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Developmental and Genetic Mechanisms for Evolutionary Diversification of Serial Repeats: Eyespot Size in *Bicyclus anynana* Butterflies

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ABSTRACT Serially repeated pattern elements on butterfly wings offer the opportunity for integrating genetic, developmental, and functional aspects towards understanding morphological diversification and the evolution of individuality. We use captive populations of *Bicyclus anynana* butterflies, an emerging model in evolutionary developmental biology, to explore the genetic and developmental basis of compartmentalized changes in eyespot patterns. There is much variation for different aspects of eyespot morphology, and knowledge about the genetic pathways and developmental processes involved in eyespot formation. Also, despite the strong correlations across all eyespots in one butterfly, *B. anynana* shows great potential for independent changes in the size of individual eyespots. It is, however, unclear to what extent the genetic and developmental processes underlying eyespot formation change in a localized manner to enable such individualization. We use micromanipulations of developing wings to dissect the contribution of different components of eyespot development to quantitative differences in eyespot size on one wing surface. Reciprocal transplants of presumptive eyespot foci between artificial selection lines and controls suggest that while localized antagonistic changes in eyespot size rely mostly on localized changes in focal signal strength, concerted changes depend greatly on epidermal response sensitivities. This potentially reflects differences between the signal-response components of eyespot formation in the degrees of compartmentalization and/or the temporal pattern of selection. We also report on the phenotypic analysis of a number of mutant stocks demonstrating how single alleles can affect different eyespots in concert or independently, and thus contribute to the individualization of serially repeated traits. *J. Exp. Zool. (Mol. Dev. Evol.) 310B:191–201, 2008.*

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Serially repeated structures such as vertebrate teeth (Jernvall, 2000; Salazar-Ciudad and Jernvall, 2002; Weil, 2003; Klingenberg et al., 2003; Polly, 2005; Plikus et al., 2005; Mitsiadis and Smith, 2006; Fraser et al., 2006), insect body segments (Williams and Carroll, '93; Akam, '98; Williams and Nagy, 2001; Jockusch et al., 2004), and butterfly wing pattern elements (Nijhout, '91; McMillan et al., 2002; Beldade and Brakefield, 2002) offer exciting opportunities to investigate morphological diversification. Such repeated elements are typically tightly integrated, both developmentally and functionally, but have in many cases evolved independently leading to morphological diversification and functional specialization. The repeated elements can vary greatly, both between lineages and also across

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the series within an individual (Nijhout, '85, '94, 2001; Brakefield, 2001; Monteiro et al., 2006). The precise genetic and developmental mechanisms that underlie such variation are an area of great interest in evolutionary developmental biology (evo-devo).

Eyespots on butterfly wings can be used to address questions about compartmentalization and the evolution of individuality in a highly integrated manner, from the genetic and developmental basis of pattern variation, to the ecological relevance of variant phenotypes and the diversity of patterns across species (Nijhout, '91; Beldade and Brakefield, 2002; Brakefield et al., 2003). *Bicyclus anynana* butterflies, an emerging model organism in evo-devo (Beldade et al., 2007) have a series of marginal eyespots on different wing surfaces, and much potential for variation in eyespot patterns. This variation is caused in part by developmental plasticity in relation to environmental conditions during pre-adult life (Brakefield et al., '96; Brakefield and French, '99), and also both to the segregation of alleles producing subtle quantitative effects and to spontaneous mutations of large effect on phenotype (Beldade et al., 2005). Furthermore, there are strong genetic correlations between the eyespots on a butterfly (Monteiro et al., '97b, '94; Brakefield, '98; Allen, 2007), but also evidence for enough developmental flexibility to allow individual eyespots to change independently, at least with respect to their size (Beldade et al., 2002b,c; Brakefield et al., 2003; Monteiro et al., 2003).

The mechanistic basis of variation in eyespot traits has been explored in different types of experimental and theoretical studies. Laboratory breeding experiments have characterized existing patterns of genetic variation and explored the potential for changes in diverse aspects of eyespot morphology, including size (Monteiro et al., '94; Wijngaarden and Brakefield, 2000; Beldade et al., 2002b,c), shape (Monteiro et al., '97b), number (Monteiro et al., 2003; Beldade and Brakefield, 2003), and color composition (Monteiro et al., '97a; Allen, 2007). Analysis of expression of candidate genes from *Drosophila* wing development has identified a number of pathways that are re-deployed during eyespot formation (Carroll et al., '94; Brakefield et al., '96; Keys et al., '99; Brunetti et al., 2001; Reed and Serfas, 2004; Monteiro et al., 2006) and can potentially contribute to quantitative variation in eyespot morphology (Beldade et al., 2002a). Theoretical models have explored potential relations between some of these genes (Evans and Marcus, 2006) and various aspects of the cellular interactions that underlie eyespot formation (Nijhout, '80; Nijhout and Paulsen, '97; Monteiro et al., 2001; Nijhout et al., 2003; Dilão and Sainhas, 2004), which have been extensively characterized experimentally using manipulations of wing primordia. Surgical damage and transplantation of pupal wing tissue has shown that the presumptive eyespot center (the focus) functions as an ''organizer''; grafting the early pupal focus into a new host region leads to eyespot loss in the donor wing and the formation of an ectopic eyespot in the host tissue. Experiments such as these have characterized the cellular interactions underlying eyespot formation in terms of a signal-response or diffusion-gradient-threshold process. Early in the pupa, a signal (morphogen) produced or degraded (French and Brakefield, '92) in the eyespot focus forms a concentration gradient around it. Depending on the concentration of morphogen experienced and on response threshold levels, the neighboring cells become fated to synthesize the different color pigments that make up the adult eyespot.

Using surgical manipulation techniques and laboratory lines with divergent phenotypes, overall changes in eyespot morphology have been assigned to modification of different components of the signal-response process (Monteiro et al., '94, '97a; Brakefield and French, '95; French and Brakefield, '95). Although changes in eyespot color composition seem solely due to changes in response thresholds, variation in overall eyespot size can be traced mostly to variation in focal signal strength (reviewed in Beldade and Brakefield, 2002). It is, however, unclear to what extent each of these two components can change locally and how that contributes to the evolutionary diversification of individual eyespots on the same wing surface.

The two eyespots on the dorsal surface of *B. anynana* forewings show a typical pattern of relative size and strong phenotypic and genetic correlations (Monteiro et al., '94; Brakefield, '98; Beldade and Brakefield, 2003; Allen, 2007). Nonetheless, butterflies with novel eyespot size combinations are maintained in different laboratory populations, either derived by artificial selection or carrying single spontaneous mutations of large effect (Beldade et al., 2002b,c, 2005; Monteiro et al., 2003). Here, we use these different populations to explore the developmental and genetic mechanisms underlying individualization of serial repeats. Transplant experiments using material from the artificial selection lines explore how the

cellular mechanisms underlying eyespot formation change locally on the wing surface, and the phenotypic analysis of eyespot size mutants gives insights into how alleles of large effect can contribute to individualization of serial repeats.

MATERIAL AND METHODS

Experimental animals and target traits

Typically, *B. anynana* butterflies have a smaller anterior (A) and a larger posterior (P) eyespot on the dorsal surface of each of forewing (Fig. 1b; wing on the right) that, unlike the ventral surface (Brakefield and French, '99), shows no evidence for plasticity in color pattern in relation to environmental conditions during development. Eyespot and wing size were measured using a digitizing tablet attached to a microscope with a camera lucida. Each eyespot is composed of a central white focus, a middle black ring, and an outer gold ring. Eyespot size was quantified as the total diameter of the eyespot along its proximal-distal midline. To correct for overall differences in wing size, eyespot size was evaluated as the ratio between eyespot diameter and the distance between two wing landmarks (cf. Beldade et al., 2002b,c).

We analyzed dorsal forewing eyespot size in butterflies reared at 27°C in the laboratory's standard conditions (cf. Beldade et al., 2002b). The developmental mechanisms underlying quantitative variation in eyespot size were analyzed in artificial selection lines with different combinations of eyespot sizes and their unselected controls (see below), and the effect of single genes on eyespot size variation was characterized in three laboratory mutant stocks (see below).

Surgical manipulations

Grafts were performed on 3–4-hour-old female pupae from selection lines differing in eyespot size on the dorsal forewing (Fig. 1d). Reciprocal transfers of foci of the posterior dorsal eyespots were performed between one directional selection (hereafter, SELECTED) individual and one unselected control (hereafter, CONTROL) individual; always using left wings (Fig. 1a and b). Four SELECTED groups were compared using individual pupae from different laboratory selection lines (Beldade et al., 2002b): two replicate lines selected for larger anterior and posterior eyespots (*AP*), one line selected for a larger anterior and a smaller posterior eyespot (*Ap*), two replicate lines selected for a smaller anterior eyespot and a larger posterior eyespot (*aP*), and a line selected for both smaller anterior and posterior eyespots (*ap*). Foci from these lines were exchanged (Fig. 1a) with those from pupae from one of the two unselected control replicate lines.

For each operation, a small square of focal epidermis plus cuticle was cut (cf. Monteiro et al., '94; French and Brakefield, '95) on both the SELECTED and CONTROL individuals, and the cut squares were immediately transferred between pupae to avoid desiccation of the excised tissue. Pupae were left at 27°C until adult emergence and adults were freshly frozen immediately after. Successful grafts (i.e. those for which the grafted tissue healed) were scored and the diameter of the induced or experimental eyespots (eP) produced in the host wing region measured. The size of the wing (W) and the native posterior eyespot (nP) for both the SELECTED and CONTROL individuals of each manipulated pair were measured on the undamaged, non-operated right wings.

Comparison of the mechanisms of eyespot formation across the phenotypically divergent groups was done using analysis of variance for eP/W (where W is wing size of the SELECTED individual) across selection groups. The diameter of the experimental eyespot produced on the CONTROL butterfly after transplantation of a focus from a SELECTED pupa from different selection groups measures differences in focal signal strength, while the diameter of the experimental eyespot produced on the SELECTED butterfly after transplantation of a CONTROL focus tests for differences in response sensitivities. Pairwise comparisons were done using Tukey HSD test with 95% family-wise confidence level.

Phenotypic characterization of spontaneous eyespot size mutants

We characterized the relative size of the dorsal forewing eyespots from three mutant stocks: *Bigeye* (*BE*) with enlarged eyespots (Brakefield et al., '96; Brakefield and French, '99; Beldade and Brakefield, 2002), *Pminus* (*P−*) with a reduced posterior eyespot (Beldade et al., 2005), and *Aminus* (*A−*) with a reduced anterior (Fig. 2c). From each mutant stock, around 100 female butterflies (numbers in Table 2) were frozen freshly after eclosion and measured for the anterior and posterior eyespots, and for wing size. As control, we used measurements from the laboratory's outbred stock females ($N = 2{,}254$) reared in similar conditions.

Eyespot diameter/wing size (A/W and P/W, respectively for the anterior and posterior eyespots) was compared across stocks (analysis of variance and Tukey HSD pairwise comparisons with 95% family-wide confidence level) and the Pearson correlation between A/W and P/W was calculated for each stock. All statistical analysis was done in R (http://www.r-project.org/).

RESULTS

Mechanistic basis of compartmentalized changes in eyespot size

From the 322 operated pairs, 202 had at least one healed graft and the non-operated wing of the SELECTED adult was undamaged, so that wing size (W) could be measured (Table 1). Some (numbers in Table 1) of the grafts scored as successful (i.e. healed), especially in association with selection groups with small or absent native eyespots (*ap* and *Ap*), produced no induced eyespot but were not excluded from the analysis to represent actual phenotypes of selection groups (Fig. 1d). In terms of the mechanisms being analyzed here, eyespots of ''size zero'' are likely to represent below-threshold signal strength and/or epidermal sensitivities and are, thus, potentially informative trait values.

Our data suggest that there are differences between selection lines in the way the focal signal and epidermal response components vary between selection groups (Fig. 1c). Experimental eyespots formed in association with transplants involving individuals with large posterior eyespots (*AP* and *aP*) generally produced larger eyespots than those involving individuals with small posterior eyespots (*Ap* and *ap*; Fig. 1d). There were significant differences across groups in the presumed effect of both focal signal strength and epidermal response sensitivities (Table 1). For the focal signal component, experimental eyespots produced from transplants of SELECTED foci into CONTROL hosts were significantly smaller for

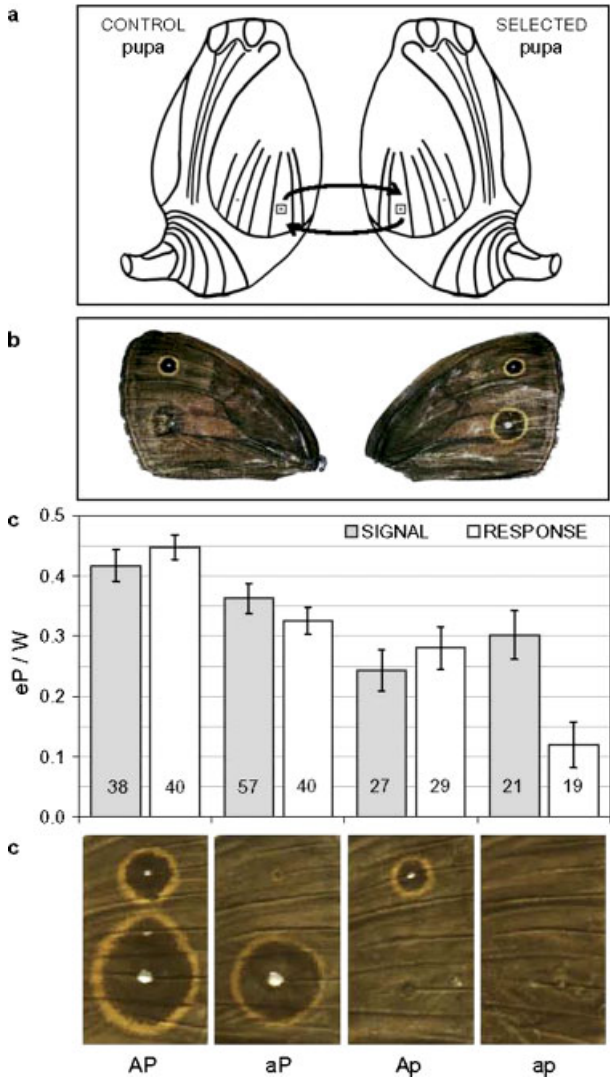

Fig. 1. Surgical manipulations used to characterize the cellular interactions underlying changes in eyespot size. (**a**) The foci from the presumptive dorsal forewing eyespots are visible in pupal wings (two dots on each pupa). Each grafting operation involved one pupa from an unselected control line (CONTROL pupa) and one from a directional selection line (SELECTED pupa) (see Methods section). Small squares of epidermis containing the focus of the posterior eyespot were reciprocally transferred between CONTROL and SELECTED pupae. Drawings of pupae after Monteiro et al. ('97a). Note that only foci from the left forewings were transplanted; the SELECTED pupa was turned in the drawing to make it visually easier to follow. (**b**) Photo of the left (operated) and right (unoperated) forewings of a single CONTROL butterfly, showing the induced and native posterior eyespots, respectively. (**c**) Mean (±standard error) eyespot diameter corrected for wing size (eP) is given for the experimental eyespots formed after reciprocal grafting of the dorsal forewing posterior eyespot foci between one SELECTED and one CONTROL female pupa. The eyespots formed on the CONTROL host reveal differences in focal signal strength between selection lines (gray columns); while those formed on the SELECTED host individuals reveal differences in epidermal response sensitivities (white). Numbers inside columns are sample sizes (i.e. all grafts for which the grafted tissue survived the transfer and healed). (**c**) Photo of a detail of the dorsal surface of a female *B. anynana* forewing showing the characteristic eyespot size phenotypes of our test selection groups: *AP*, *aP*, *Ap*, and *ap* (see Methods).

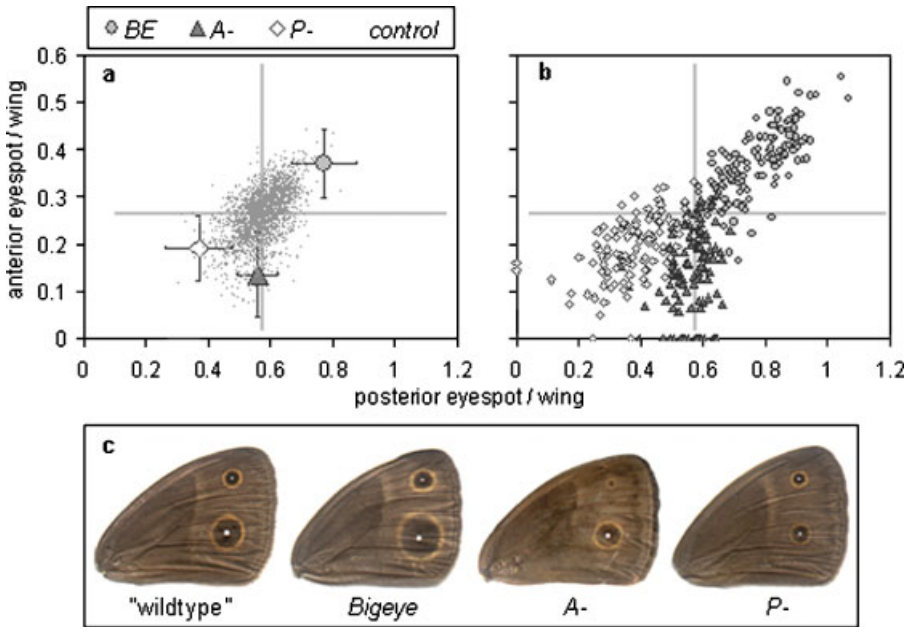

Fig. 2. Dorsal eyespot size phenotype of different *Bicyclus anynana* stocks: outbred stock (the ''wildtype'' pattern) and three mutant stocks. (**a**) Values for eyespot/wing size for the anterior and posterior dorsal forewing eyespots in 2,254 butterflies from the laboratory stock population and mean (±standard deviation) phenotypes for the mutant stocks *Bigeye* (*BE*), *A−*, and *P−*. (**b**) Eyespot/wing phenotypes for the mutant stocks; *BE* ($N = 134$), *A−* ($N = 114$), and *P−* ($N = 100$). On both panels the gray horizontal and vertical lines indicate mean phenotypes for the control stock population. (**c**) Photos of dorsal surface of the forewing of butterflies from the four target stocks.

TABLE 1. Differences across selection groups in wing size, relative size of native posterior eyespot, and relative size of experimental eyespot

SELECTED[a]	W *SELECTED* (mm)	nP/W *CONTROL*	nP/W *SELECTED*	eP/W *CONTROL*[b]	eP/W *SELECTED*[c]
AP	5.572 (0.246) $n = 52$	0.656 (0.058) $n = 55$	0.983 (0.077) $n = 52$	0.417 (0.160) $n = 38$ [2]	0.448 (0.127) $n = 40$ [1]
aP	5.547 (0.322) $n = 65$	0.660 (0.082) $n = 72$	0.873 (0.079) $n = 65$	0.363 (0.189) $n = 57$ [9]	0.325 (0.139) $n = 40$ [5]
Ap	5.541 (0.307) $n = 35$	0.661 (0.063) $n = 38$	0.409 (0.089) $n = 35$	0.243 (0.179) $n = 27$ [8]	0.280 (0.191) $n = 29$ [8]
ap	5.793 (0.266) $n = 29$	0.673 (0.077) $n = 35$	0.027 (0.063) $n = 29$ [24]	0.302 (0.185) $n = 21$ [5]	0.120 (0.167) $n = 19$ [12]
F[d]	5.6224* (3, 177)	0.3959 (3, 196)	1207.9** (3, 177)	5.567* (3, 139)	20.769** (3, 124)

[a]Comparison of wing (W) and eyespot size phenotypes (native posterior eyespot, nP, and experimental eyespot, eP) across selection groups. In each cell is displayed the mean values, standard deviation (in brackets) and sample size for different traits in the four selection groups. In square brackets next to the sample size is the number therein relative to native or experimental eyespots of ''size zero''.
[b]The experimental eyespot produced on the *CONTROL* host is used as a measure of signal strength.
[c]The experimental eyespot produced on the *SELECTED* host is used as a measure of epidermal threshold sensitivity.
[d]Analysis of variance F for differences across selection groups. Stars indicate statistical significant level: $*P < 0.005$, $**P < 0.0001$ (for nP/W in *UC*, $P = 0.7561$). Numbers in brackets are degrees of freedom of analysis of variance fixed factor and residuals, respectively. Statistical analysis done in R.

Ap donors relative to both *AP* and *aP* donors, and all other pairwise comparisons did not show significant differences (Tukey HSD pairwise comparisons with 95% family-wise confidence level). For the epidermal response component, pairwise comparisons revealed significant differences for all groups except between the two groups resulting from antagonistic selection on the two target eyespots (*aP* and *Ap*).

Contribution of single genes to localized changes in eyespot size

Butterflies from the mutant stocks show altered patterns of variation in eyespot size and differences in how compartmentalized the allelic effects are. All four stocks compared differ significantly in size of both A and P eyespots relative to wing size (Table 2 and Tukey HSD pairwise comparisons),

TABLE 2. Differences across laboratory stocks in wing size and relative eyespot size

Stock	W (mm)	A/W	P/W
Wt	6.226 (0.280)	0.263 (0.054)	0.573 (0.065)
A−	6.176 (0.281)	0.135 (0.091)	0.559 (0.064)
P−	5.844 (0.282)	0.191 (0.068)	0.371 (0.107)
BE	6.092 (0.278)	0.370 (0.073)	0.773 (0.106)
ANOVA F	67.086** (3, 2598)	393.67** (3, 2598)	649.83** (3, 2598)

Comparison of wing (W) and eyespot size phenotypes (anterior eyespot, A, and posterior eyespot, P) across laboratory stocks: *wt* ($n = 2254$), *A−* ($n = 114$), *P−* ($n = 100$), and *BE* ($n = 134$). In each cell is displayed the mean value (standard deviation) for the different traits. Stars indicate statistical significant level of ANOVA (numbers in brackets are degrees of freedom for fixed factor and residuals, respectively) testing for differences across groups: $^{**}P < 0.0001$. Statistical analysis done in R.

and also in the relative magnitudes of the phenotypic effects for the two target eyespots (Fig. 2, Table 2). The three mutant alleles cause phenotypic differences up to three standard deviation units of the unselected control stock (CSD), but the magnitude of the detected effects is not the same for the two target eyespots (Table 2). *BE* females have both eyespots larger than females from any of the other stocks, but appear to show a mildly stronger effect on the size of the posterior eyespot. The difference in mean eyespot diameter/wing size phenotypes between *BE* and control females is of 2.0 CSD for the anterior eyespot and 3.1 CSD for the posterior eyespot. Both *A−* and *P−* females, in contrast, have smaller dorsal eyespots than control individuals, but opposite relative magnitude of effects on the two eyespots (Fig. 2). The anterior eyespot in *A−* butterflies is 2.4 CSD smaller than in control individuals, while the effect on the posterior eyespot is very small (0.2 CSD). Conversely, *P−* produces a greater decrease in the posterior eyespot (3.1 CSD) than in the anterior (1.3 CSD).

The compartmentalization of allelic effects is also reflected in the phenotypic correlations between A/W and P/W (Pearson correlation coefficients $wt = 0.52^*$ [0.49, 0.54], $P- = 0.52^*$ [0.39, 0.65], $A- = 0.15$ [0.03, 0.33], and $BE = 0.73^*$ [0.64, 0.80], where numbers in square brackets are the 95% confidence intervals and stars indicate that the estimated correlation is significantly different from zero with $P < 0.0001$ (for $A-$, $P = 0.103$)).

DISCUSSION

We used different laboratory populations of *B. anynana* butterflies to explore the genetic and developmental basis of compartmentalized changes in serially repeated structures. These butterflies typically have eyespots along the margins of different wing surfaces and much genetic variation for different aspects of eyespot patterns (Beldade et al., 2005). Eyespots are serially repeated homologous pattern elements which have been shown to be developmentally and genetically coupled in *B. anynana* (discussed in Brakefield, '98, 2001). However, despite the strong correlations among eyespots (Monteiro et al., '94; Monteiro et al., '97a; Allen, 2007), independent changes in eyespot size can be produced by artificial selection on segregating quantitative allelic variation (Beldade et al., 2002b,c), and based on induced mutations of large phenotypic effect (Monteiro et al., 2003). Here, we compared the developmental basis of localized changes in eyespot size between artificial selection groups, and characterized the phenotypic variation associated with three spontaneous mutations affecting *B. anynana* eyespot size. We discuss how the different components of the signal-response developmental process that underlies eyespot formation change in a more or less compartmentalized manner, and how mutant alleles with different effects on individual eyespots can alter the strength of the correlations between them and potentially favor their developmental and evolutionary independence (discussed in Paulsen and Nijhout, '93).

Developmental basis of localized changes in eyespot size

Transplant experiments such as those used here are a well established method for dissecting eyespot formation into signal and response components and for assigning variation in eyespot morphology to variation in each of these components (French and Brakefield, '92; Monteiro et al., '94; French and Brakefield, '95; Brakefield and French, '95). The tissue that is cut and transplanted into a new location induces the production of experimental eyespots whose properties depend

on the strength of the signal produced from grafted focus and on the response sensitivity thresholds (to focal signal and potentially also to wounding; see below) of the host tissue. To minimize experimental error associated with difficulties in scoring experimental eyespots (e.g. assessing whether grafted tissue healed properly, and quantifying the size of irregularly-shaped eyespots), we were aggressive in scoring grafts as successful (of 322 pairs of manipulated pupae, 202 produced at least one adult that contributed to our dataset).

Our analyses show that localized antagonistic changes in eyespot size on the same wing surface rely mostly on localized changes in the focal signal, while concerted changes depend greatly on changes in response sensitivities (Fig. 1). For example, the small posterior eyespots in group *Ap* seem to be produced in response to a rather weak focal signal in an area of the wing where the epidermal response sensitivities do not differ significantly from those of *aP* individuals with a much larger native posterior eyespot. In contrast, whereas the signal has also diverged between *AP* and *ap* groups, the difference in eyespot size between them appears to be largely attributable to differences in the properties of the epidermal response to signal. These results suggest that the response component of the cellular process of eyespot formation might be more resistant to change in a localized manner (i.e. giving different properties in different regions on the same wing surface) than the focal signal component.

Comparison of two studies on the developmental dissection of eyespot size

In contrast to previous reports that changes in eyespot size are mostly the result of changes in focal signal and only to a small extent to changes in response thresholds (Monteiro et al., '94), we found that the response component was important in explaining variation across test groups. The groups that were analyzed in the two studies have some crucial differences that are potentially relevant for explaining the contrasting results. First, they differ in terms of the compartmentalization of changes in eyespot size: Whilst the lines tested by Monteiro et al. ('94) had direct artificial selection on the posterior eyespot alone and showed correlated changes in the anterior eyespot, the ones used here targeted both eyespots simultaneously (Beldade et al., 2002b). Secondly, they also differ in that Monteiro and colleagues applied artificial selection on both male and female butterflies, while our test groups were derived from selection on females only: Since realized heritabilities for posterior eyespot size are higher for males relative to females (Monteiro et al., '94), the two studies might differ in the extent to which potential male-specific additive genetic variation is contributing to end phenotypes. Finally, the two studies differ in terms of how much phenotypic divergence occurred between lines: Because the selection that derived the lines tested here was much more extensive (number of generations and consequently, phenotypic divergence), it might have extended to genes potentially not involved in an initial response to selection (see the next section).

Temporal pattern of selection on signal-source and response-threshold components

Theoretical models simulating butterfly eyespot evolution and development have predicted that the genes involved in the different phases of eyespot formation (signal source versus response thresholds) have distinct patterns of response to selection. The signal-type (or ''source'') genes have the highest initial correlation with eyespot size phenotype and respond more rapidly to selection, while response-type (or ''threshold'') genes become highly correlated to phenotype, and subject to effective selection, only after allelic variation at the first set of genes has gone to fixation (Nijhout and Paulsen, '97). This differential timing of response to selection might be very relevant in explaining important aspects of our results: (1) the fact that less extreme phenotypes (being those tested by Monteiro and colleagues in relation to ours, or those of *aP* and *Ap* groups relative to *AP* and *ap*) show relatively little change in response thresholds, and (2) the finding of a relatively strong focal signal from *ap* donors (rather extreme example in Fig. 1b, left wing), which themselves have very small, and often absent, posterior eyespots (Table 1). Less extreme phenotypes might not have reached fixation for ''source'' allelic variation and thus not have involved much selection on ''threshold'' variation. In contrast, it is conceivable that after fixation of ''weak-signal'' alleles during an initial phase of selection in the *ap* group, further reduction in eyespot size would have involved fixation of alleles decreasing the ability of the epidermal cells to respond to the weak signal. Once the response ability is completely eliminated,

however, any mutations restoring focal signal become irrelevant and can potentially accumulate in lines selected for small eyespots. Still, because different types of evidence suggest that *ap* wings produce no active foci (the focal marks on the pupal cuticle are hard to detect (if visible at all), and the expression of genes typically associated with focus establishment (Brakefield et al., '96; Keys et al., '99) is greatly down-regulated in *ap* larval and pupal wing discs (Beldade et al., 2002a, 2005), the experimental eyespots generated by grafted *ap* foci beg further analysis.

Focal signaling in ap butterflies and epidermal response to wounding

Further studies will be necessary to characterize the details of focal signal and epidermal response properties in *ap* and other groups. These include the analysis of expression pattern of other genes known to be involved in focus establishment (e.g. *Notch*; Reed and Serfas, 2004) and focal signaling (e.g. *wg*; Monteiro et al., 2006), and the characterization of epidermal response sensitivity to tissue damage. Wounding pupal wings at around 12–18 hr after pupation (Brakefield and French, '95) is known to induce production of ectopic eyespots, presumably because wounding and focal signaling share signaling molecules (Monteiro et al., 2006). A component of epidermal sensitivity to wounding is, thus, an unavoidable aspect of focal transplant experiments in butterflies and might help explain why transplanted *ap* foci induced eyespots in CONTROL hosts. However, response to wounding cannot explain the differences in focal signal strength across SELECTED test groups as our comparison was done by grafting test foci into a ''constant'' CONTROL host (Table 1). Differences in epidermal sensitivities, on the other hand, might include a component of sensitivity to focal signaling and a one of sensitivity to wounding, but it seems quite unlikely that these are actually different properties (Monteiro et al., 2006). Transplants of both focal and non-focal tissue will be necessary to address this distinction.

Single genes and the genetic decoupling of serial repeats

The differentiation across serial homologues can proceed through the acquisition of genetic variants that affect traits singly and lower the correlations between them (Riska, '86; Brakefield and French, '93; Paulsen and Nijhout, '93). Such genes can potentially be important in promoting an independence of evolution from the genetic covariances found at the level of quantitative genetics (Brakefield and French, '93; Paulsen, '94). *A−* might be one such gene as suggested by the observations that: (1) it produces smaller anterior eyespots but the size of the posterior eyespots are not significantly different from control butterflies, and (2) the correlation between eyespot sizes is not significantly different from zero. This contrasts with *P−* which does have a stronger effect of the posterior eyespot, but also clearly affects the anterior eyespot and does not seem to change the strength of the correlation between the two dorsal forewing eyespots. It also contrasts with *BE* that affects both eyespots and leads to a stronger correlation between anterior and posterior eyespot size.

The genes underlying independent evolution of serial repeats

How important alleles such as those characterized here are for evolutionary change in *B. anynana* natural populations is an exciting issue that remains unaddressed. We have shown evidence for the potential for independent evolution of the two dorsal forewing eyespots in *B. anynana* both based on standing quantitative variation and on alleles of large effect, and how these localized changes can be achieved in terms of the signal and response components of eyespot formation. We have argued that the potential for independent evolution of serially repeated eyespots in *B. anynana* might be the result of an history of selection favoring their genetic independence (Beldade et al., 2002c). Alleles such as *A−* might have played an important role in conferring developmental and evolutionary independence to homologous pattern elements, and enabled the spectacular diversification in butterfly wing patterns found across species (Nijhout, '94, 2001). The identity of the loci responsible for the extreme phenotypes generated by selection and found in stocks of single mutants remains largely unresolved. Previous work implicated the gene *Dll* in quantitative variation in eyespot size and showed a clear pattern of eyespot- and gender-specific effects of this gene (Beldade et al., 2002a). The development of genomic resources for *B. anynana* (Marcus et al., 2004; Ramos et al., 2006; Beldade et al., 2006, 2007; Long et al., 2007) will hopefully soon allow us to

TABLE 3. Wing pattern genes from EST project in Bicyclus anynana

Gene	GenBank[a]	Blastx.Dmel[b]	Blastn.lep[c]	blastx.Bmori[d]	tblastx.bbase[e]	ORF[f]
Apc	DY770932	CG1451 [6E−18]		CH379808 [2E−58]		1-506
ci	DY767768 DY767378 DY765949		AY429264 [3E−11]	CH389242 [5E−11]	PIC02500 [5e-12]	
cno	DY767972 DY765414	CG2534 [1E−57]		CH381188 [2E−73]		32-255
En	DY764191			CH392703 [1E−35]		21-407
nkd	DY768787	CG11614 [2E−25]	DT668093 [7E−79]	CH381417 [9E−64]	HEC04121 [2e−72]	1-588
Nle	DY772470	CG2863 [1E−69]		CH386214 [1E−100]	AMC00331 [3e−22]	2-601

Bicyclus anynana ESTs (Expression Sequence Tags) generated, assembled and annotated in (Beldade et al., 2006).
[a]GenBank accession numbers for ESTs corresponding to each gene object: *APC-like* (*Apc*), *cubitus interruptus* (*ci*), *canoe* (*cno*), *Engrailed (En), naked cuticle (nkd)*, and *Nle (Notchless)*. Annotation of assembled gene objects was done using BLAST analysis to more relevant gene collections (Evalues in square brackets).
[b]FlyBase CG number of best hit of BLASTX to *Drosophila melanogaster* peptides.
[c]GeneBank accession number of best hit for BLASTN to collection of lepidopteran nucleotide sequences.
[d]GeneBank accession number of best hit for BLASTX to collection of *Bombyx mori* peptides (see Beldade et al., 2006 for b–d).
[e]ButterflyBase (www.butterflybase.org) gene object number of best hit of TBLASTX to all lepidoptera ESTs except *Bombyx mori* as assembled in ButterflyBase (Papanicolaou and others, 2005).
[f]Predicted coding nucleotides using NCBI's ORF finder on the reverse complemented consensus sequences for each gene. Because ESTs were sequenced from the 3′ end (Beldade et al., 2006), predicted peptides correspond with the carboxyl end of the protein.

test the contribution and specific effects of many other candidate genes, including those of the *hedgehog* (Keys et al., '99; Brunetti et al., 2001), *wingless* (Carroll et al., '94; Monteiro et al., 2006), *Notch* (Reed and Serfas, 2004), and *TGF-â* (Monteiro et al., 2006) pathways which have been implicated in eyespot formation (see examples in Table 3).

Compartmentalization of gene effects and developmental properties

We have tested the contribution of different aspects of the underlying developmental mechanism, and of single alleles of large effect to compartmentalized changes in eyespot size in *B. anynana*. The results of transplant experiments using artificial selection lines suggest that while localized quantitative antagonistic changes in eyespot size rely mostly on localized changes in focal signal, concerted changes of serially repeated eyespots depend greatly on changes in response sensitivities. This contrast reflects potential differences between the signal-response components of eyespot formation in the degrees of compartmentalization and/or in the temporal pattern of response to the artificial selection that generated our test groups. Our phenotypic characterization of eyespot size variation across mutant stocks, on the other hand, revealed the potential for single loci to affect serially repeated traits individually and thus contribute to their evolutionary diversification.

The study of *B. anynana* wing patterns enables the integration of different types of analysis of the genetic (e.g. Beldade et al., 2002a; Monteiro et al., 2006), developmental (e.g. Monteiro et al., '94; Monteiro et al., '97a), and physiological (Brakefield et al., '98) basis of variation in phenotypes with tests of the adaptive value of variant morphologies (Robertson and Monteiro, 2005; Frankino et al., 2005) and a comparative analysis of variation across species (Brakefield and Roskam, 2006). Such integration promises to give important insights into the diversification of serial repeated traits and the evolution of individuality (e.g. Akam, '98; Ohazama and Sharpe, 2004).

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