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Developmental constraints versus flexibility in morphological evolution

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Evolutionary developmental biology has encouraged a change of research emphasis from the sorting of phenotypic variation by natural selection to the production of that variation through development[1]. Some morphologies are more readily generated than others, and developmental mechanisms can limit or channel evolutionary change[2]. Such biases determine how readily populations are able to respond to selection[3], and have been postulated to explain stasis in morphological evolution[4] and unexplored morphologies[5]. There has been much discussion about evolutionary constraints[6–8] but empirical data testing them directly are sparse[9,10]. The spectacular diversity in butterfly wing patterns[11] is suggestive of how little constrained morphological evolution can be. However, for wing patterns involving serial repeats of the same element, developmental properties suggest that some directions of evolutionary change might be restricted[12,13]. Here we show that despite the developmental coupling between different eyespots in the butterfly *Bicyclus anynana*, there is great potential for independent changes. This flexibility is consistent with the diversity of wing patterns across species and argues for a dominant role of natural selection, rather than internal constraints, in shaping existing variation.

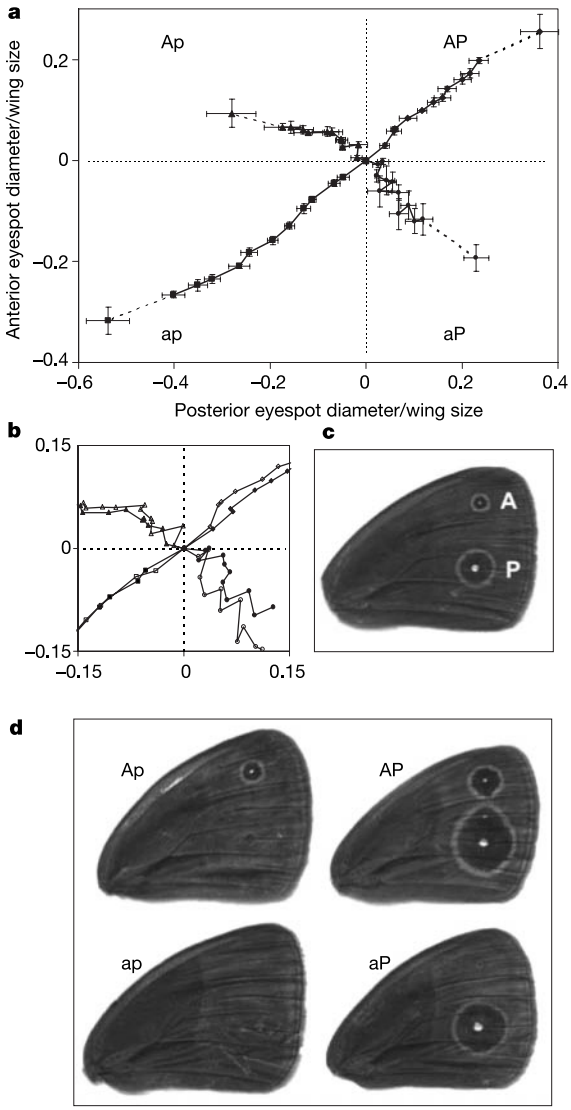

Figure 1 Response to artificial selection on the size of the dorsal forewing eyespots of *B. anynana*. **a**, Eyespot diameter/wing size relative to unselected control values are given for the different directions of selection. AP and ap are coupled directions; Ap and aP are uncoupling directions. Each point represents the mean (±standard error) for the two replicate lines for each generation. Solid lines join points covering the first 11 consecutive generations, all starting from the same original population (centre of graphic, G0). Broken lines join the points for G11 and G25 phenotypes. **b**, Enlargement of the central area of **a** showing the behaviour of individual replicate lines (filled and open symbols). **c**, Typical dorsal surface of forewing of unselected female showing the anterior (A) and posterior (P) eyespots. **d**, Representative G25 phenotypes (most ap females have no eyespots and many AP females have extra, satellite eyespots). Responses in males were comparable to those in females.

The different pattern elements on butterfly wings, including the eyespots, are apparently generated by an extremely flexible developmental system that allows independent variation and evolution of these elements[14]. Nonetheless, pattern elements belonging to the same homologous series have frequently been found to be positively correlated within different species[15,16]. Genetic covariances describe potential constraints arising from shared developmental pathways[17] that might limit wing pattern evolution[18]. In *B. anynana*, eyespots are characterized by a conserved pattern of relative size and are formed by a common developmental mechanism. Each eyespot is centred around a group of organizing cells[19] with a characteristic expression of several developmental genes[20–22]. Furthermore, allelic combinations favoured by artificial selection for their effect on one eyespot generally affect other eyespots in the same direction[16,23]. This coupling of eyespots might impose constraints on the evolution of wing patterns, at least in the short term, so we can predict that concerted changes will be more readily produced than opposing ones[12].

Artificial selection can be a powerful way of exploring the space of possible phenotypes[2,24,25]. In this study we used this approach to examine how readily the pattern of relative size of the anterior and posterior eyespots on the dorsal forewing of *B. anynana* (Fig. 1c) can be changed on the basis of standing genetic variation present in a laboratory population. Despite the positive genetic correlation between eyespot sizes[16], we obtained substantial responses to selection in different directions (Methods), both when the two eyespots were selected to change in a concerted or 'coupled' manner (both larger or both smaller, lines 'AP' and 'ap', respectively), and in opposite or 'uncoupling' directions (lines 'Ap' and 'aP') (Fig. 1). Directional selection was applied in two periods, from generations 0 to 11 and from G19 to G25 (with relaxed selection in between). Responses to selection were rapid, gradual and similar across replicate lines (Figs 1 and 2). All directions have produced butterflies with strikingly different ratios of eyespot size to wing size for both eyespots (Fig. 2), and by G25 almost all butterflies had extreme phenotypes not represented in the base population. These two domains of wing morphology in *B. anynana* have been able to respond independently to selection in a manner comparable to experiments with *Drosophila* wing compartments[25]. Despite this autonomy, we have obtained strong correlated responses across wing surfaces with ventral eyespots showing substantial, albeit less extreme, changes in the different directions (results not shown).

The observation that the 'uncoupling' phenotypes are not as extreme as the 'coupled' phenotypes (Fig. 1) is not in itself evidence for constraints. Positive correlations between the size of the target eyespots ($r_{\text{Pearson}} = 0.52 \pm 0.02$ in G0 females[26]) result in higher phenotypic variation along the coupled axis. Comparing changes in eyespot size relative to cumulated selection differential across directions shows no clear evidence that response in the uncoupling lines is more difficult than in the coupled lines (Table 1; compare rates of response for the posterior eyespot). Nevertheless, there are differences in the behaviour of the two types of lines. For both periods of directional selection, the anterior eyespot responds more slowly in both Ap and aP lines relative to AP and ap (Table 1). In particular, the relative lack of response in Ap after G6 (Fig. 1) presumably reflects exhaustion of genetic variation that increases the anterior eyespot with no (or opposite) effect on the posterior (although alleles that increase both eyespots simultaneously are still present; see response to selection imposed on the anterior eyespot alone, A in Fig. 2). Furthermore, our results show wider differences between replicate lines for the uncoupling directions, and a clear contrast between an apparent step-like progression in the uncoupling directions (in each generation one eyespot responding more than the other) versus a more linear progression in the coupled directions (Fig. 1b). Similar 'erratic responses' have been reported for antagonistic selection applied to positively correlated traits in other organisms[27].

Despite these indications of tension for uncoupling changes, our results clearly demonstrate great potential for independent changes of eyespot size in *B. anynana*. This potential is apparently not explored in this species, perhaps owing to stabilizing selection on the pattern. We examined the potential fitness disadvantages of uncoupled phenotypes by monitoring changes under relaxed selection (G11–G19). Such disadvantages should result in more rapid declines in frequency of extreme Ap and aP relative to AP and ap phenotypes. However, all lines reverted gradually towards control values with no clear distinction between coupled and uncoupling directions (Fig. 2). Nonetheless, even if in laboratory conditions there are no substantial fitness disadvantages in having an uncoupled phenotype, these may exist in nature, where sexual selection and interspecific interactions are likely to be more important.

Overall, our results show great flexibility in the formation and

Table 1 Rate of response to selection on *B. anynana* eyespot size

	G0–G11		G19–G25	
Direction	Anterior	Posterior	Anterior	Posterior
AP	0.331[a]	0.301[c]	0.281[d]	0.308[e]
ap	0.295[a]	0.356	0.252†[d]	0.251[e]
Ap	0.257[a,b]	0.464	0.196†[d]	0.369[e]
aP	0.237[b]	0.253[c]	0.126†[d]	0.355[e]
A	NA	NA	0.247[d]	NA
ANCOVA	9.84***	24.11***	3.18*	3.96**
	(3/40)	(3/40)	(4/25)	(3/20)

Slopes of the regression lines of response to selection on cumulated selection differential are given for each target eyespot during each period of directional selection (see Methods). All regression coefficients are significantly different from zero with $P < 0.0005$, except those marked with† ($P < 0.025$). Realized heritabilities for the first period of directional selection range from 0.5 to 0.9. Analysis of covariance (ANCOVA) F values (and interaction/error degrees of freedom) are given for the interaction effect of direction with cumulated selection differential on eyespot size/wing size; *** $P < 0.0005$, ** $P = 0.023$, * $P = 0.03$. Superscript characters indicate pairs of values, for each eyespot, that are not significantly different under Tukey's pairwise comparisons at 5% error rate. NA, not applicable.

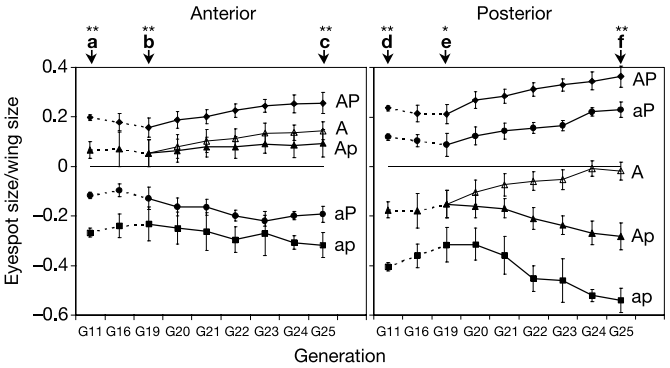

Figure 2 Response to relaxed selection (G11–G19) and additional directional selection (G19–G25). Average eyespot size/wing size values across replicate lines (±standard error) are given relative to unselected control values (horizontal line) for the different directions. Directional selection produced butterflies with highly divergent phenotypes and after eight generations under relaxed selection, almost all lines reverted to values closer to the unselected control. Characters above arrows correspond to the following analysis of variance (ANOVA) F values (with degrees of freedom for factor/error) for the effect of direction on phenotype at G11, G19 and G25: **a**, 174.12 (4/6); **b**, 73.24 (4/5); **c**, 169.94 (5/6); **d**, 161.64 (4/6); **e**, 28.45 (4/5); and **f**, 119.16 (5/6). In all cases there were significant differences in eyespot size/wing size across directions (asterisk, $P = 0.001$; double asterisk, $P < 0.0005$). Tukey's comparisons showed that all pairs of lines had significantly different eyespot size/wing sizes at a 5% error rate, with the following exceptions: **b**, Ap = UC; **c**, Ap = A; **e**, AP = aP = UC, ap = Ap; and **f**, AP = aP, UC = A.

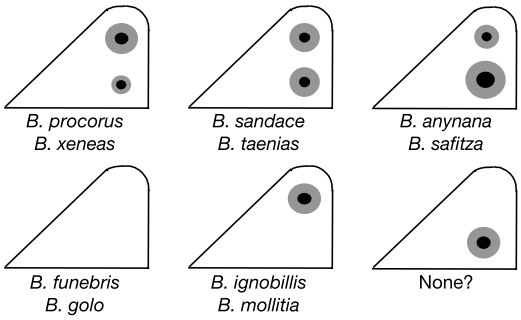

Figure 3 Diversity of eyespot patterns across the genus *Bicyclus*. Examples are shown of relative size phenotypes of the dorsal forewing eyespots of females from among the 80 or so *Bicyclus* species[30]. Most patterns of relative eyespot size have been explored at a macroevolutionary scale, but that of a large posterior and no anterior eyespot appears not to be represented in any extant species[30]. The pattern, however, eventually became frequent in both our aP selected lines of *B. anynana*. This suggests that its absence in the genus is explained by no history of selection in its favour rather than by an inability to generate it.

evolution of a morphological pattern for which developmental and genetic characterization had previously suggested the potential for constraints. This flexibility is consistent with the distribution of eyespot size phenotypes in the genus *Bicyclus* (Fig. 3). Standing genetic variation within a laboratory population of one species is sufficient to account for the production of all phenotypes found within the genus, and even one which is not explored in any extant species.

It has been proposed that the great flexibility in wing pattern formation in butterflies follows from the compartmentalization of individual pattern elements within vein-bounded wing regions[14,28]. Our results provide experimental support for this proposal, and show that the developmental properties of eyespot formation are unlikely to constrain any process of adaptive radiation in the pattern of relative size of butterfly eyespots. Natural selection, together with population-level properties[29], rather than the generation of phenotypic variation, is likely to dominate in shaping the evolution of morphology that has led to the spectacular diversity of butterfly wing colour patterns. □

Methods

Target traits and directions of selection

Typically *B. anynana* butterflies have a small anterior and a large posterior eyespot on the dorsal surface of their forewings (Fig. 1c). We selected on the ratios between eyespot diameters (anterior, *A*, and posterior, *P*) and a measurement of wing size (*W*, the distance between two wing landmarks). Starting from a single outbred laboratory stock, we derived three groups of lines selecting on different combinations of eyespot/wing sizes: (1) selection for one eyespot to become larger and the other smaller, the 'uncoupling' directions (large anterior and small posterior eyespots, 'Ap', or small anterior and large posterior eyespots 'aP'), (2) selection on both eyespots to change in a concerted manner (that is, both larger, 'AP' or both smaller, 'ap'), the 'coupled' changes, and (3) the unselected controls (UC). We derived two replicate lines for each mode of directional selection and three replicate UC lines. Selection was done on the basis of an additive combination of the rank values of A/W (R_A) and P/W (R_P); $R_A + R_P$ for the coupled and $R_A - R_P$ for the uncoupling lines. The laboratory stock and the maintenance of the butterflies was described in previous studies[16,23].

Selection procedure

This experiment was divided into three consecutive phases: (1) 11 generations of directional selection of similar intensity for all lines (G0 to G11); (2) eight generations under relaxed selection (G11 to G19); and (3) six additional generations of directional selection with doubled intensity for the uncoupling lines (G19 to G25).

A total of 2,254 female butterflies were measured at G0 and 45 of these were randomly selected to lay eggs that produced the next generation of one UC replicate line. The remaining butterflies were randomly split into two groups from which the two sets of replicate lines for all other directions were derived (first the UCs and next the directional selection lines). In subsequent generations, between 150 and 200 females were measured per line. The selected females were put with about 50 random males and allowed to lay eggs. To increase selection intensity, the number of parents was progressively reduced in the course of the experiment (no indication of inbreeding depression was observed). From G1 to G5 we selected 40 females per line; from G5 to G8, 35 females and from G8 to G11, 30. After G11 one UC line was discontinued and all other lines were maintained under relaxed selection with about 200 adult females reared per line per generation and 50–100 taken randomly as parents. After G19, the coupled lines and the UCs were maintained under a selection regime similar to that used in the first phase, while selection intensity for the uncoupling lines was doubled (330–450 females measured and 30 selected per line per generation). During this second period of directional selection we included another set of two replicate lines derived from each G19 Ap line and selected to increase the value of A/W with no selection on P/W (A lines).

Statistical analysis

To test for differences in phenotype between selection directions we performed analyses of variance (ANOVA)[26] on G11, G19 and G25 eyespot/wing phenotypes using the mean values of the two (or three for UCs) replicate lines for each direction (Fig. 2). When ANOVAs showed evidence of a significant effect of direction on phenotype, Tukey pairwise comparisons were performed.

Least-square regressions were fitted to the average points for eyespot size/wing size ratios (relative to UC values) on cumulated selection differential for each direction in each period of directional selection (Table 1). Average points across replicate lines were used because there were no significant differences between replicates (as tested with an analysis of covariance, ANCOVA[26]). Realized heritabilities are estimated as twice the absolute values of the slopes of the regression lines (selection done on females only). ANCOVAs were used to compare the slopes of the regression lines using 'direction' as a fixed factor and 'cumulated selection differential' as a covariate[26]. When these analyses showed a significant effect of the interaction between these two factors on eyespot size/wing size, Tukey pairwise comparisons were performed.

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Association of dwarfism and floral induction with a grape 'green revolution' mutation

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The transition from vegetative to reproductive growth is an essential process in the life cycle of plants. Plant floral induction pathways respond to both environmental and endogenous cues and much has been learnt about these genetic pathways by studying mutants of *Arabidopsis*[1,2]. Gibberellins (GAs) are plant growth regulators important in many aspects of plant growth and in *Arabidopsis* they promote flowering[3–5]. Here we provide genetic evidence that GAs inhibit flowering in grapevine. A grapevine dwarf mutant derived from the L1 cell layer of the champagne cultivar Pinot Meunier produces inflorescences along the length of the shoot where tendrils are normally formed. The mutated gene associated with the phenotype is a homologue of the wheat 'green revolution' gene *Reduced height-1* (ref. 6) and the *Arabidopsis* gene *GA insensitive* (*GAI*)[7]. The conversion of tendrils to inflorescences in the mutant demonstrates that the grapevine tendril is a modified inflorescence inhibited from completing floral development by GAs.

Grapevine (*Vitis* sp.) is one of the world's major perennial horticultural crops. It is a vine, and under natural conditions tendrils are used to support a tree-climbing habit to reach high sunlight levels for flowering[8]. A small number of *Vitis vinifera* cultivars dominate wine production in the world owing to their reputation for producing premium quality wine, and in France the Champagne region has become famous for its sparkling wine. Pinot Meunier, Pinot noir and Chardonnay are the only three cultivars authorized to be grown for champagne production; together the black berry cultivars, Pinot Meunier and Pinot noir, represent 74% of the planted vines. Pinot Meunier is a cultivar of ancient origins and has long been considered a periclinal mutant of Pinot noir. It is distinguished from Pinot noir in having tomentose (densely covered with trichomes) shoot tips and expanding leaves[9,10]. All grapevine cultivar propagation is vegetative, and novel phenotypes, like that of Pinot Meunier, arise by somatic mutation.

The apical meristem of the grapevine shoot is organized into two distinct layers designated L1 (outermost) and L2 (ref. 11). Plants have been regenerated from the L1 and L2 cell layers of Pinot Meunier by passage through somatic embryogenesis, and whereas those from the L2 cell layer were phenotypically indistinguishable from Pinot noir, the plants regenerated from the L1 cell layer displayed the tomentose phenotype of Pinot Meunier and were dwarfed[12]. When grown under glasshouse conditions favourable for floral induction, the L1 dwarf plants produced inflorescences and bunches along the length of the shoots (Fig. 1a, c) where the L2 plants (and Pinot Meunier) had a normal phenotype and produced tendrils (Fig. 1b, d). Inflorescences and tendrils in grapevines are derived from meristematic structures called uncommitted primordia (Fig. 1e), which develop from shoot meristems and are found opposite two of every three leaves[13]. Uncommitted primordia formed on actively growing shoots develop into tendrils (Fig. 1b, f), whereas those in latent buds develop into inflorescences. Latent buds are formed during spring and summer and experience a winter dormancy before bud burst and flowering (Fig. 1d). In the L1 dwarf plants this process is not necessary and uncommitted primordia differentiate into inflorescences on actively growing shoots (Fig. 1a, g).

The dwarf stature of the L1 plants was consistent with altered levels of GAs or an altered response to GAs. The application of GAs and inhibitors of GA biosynthesis has been shown to modify grapevine tendril and inflorescence development[14–16]. We concluded, on the basis of the following, that the L1 plants had an altered GA response and that this is associated with a mutated gene similar to the *Arabidopsis* gene *GAI*[7,17], a negative regulator of GA response. First, the L1 plants did not respond when GA was applied, indicating that it was not a GA-deficient dwarf. Second, the L1 mutant accumulated fourfold more GA_1 and 12-fold more GA_4 in leaves than the L2 plant (data not shown). Elevated levels of

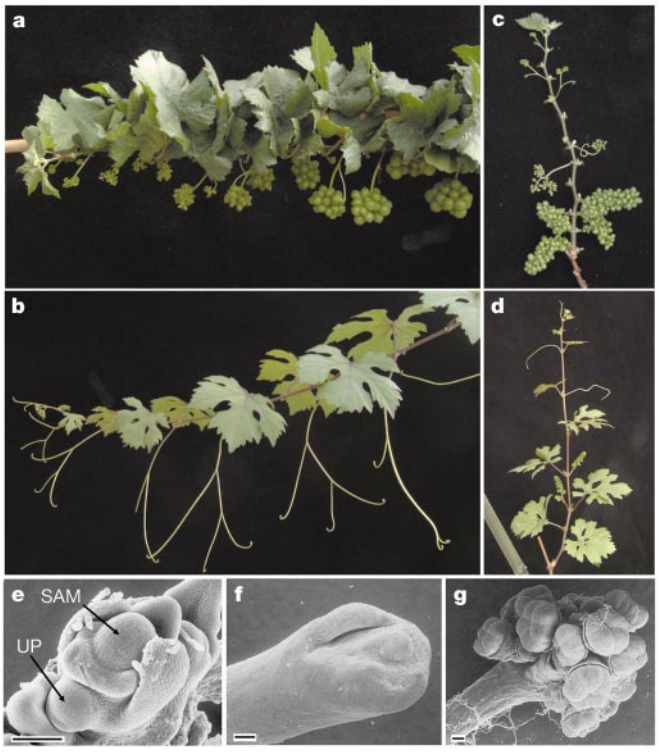

Figure 1 The L1 plant produces inflorescences instead of tendrils. **a**, Main shoot of an L1 plant. **b**, Main shoot of an L2 plant. **c**, Shoot from a latent bud of an L1 plant (leaves removed). **d**, Shoot from a latent bud of an L2 plant. **e**, Scanning electron micrograph of a shoot meristem from a wild-type latent bud showing an uncommitted primordium (UP) that has separated from the shoot apical meristem (SAM). **f**, Scanning electron micrograph of a tendril tip from an L2 plant. **g**, Scanning electron micrograph showing flowers at the tip of a tendril-like structure from an L1 plant. Scale bar in **e**–**g**, 100 μm.

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