorsal and ventral eyespots are already quite independent. Ventral eyespot development shows plasticity in size relative to rearing temperature and hormonal regulation, whereas dorsal eyespots do not (Brakefield et al. 1998; Brakefield and French 1999). Furthermore, across-species comparisons have shown that the genetic correlations between butterfly wing pattern elements can change over time (Paulsen 1994, 1996).

The developmental and evolutionary independence between eyespots is likely to be related to the compartmentalization of each of these serially homologous pattern elements within individual wing cells (Nijhout 1994). This individualization might involve the existence of compartment-specific genetic compositions that modulate the expression of the eyespot-forming genes (Nijhout 2001; Beldade et al. 2002c; McMillan et al. 2002; Monteiro et al. 2003). Eyespot-specific effects have been reported in *B. anynana* for genes involved in the presence/absence of eyespots both on the forewing (*Spotty* mutant shown in Brakefield et al. 1996) and hindwing (mutant 3–4 described in Brakefield 2001; McMillan et al. 2002) and for the *Distal-less* gene known to contribute to variation in eyespot size (Beldade et al. 2002a). Accumulating data from gene mapping studies in different organisms

have shown that many genes affecting correlated traits do have trait-specific magnitude of effects (e.g., Doebley and Stec 1991; Long et al. 1995; Juenger et al. 2000).

Evolutionary history of eyespot individuality

We propose that the potential for evolutionary change in butterfly eyespots is the result of a combination between their origin as highly coupled serial repeats and a legacy of natural selection favoring eyespot individuality. The genetic integration between morphological traits might evolve due to functional coupling between traits or may be a consequence of a shared (ancestral) developmental mechanism. Morphological integration is expected to evolve for traits that collectively serve a common functional role (Cheverud 1996; Wagner 1996). The fact that wing color patterns are involved in visual communication might explain the stronger correlations found in *B. anynana* between eyespots on the same wing surface (Monteiro et al. 1994, 1997; Brakefield et al. 1998). On the other hand, the ancestral state in eyespot patterns is probably one where serially repeated homologous elements are highly developmentally coupled but subsequent evolution might have favored their genetic decoupling (Beldade et al. 2002c). From an adaptive point of view, it might be useful to be able to change the morphology of particular eyespots and different features of the morphology of an individual eyespot independently. Studies in other organisms have shown that different features of an animal color pattern can indeed have a variety of functions (Kuwamura et al. 2000; Badyaev et al. 2001). Given the diversity found in butterfly wing patterns (both across and within species), it seems likely that evolutionary history has favored the developmental independence of different eyespots and eyespot features. The organization of the eyespot pattern in *B. anynana* probably reflects some vestiges of the common developmental origin of eyespots in ancestral nymphalidae in combination with the effects of a history of natural selection in favor of some degree of eyespot independence (Beldade et al. 2002c). The resulting individualization has potentially rendered butterfly wing pattern formation an extremely flexible system and enabled the spectacular evolutionary diversification of these patterns of color (Nijhout 1994, 2001).

This work illustrates how a morphometric analysis can contribute to revealing and interpreting different levels of genetic integration. These are essential to understanding the constraints or biases in morphological evolution (Cheverud 1984; Brakefield 2001) and the processes underlying the generation of phenotypic variation (Stern 2000), both fundamental issues in contemporary evolutionary developmental biology (Arthur 2002).

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