Development, plasticity and evolution of butterfly eyespot patterns

Paul M. Brakefield*, Julie Gates†, Dave Keys†, Fanja Kesbeke*, Pieter J. Wijngaarden*, Antónia Monteiro*‡, Vernon French‡ & Sean B. Carroll†

* Institute of Evolutionary and Ecological Sciences, University of Leiden, Kaiserstraat 63, Postbus 9516, 2300 RA Leiden, The Netherlands
† Howard Hughes Medical Institute and Laboratory of Molecular Biology, University of Wisconsin, 1525 Linden Drive, Madison, Wisconsin 53706, USA
‡ Institute of Cell, Animal and Population Biology, University of Edinburgh, Kings Buildings, West Mains Road, Edinburgh EH9 3JT, UK

The developmental and genetic bases for the formation, plasticity and diversity of eyespot patterns in butterflies are examined. Eyespot pattern mutants, regulatory gene expression, and transplants of the eyespot developmental organizer demonstrate that eyespot position, number, size and colour are determined progressively in a developmental pathway largely uncoupled from those regulating other wing-pattern elements and body structures. Species comparisons and selection experiments suggest that the evolution of eyespot patterns can occur rapidly through modulation of different stages of this pathway, and requires only single, or very few, changes in regulatory genes.

ANIMAL colour patterns are often adaptations to different environments and predators, and their diversity represents one important form of morphological evolution[1,2]. A key challenge in evolutionary biology is to understand how phenotypic diversity is generated by the modification of developmental pathways. Butterfly wings are decorated with a great variety of scale colour patterns, which are subject to strong natural selection and are amenable to genetic and developmental study[3]. Extensive analyses of the monophyletic Lepidoptera indicate that roughly 15,000 species wing patterns have evolved within ~100 Myr by modifications to a common set of pattern elements. Dramatic diversity in wing pattern also occurs within some butterfly species, such as the African satyrine *Bicyclus anynana*, which shows phenotypic plasticity whereby individuals of the same genotype develop into alternative phenotypes through hormone-mediated responses to the environment[3–10]. Understanding the regulation and evolution of plasticity may offer general insights into the genetic and developmental bases of morphological evolution[11–14].

One important pattern element on butterfly wings, the eyespot, arises through the establishment of an inductive organizing centre (the focus) at a specific location on the developing wing[15]. Transplantation of the focus can induce surrounding host cells to form an eyespot in an ectopic site[15,16]. The focus is proposed to be a signalling source for a morphogen, the levels of which determine the pigmentation of surrounding cells[3,15]. Recent investigations have indicated that regulatory genes, such as the *Distal-less* homeobox gene, are specifically expressed in the eyespot focus[17].

One advantage for studying the eyespot focus is that the colour patterns are far less constrained than those regulated by other organizers acting earlier in development. Eyespots vary tremendously in number and pattern, reflecting the subtle, varying and strong visual selective pressure under which butterflies live. To understand how these patterns are generated and diverge, we first define the developmental pathway that regulates eyespot formation. We then examine how this developmental pathway is modulated in species bearing different numbers of eyespots, or no eyespots at all. Finally, we analyse in detail the genetic and developmental bases for the phenotypic plasticity of eyespot size in alternative seasonal forms and artificially selected lines of *B. anynana*.

The eyespot developmental pathway

We have performed molecular, genetic and transplantation studies which suggest that eyespot position, number, size and colour are determined progressively in four stages. The first three stages of eyespot formation involve the placement of, and signalling from, the organizing focus, and are accurately reflected by the expression of the *Distal-less* (*Dll*) regulatory gene (Fig. 1*a–c*). An antibody to the *Dll* protein was used to follow the dynamics of *Dll* expression in *B. anynana* during the late larval and early pupal stages, when eyespots are determined. In the imaginal discs of

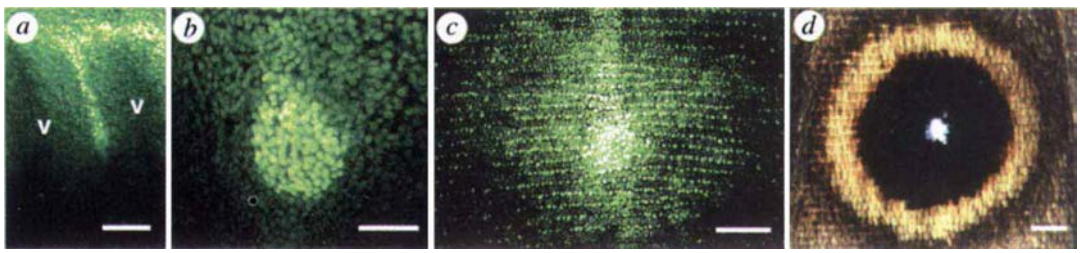

FIG. 1 Expression of Distal-less protein marks the eyespot foci and reveals three stages of eyespot formation. *a–c*, Ontogeny of the *Dll* protein pattern within a single wing subdivision and eyespot field of *B. anynana*. *a*, *Dll* expression in wing disc of mid-fifth instar larvae is highest in the middle of each wing subdivision (V, veins). *b*, By the late fifth instar, the protein accumulates at high levels in the future centre of the eyespot. *c*, By 24 h after pupation, when scale-forming cells (large nuclei) are aligned into rows, *Dll* is expressed in the larger scale-forming cells and the epithelial cells in the centre of the field which will form a large portion of the future eyespot field (*d*). Scale bars: *a*, *b*, 100 μm; *c*, 50 μm; *d*, 0.5 mm.

early fifth-instar larvae, *Dll* is expressed in a broad distal band and at high levels in stripes down the midline of each subdivision (stage I, the larval prepattern; Fig. 1*a*). At the proximal tips of these stripes, the *Dll* pattern begins to enlarge. In each subdivision of the hindwing imaginal disc (but in only two subdivisions of the forewing), the stripes then resolve into stable circular spots of *Dll* expression by the late fifth-instar stage (stage II, focal determination; Figs 1*b*, 2*a*). In the early pupa, strong *Dll* expression expands to encompass a broader circular field of the scale-forming cells, which are now aligned in rows and protrude above the other epithelial cells (Fig. 1*c*). We believe this expanded domain reflects a response to signalling from the epithelial focus, which remains visible at the centre of the field of *Dll*-expressing cells (stage III, focal signalling; Fig. 1*c*). We deduce that the *Dll* spots must include the inductive focus, because grafts containing them induce ectopic eyespots (see Fig. 3 below). The positions of these spots of *Dll* expression correspond to the central regions of the eyespots that form several days later when scale colour differentiation occurs (stage IV, differentiation; Fig. 1*d*). Because *Dll* expression reveals the larval prepattern, accurately marks the position and number of foci, and reflects signalling from the focus, we have used it to analyse eyespot formation in mutant butterflies and different species, morphs and selected lines.

The genetics of eyespot patterns

To better define the stages at which eyespot formation is regulated, and to identify some of the genetic determinants involved, we have begun to isolate and analyse mutants with altered eyespot patterns. Three spontaneous autosomal dominant *B. anynana* mutants (*Cyclops*, *Spotty* and *Bigeye*) selectively affect eyespot position, number and size, and perturb three different stages of eyespot formation.

Heterozygotes for *Cyclops* are dramatically altered in the position and number of eyespots on the hindwing (homozygotes die as embryos). Wild-type hindwings normally bear seven distinct eyespots (Fig. 2*b*), but *Cyclops* heterozygotes have abnormal wing venation and display a range of phenotypes from a loss and/or fusion of one or two eyespots (data not shown) to the formation of one large elliptical eyespot and the loss of the other eyespots (Fig. 2*f*). However, even in severely disrupted hindwings, pattern elements that are proximal or distal to the eyespots are largely unaffected (Fig. 2*f*).

To determine when and how development is changed in the *Cyclops* mutant, we examined *Dll* expression in mutant imaginal discs. The broad distal band of *Dll* expression seems normal, but the larval prepattern (stage I) and the foci (stage II) are severely disrupted. Specifically, regular larval midline stripes of *Dll* expression are not observed (data not shown), and individual focal spots are absent or greatly elongated to four or five times their normal cell number (Fig. 2*e*). The distortion of the *Dll* pattern and adult eyespot shape and venation suggests that the *Cyclops* mutation might involve a gene required for some aspect of patterning along the anteroposterior axis.

Heterozygotes and homozygotes for the *Spotty* mutation[18] display two additional eyespots on the adult forewing (compare Fig. 2*d* and 2*h*), but other elements of the forewing and hindwing pattern appear normal. In forewing imaginal discs of *Spotty* homozygotes, no disruption of the larval prepattern was evident (stage I), but extra *Dll*-expressing focal spots were detected in the larval fifth-instar disc (stage II; data not shown) and in the pupa (Fig. 2*g*), demonstrating that the *Spotty* mutation causes the establishment of specific extra foci during forewing development.

Homozygotes for the *Bigeye* mutation display a dramatic enlargement of eyespot size, with the most pronounced effects on the ventral hindwing (Fig. 2*j*) and ventral forewing. Analysis of *Dll* expression reveals no change in the number or size of the *Dll*-expressing foci in larval (Fig. 2*i*) or early pupal wings (data not shown). Thus the effect of *Bigeye* on eyespot size is likely to be downstream of the establishment of and signalling from the focus (after stage III) and may involve the response to focal signalling.

Positional information and eyespot colour

The above results demonstrate that discrete genes influence eyespot position, number and size, but other determinants must influence the ultimate colour pattern of the eyespots induced by different foci both within and between species. In experiments with a different butterfly species, *Precis coenia*, in which we surgically transplanted small pieces of early pupal wing tissue containing the *Dll*-expressing cells to ectopic sites, the colour pattern of surrounding host cells was found to depend on their proximodistal position.

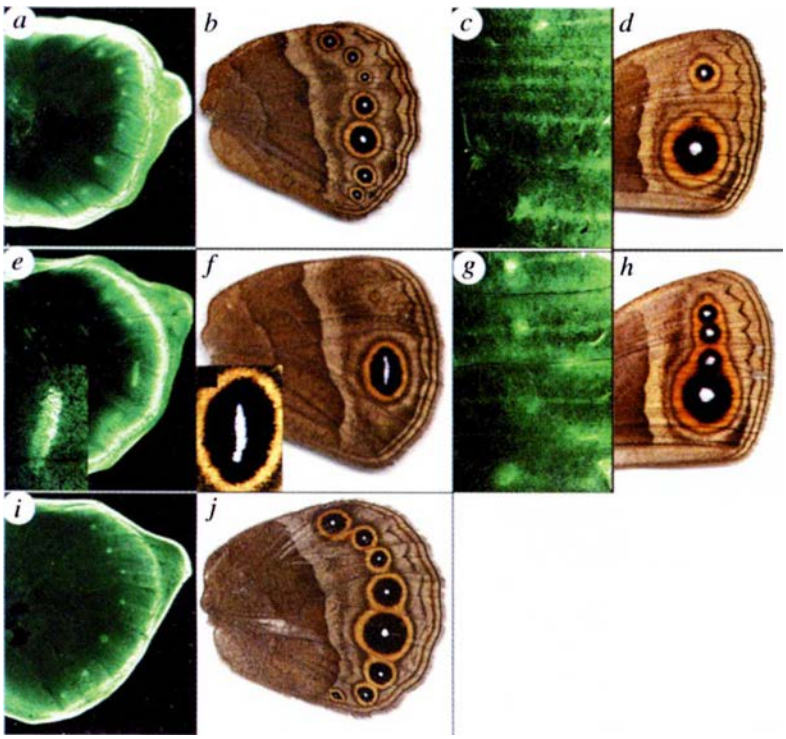

FIG. 2 The *Cyclops*, *Spotty* and *Bigeye* mutants affect eyespot position, number and size at three different stages in the eyespot developmental pathway. *a*, *Dll* expression in a late-fifth instar *B. anynana* ventral hindwing imaginal disc is in a broad distal band and at high levels in seven spots which correspond to the future position of the seven eyespots (*b*) that form on the adult ventral hindwing. *c*, Wild-type *B. anynana* forewing (ventral view) 24 h after pupation. Two *Dll* spots are visible as scale cells form (arrows); these correspond to the future position of the two eyespots that form on the adult forewing (*d*). *e*, *Dll* expression in a *Cyclops* mutant hindwing imaginal disc is in all distall cells, as in the wild type, but short stripes of high-level *Dll* expression arise in place of the circular spots (inset). *f*, The strong *Cyclops* mutant phenotype involves the formation a large elliptical eyespot with an elongated white focus (inset) and the loss of other eyespots (compare with *b*)). The marginal and submarginal bands and the proximal band (which are elements of different patterning systems to the eyespots; see ref. 3) are largely unaffected. *g*, In *Spotty* mutants, two additional *Dll* spots form between the wild-type spots, in the future position of the additional eyespots that form on the adult forewing (*h*). *i*, *Dll* expression in the *Bigeye* mutant wing disc is indistinguishable from the wild-type; *j*, much larger eyespots form on the ventral hindwing of *Bigeye* adults (compare to *b*).

When *P. coenia* focal grafts were transplanted to a nearby distal position (see Fig. 3*a* for location of grafts), an ectopic eyespot was induced that had buff-coloured scales surrounding the graft (Fig. 3*b*; see also ref. 15). By contrast, when a focal graft was transplanted to a more proximal site in the same wing subdivision, a ring of scales coloured bright orange was induced around the graft (Fig. 3*c*). Control non-focal grafts had no effect when transplanted into these positions (data not shown). These results indicate that the colour pattern response to a focal signal depends on the position of the responding cells (see also ref. 16).

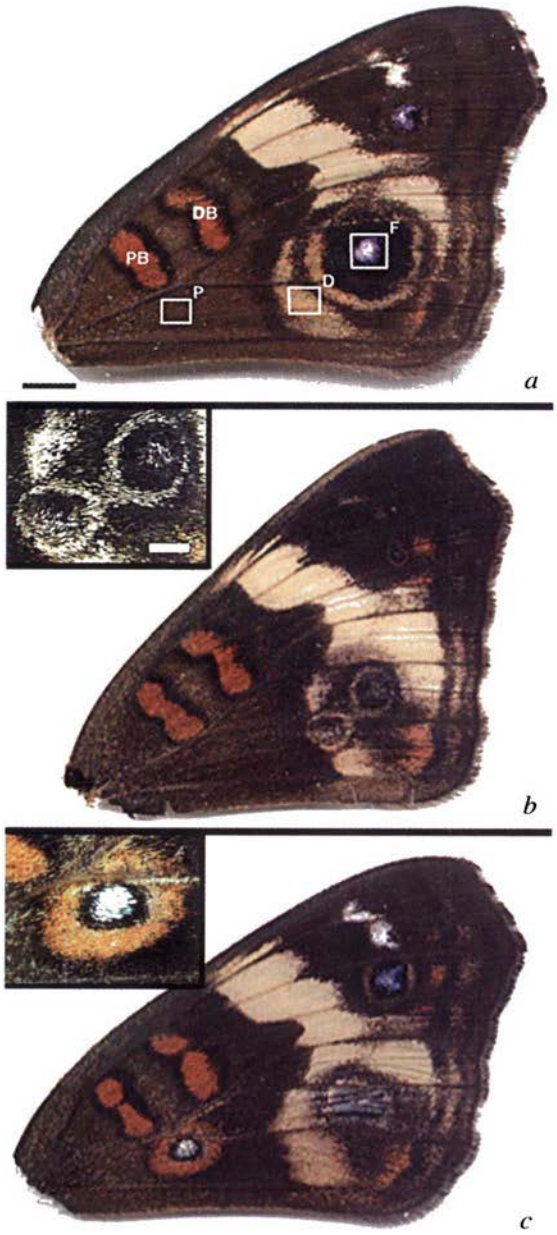

FIG. 3 The colour pattern formed by cells responding to focal signalling depends on their position. *a*, A wild-type adult *P. Coenia* forewing. The relevant pattern elements and wing positions are the posterior eyespot bearing a white focus (F) at its centre, the orange and black bands of the central symmetry system (PB and DB), the proximal site (P) posterior to the PB, and the more distal site (D) adjacent to the eyespot. *b*, *c*, The patterns formed within and around grafts of dorsal wing tissue that were transplanted 3 h after pupation to different sites within the same wing subdivision. *b*, Reciprocal grafts between the F and D sites. Transplantation of the focus to the ectopic site induces circular pigmentation (mostly buff-coloured scales) around the graft. A diminished eyespot still forms at F because the focus was removed after induction was initiated. Inset, detail, c, A graft from the F site to the P site induces a brilliant orange-and-black eyespot. The graft from the P site is not induced because the focus has been removed. Inset, detail.

Different species patterns diverge early

The four stages of the eyespot developmental pathway each represent a potential point of divergence in eyespot patterns between wing subdivisions or wing surfaces within a species or between different butterfly species. We examined the dynamics of *Dll* expression to determine the point in development at which patterns diverge in several unrelated butterfly species, including some with different numbers of eyespots and others without eyespots. In *P. coenia*, a species that has two eyespots on the adult hindwing (Fig. 4*b*) and forewing, stripes of *Dll* expression form on the midline of each wing subdivision, just as in *B. anynana*. However, in *P. coenia* only two stripes give rise to stable foci (Fig. 4*a*) and eyespots, while the other stripes fade away (data not shown) and no foci or eyespots form. Thus the two eyespot-bearing species have very similar prepatterns, but diverge at the focal determination stage (stage II).

By contrast, *Danaus plexippus* (the monarch butterfly) has no adult eyespots (Fig. 4*d*), and no midline stripes or spots are detectable in the imaginal discs (Fig. 4*c*). However, the broad distal band of *Dll* expression is present, as in other butterfly wing discs and *Drosophila* wing discs[17]. These results suggest that, although the broad distal band of *Dll* expression is widely conserved, only species with eyespots display a larval prepattern of midline stripes of *Dll* expression, which is refined into discrete spots within specific wing subdivisions. This indicates that the development of species with eyespots diverges from those without before the larval prepattern forms (before stage I).

Seasonal polyphenism in *B. anynana*

In some butterfly species, large differences in eyespot patterns can occur between genetically identical individuals. *B. anynana* has two seasonal forms (Fig. 5*a*, *b*). The wet-season form has conspicuous ventral eyespots, submarginal 'chevron' markings, and a prominent pale medial band. These pattern elements are greatly reduced or absent in the dry-season form, which is more uniformly

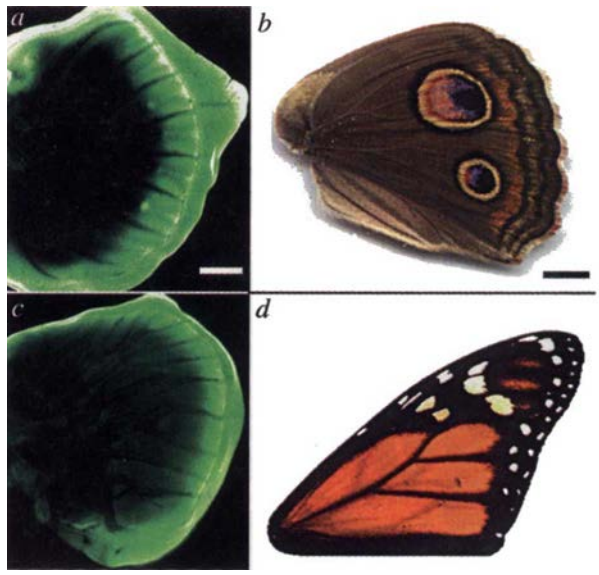

FIG. 4 Only eyespot-bearing species display a prepattern of *Dll* expression. *a*, Fifth-instar *P. coenia* hindwing imaginal disc exhibits two spots of *Dll* expression which correspond to the future position of eyespots on the adult hindwing (*b*). *c*, The fifth instar imaginal disc of *Danaus plexippus* does not exhibit any spots of *Dll* expression, but does express *Dll* in all distal cells, as in other species. *d*, The adult *D. plexippus* wing does not bear eyespots.

brown[4,19]. This plasticity is an adaptive response to seasonal climates in Africa[4]. The dry-season butterflies rest on dead, brown leaf litter and are highly cryptic; any conspicuous markings on the exposed ventral wing surfaces can attract predatory lizards. These butterflies must persist as inactive adults for the duration of the dry season, before egg laying can begin with the rains. In contrast, eyespots are favoured in the highly active butterflies within the green herbage layer of the wet season, because they can deflect attacks by birds or lizards away from the vulnerable body. Survival analyses of marked cohorts of each form in each season have supported this hypothesis (N. Reitsma, G. H. Engelhard and P. M. Brakefield, unpublished data).

In field populations of *B. anynana* butterflies, larvae growing in hot, wet conditions develop into adults of the wet-season form, whereas the cohort of larvae growing in the transition from wet to dry season, when the temperature is declining, develop into the cryptic, dry-season butterflies[19,20]. By rearing animals in controlled conditions in the laboratory, we have demonstrated a clear relationship between rearing temperature and wing phenotype that can be described graphically in the form of a norm of reaction[21] (Fig. 6*a*). Development of the larvae at 23 °C or warmer yields the wet-season form, whereas around 17 °C produces the dry-season form. Laboratory temperature-shift experiments show that the sensitive period is the late larval stage, especially the final instar, when the initial stages of eyespot development are occurring in the wing imaginal disc[22].

Genetic basis of phenotypic plasticity

There is variation within our laboratory population in the expression of phenotypic plasticity. We studied the genetic basis of variation in eyespot size by applying artificial selection on the phenotypically plastic ventral hindwing. LOW and HIGH lines were established by selecting as parents the individuals that were most similar to the dry-season form or the wet-season form, respectively, when reared at intermediate temperatures. Smooth and rapid responses to selection were observed in each line, consistent with estimates of high realized heritability from other selection experiments on eyespots[23,24]. There were dramatic changes in wing pattern across all temperatures after 16 (HIGH) or 20 (LOW) generations (Fig. 6), leading to a divergent pair of lines with well separated 'bundles' of reaction norms for individual families. The LOW (Fig. 5*c*, *d*) and HIGH (Fig. 5*e*, *f*) selected lines now produce butterflies resembling the dry- and the wet-season forms, respectively; thus they have lost the ability to express both seasonal forms. For example, at 17 °C, HIGH-line individuals (Fig. 5*e*) produce eyespots similar to those of unselected individuals reared at 23 °C (Fig. 5*b*; the wet-season form).

To characterize the genetic basis of divergence in eyespot size and, in particular, to estimate the minimum number of genes involved, we analysed the phenotypic variance of offspring of controlled crosses of the LOW and HIGH selected lines. Wright's method[25] relates the difference in the means of two inbred lines to the variance of their F_2 and backcross populations. We used Lande's[26] modifications of Wright's method to estimate that a minimum of five or six genes contribute to the large difference in eyespot size in each sex between the selected lines (Table 1). Because of the assumptions of normal distributions, additive gene action, unlinked loci and equal allelic effects at all loci, such estimates need to be interpreted with caution[27,28], but deviations from the assumptions will usually cause an underestimate of the number of genes[28]. The large sample sizes and the consistency across the estimates based on different combinations of the crosses strengthen our conclusion that the lines differ in a minimum of five or six loci.

Late regulation of phenotypic plasticity

The existence of polyphenic adult colour patterns in the wet-season and dry-season forms of *B. anynana* demonstrates that the eyespot developmental pathway is plastic and under environmental control. The divergent phenotypes of the HIGH and LOW selected lines show that the nature of this plasticity can be changed through selection to yield different genotypes specifying large or small eyespots in response to a common environment. The environmental and genetic control of the differences in eyespot size in *B. anynana* might operate at any stage; for example, focus formation could be delayed in the dry-season form, or the response to focal signalling could be diminished.

To examine how eyespot development differs between the seasonal forms and selected lines of *B. anynana*, we assayed *Dll* expression in the late larval and early pupal stages (stages II–III), because these include the period during which signalling from the focus is initiated[15–17]. Late fifth-instar larvae display no significant divergence in area of focal *Dll* expression between the wet- and dry-season forms of the unselected stock or between HIGH and LOW lines (Fig. 7*a–d*; *P* is not significant by two-way analysis of variance (ANOVA)). This demonstrates that, in terms of the expression of this molecular marker, neither the environmental modulation leading to the divergence of the wet- and dry-season forms, nor the genetic differences between the HIGH and LOW lines, affect the first two stages of eyespot development.

By 24 h after pupation, however, eyespot development has clearly diverged between stock individuals reared to produce the wet- and dry-season adult forms. The areas of *Dll* expression in each focus and in the surrounding scale-forming cells are much

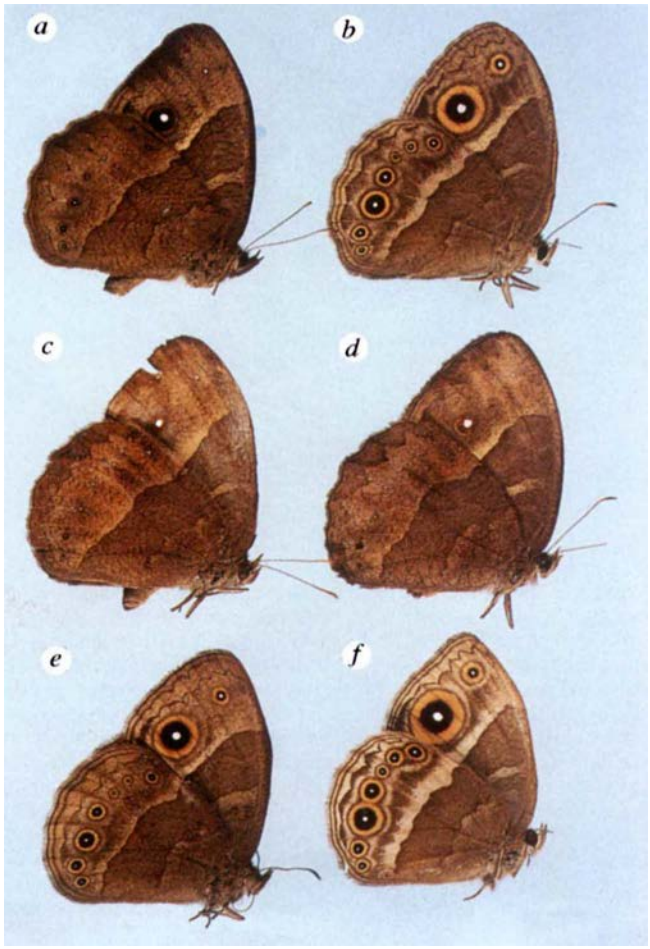

FIG. 5 *B. anynana* butterflies illustrating seasonal polyphenism in wing pattern and the response to artificial selection. All butterflies shown are representative females displaying their right ventral wings (as they would at rest). *a*, *c*, *e*, Reared at 17 °C; *b*, *d*, *f*, reared at 27 °C. Top row, dry-season form (*a*) and wet-season form butterflies (*b*) reared from the unselected stock; middle row, *c* and *d* are both LOW-line butterflies; bottom row, *e* and *f* are both HIGH-line butterflies.

larger in the wet-season form (Fig. 7*e*) than in the dry-season form (Fig. 7*f*; $P < 0.001$ by two-way ANOVA). The environmental rearing conditions must be responsible for this difference in gene expression. Similarly, HIGH-line pupae also express *Dll* in larger foci and greater numbers of surrounding scale-forming cells (Fig. 7*g*) than are seen in the LOW line (Fig. 7*h*; $P < 0.001$ by two-way ANOVA). As these lines are reared in the same environment, genetic differences must underlie these differences in *Dll* expression. Hence, by the time of signalling from the foci (stage III), environmental and genetic influences have clearly begun to affect the course of eyespot development.

The divergence in eyespot size, which is environmentally regulated in the seasonal forms and genetically controlled in the selected lines, has a common developmental basis. For *B. anynana*[29] and other species[9,10,30], the mechanisms that mediate seasonal polyphenism in response to environmental cues have been shown to involve ecdysteroid hormones. The separable environmental and genetic effects on the eyespot developmental pathway indicate that: these mechanisms act to modulate the expression of regulatory genes such as *Dll*; the wild-type population must contain allelic variation for genes which influence this modulation; and selection can change allele frequencies to produce genetically divergent lines which differentially regulate the same developmental pathway and markedly transform the response to an environmental gradient.

Evolution of butterfly wing patterns

Our molecular, genetic and transplantation studies suggest that eyespot pattern is determined progressively and is regulated in four stages (Fig. 8, top). The determination of the position and number of signalling sources (foci) involves the initial deployment in the mid-fifth instar imaginal disc of regulatory genes (such as *Dll*) in a molecular prepattern within each wing subdivision (Fig. 8*a*). In specific subdivisions, the *Dll*-expressing foci are then

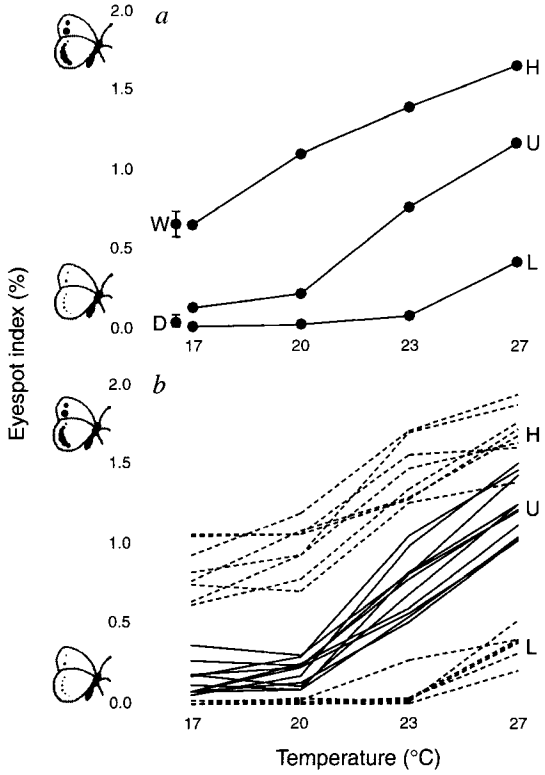

FIG. 6 Response of eyespot size to environmental temperature and artificial selection. *a*, The reaction norm (RN) underlying seasonal polyphenism in *B. anynana* and in the selected lines. Index of eyespot size is the percentage of the area of the hindwing which is covered by the black centre of the largest hindwing eyespot (spot 5). Middle line, the average RN for the unselected stock (U). Top line, RN for HIGH line (H) after selection. Bottom line, RN for LOW line after selection. The maximum value for the standard error for each data point was 0.05%, with all pairwise comparisons across lines or the stock being highly significant ($P < 0.001$). Although the direct target of selection was the size of the fifth eyespot on the ventral hindwing, analysis demonstrated that divergence occurred for all ventral eyespots, the median band and principal components describing overall plasticity in the wing pattern (see also Fig. 5). Results are consistently similar for males and females. Boxed points show eyespot index means for wild-collected butterflies of wet- (W) and dry-season (D) forms, which went through larval development at about 19 °C and 23°, respectively[19,20]. Bars represent 95% confidence intervals. *b*, Sets of RNs for female full siblings from the low-line high-line and unselected stock, as estimated from a split-family experiment. Each RN is based on four subsamples (N is usually >4) from a single family reared at each of the four standard temperatures. Multiple ANOVA of this data shows a highly significant interaction between family and temperature for the unselected stock ($F = 2.86$; d.f. 36, 481; $P < 0.001$). This interaction between genotype and environment has been much reduced in the LOW line ($F = 0.51$; d.f. 21, 110; P is n.s.) and HIGH line ($F = 1.54$; d.f. 21, 206; P is n.s.). Similar results were obtained for males.

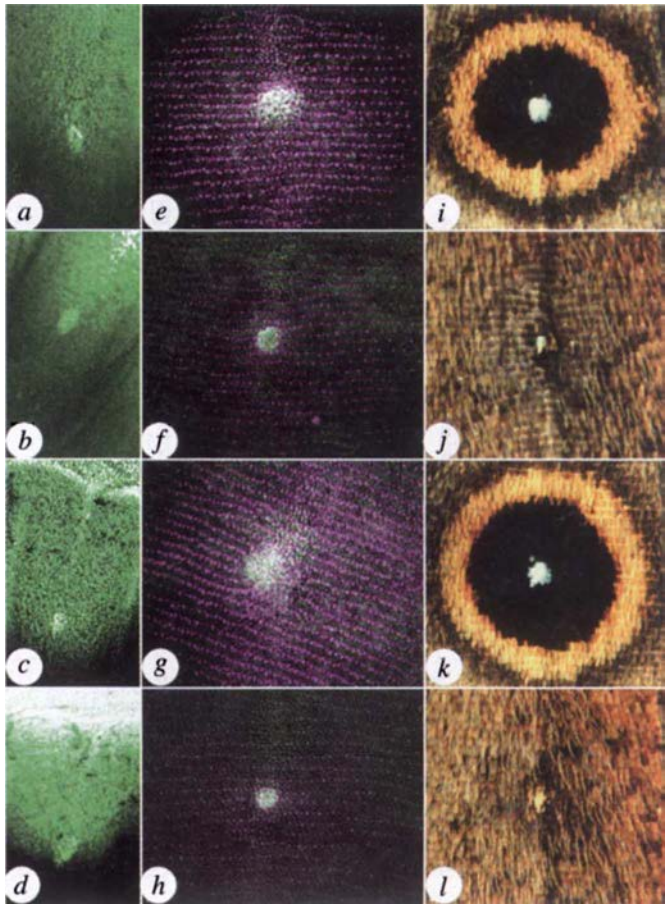

FIG. 7 Divergence in *B. anynana* eyespot development is evident by the time of signalling from the focus. *a–d*, In the late-fifth instar, *Dll* expression in wing disc epithelial cells is predictive of the locations of the eyespot centres on the adult wing. At this stage, in the ventral hindwing (representative fourth wing subdivision shown), the wet-season form (*a*) and dry-season form (*b*) of the unselected stock are similar in both the number and area of cells expressing high levels of Dll. *Dll* expression in the HIGH line (*c*) and LOW line (*d*) is also similar at this stage. *e–h*, By 24 h after pupation, the time of signalling from the focus, the *Dll* expression patterns have clearly diverged. The number and area of *Dll* expressing epithelial cells at the focus, and of scale-forming cells surrounding them, are greater in the wet-season form (e) than in the dry-season form (*f*) of the unselected stock. Similarly, the HIGH line (*g*) displays more *Dll*-expressing epithelial and scale-forming cells then the LOW line (*h*). *i–l*, Representative examples of adult fourth eyespots of the wet-season (*i*) and dry-season (*j*) forms, and the HIGH line (*k*) and LOW line (*l*).

TABLE 1 Estimates of number of genes

	Estimate 1	Estimate 2	Estimate 3	Estimate 4
Males*	6.08 ± 1.13	6.34 ± 1.17	4.81 ± 1.28	9.30 ± 2.62
Males†	9.88 ± 2.78	9.06 ± 2.09	6.41 ± 1.99	15.45 ± 6.91
Females*	10.79 ± 3.47	8.48 ± 1.91	9.74 ± 4.87	7.50 ± 1.80

Estimates of the minimum number of genes regulating the difference in fifth hindwing eyespot size between the LOW and HIGH selected lines are given with their standard errors. The values for each sex were obtained by analysis of different combinations of the parental lines and of crosses between them: estimate 1 includes F_1 hybrids and F_2; 2, includes LOW, HIGH, F_1 and F_2; 3 includes F_2, backcrosses to LOW and to HIGH; 4 includes LOW, HIGH, F_1 and both backcrosses. See Supplementary Information for details of rearing conditions, eyespot measurement and data analysis.

* Untransformed data.

† Use of the best power transformation (0.566).

established (Fig. 8*b*), after which induction from the focus is mediated by signals to surrounding cells (Fig. 8*c*). This could involve one graded morphogen[2,15] or a relay system involving multiple signals acting sequentially across the radius of the eyespot field[16]. Surrounding cells respond by eventually producing different pigmented scale types, according to the level or type of signal they receive (Fig. 8*d*), and their location within the wing (Fig. 3).

The great diversity of butterfly eyespot patterns reflects both the flexibility and modularity of individual stages of this developmental pathway. This flexibility is shown by both the plasticity and the rapid response to selection seen in *B. anynana*, and the pathway's modularity was dramatically illustrated by the specific effects of single-gene mutants. We have demonstrated how differences in the presence or absence (for example, *P. coenia* versus *D. plexippus*), number (*P. coenia* versus *B. anynana*), size (*B. anynana* wet- versus dry-season forms) and colour (*P. coenia* versus *B. anynana*) of eyespots can arise between species or between wing surfaces or subdivisions at discrete stages of this developmental pathway (Fig. 8; compare top and bottom panels at each stage).

The selection experiments on *B. anynana* rapidly produced dramatic differences in eyespot size which were due to genes of small phenotypic effect. The mutants described here show that genes also exist with large phenotypic effects on eyespot development (including size) that have no perceptible effect on other wing or body patterns. It is likely that the evolution of eyespot patterns in nature involves both genes with large and small effects on eyespot development. For example, in a closely related species, *B. safitza*, individuals with four forewing eyespots, very similar to the *B. anynana Spotty* mutants, occur with significant frequency in Malawi (P. M. Brakefield, unpublished data). These observations suggest that the regulation of the eyespot developmental pathway is such that eyespot patterns can evolve rapidly and independently of other wing-pattern elements and body structures. Eyespots respect positional coordinates that are established by a more general regulatory mechanism in that they are always centred in the midline between wing veins (and *Cyclops* demonstrates that disruption of these coordinates can perturb eyespot formation). However, the proximodistal position, number, size and colour of eyespots seems to be largely uncoupled from other patterns and structures, and can evolve rapidly through a relatively small number of changes in regulatory gene interactions.

These results in butterflies may have general implications for the evolution of colour patterns in animals. The diversity of colour patterns in other speciose taxa, such as fish, snakes, birds and various insect orders, may involve analogous developmental pathways that are uncoupled from those that control the formation of body structures. Indeed, single or small numbers of loci control dramatic features of colour pattern in snakes[31,32], land snails[33] and fish[34]. The ability to manipulate eyespot patterns makes butterflies more accessible experimentally, but it will be important to learn how colour patterns in other taxa develop and evolve. □

Methods

Antibody production and immunohistochemistry. Antibodies were raised and affinity purified against the 198 amino-acid N-terminal portion of the *P. coenia* Dll protein expressed in *Escherichia coli*. Imaginal discs were fixed in 0.1 M PIPES, pH 6.9, containing 1 mM EGTA, 2 mM $MgSO_4$, 1% Triton X-100, and 5 mg ml^{-1} bovine serum albumin (BSA) for 1–2 h at 4 °C and incubated with primary antibody for 24–48 h at 4 °C. The discs were then washed four times for 20 min each at 4 °C, incubated for 2 h at 4 °C with fluorescein-conjugated secondary antibody, washed and incubated overnight, then mounted in 0.1 M Tris, pH 8.8, containing 10% glycerol. Images were gathered on an MRC 600 laser scanning confocal microscope.

Focal transplants. All manipulations were performed on 3-hour-old *P. coenia* pupae that were cultivated with a regular light–dark cycle. Landmarks for the foci present on the overlying pigmented pupal cuticle[15] and pupal trachae were used to guide the excision and transplantation of small squares of tissue. The donor grafts were rotated 90° to identify donor from host tissue; this changes the scale orientation on tissue versus the surrounding host scales.

***Bicyclus* husbandry and selection for eyespot size.** The stock was established from more than 80 gravid females collected in 1988 from near Nkhata Bay in Malawi. It is maintained at an adult population size of 600–800 with some overlap of generations. Effective population size is one half of census number. All *Bicyclus* individuals were reared under a 12 h:12 h light:dark cycle. Wet-season forms of the stock were reared at 27 °C, dry-season forms at 17 °C.

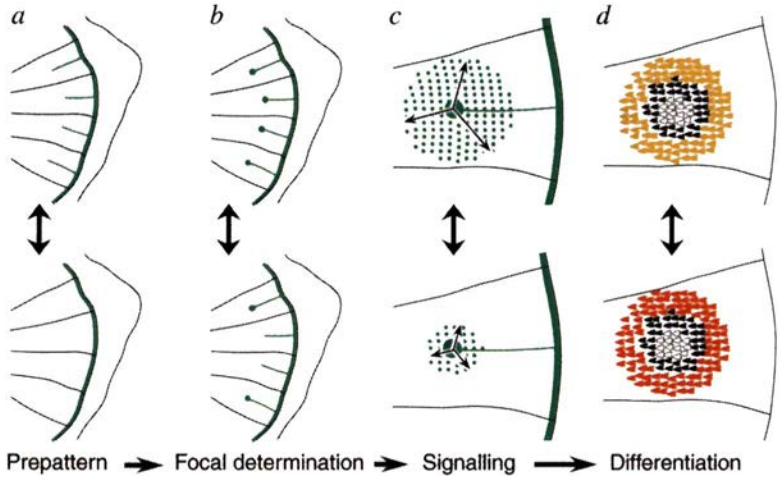

FIG. 8 Regulation of eyespot formation and diversity. Eyespot formation and diversity is regulated in four stages. Note the progressive specification of the eyespot pattern and the differences that arise in this developmental pathway between species (contrast top and bottom rows). *a*, In the larva, a prepattern of gene activity creates the potential focal pattern reflected by early *Dll* expression, which occurs only in eyespot-bearing species. *b*, Foci are determined and *Dll* expression is stabilized only in specific wing subdivisions; the number of foci differs between wing surfaces and butterfly species. *c*, In the pupa, signalling from the focus (green circle) induces surrounding cells; different sized eyespots are controlled by the size of the focus. *d*, Induced cells later differentiate into scales of different colours depending on their distance from the focus and their position in the wing.

LOW and HIGH lines were raised at 20 °C except during selection. Truncation selection was applied in each line after measurement of wing length and eyespot diameter. Selection for the first ten generations was applied to butterflies reared at 20 °C. Because of a reduction in phenotypic variation, selection was then continued by rearing the LOW line at 23 °C for ten generations, and the HIGH line at 18 °C for six generations. We measured 300–400 butterflies of each sex in each generation. At least 40 females were used as selected parents in each line after being held in a mating cage with about 100 of the most extreme males. Reaction norms were estimated using replicated subsamples of eggs reared at the four temperatures (other conditions were maintained as standard). Wing characters were measured with high repeatability using an image analysis system.

Crosses to estimate gene number. The black diameter of the fifth eyespot (corrected for wing length) was measured with a binocular microscope fitted with a micrometer. All reciprocal crosses were made and reared separately. Crosses with pooled sample sizes in parentheses (male:female) were: parental LOW (123:113) and HIGH (105:137); F_1 (206:209); F_2 (302:284); backcrosses to LOW (516:444), and to HIGH (607:581). Several crosses in females show departures from normality which are resistant to data transformation. Two crosses in males also depart from normality, but application of Taylor's power law reduces this to a single cross, the F_2.

For further experimental methods and statistical analyses, see Supplementary Information.

Received 29 August; accepted 21 October 1996.

1. Cott, H. B. *Adaptive Coloration in Animals* (Methuen, London, 1940).
2. Endler, J. A. *Natural Selection in the Wild* (Princeton Univ. Press, 1986).
3. Nijhout, H. F. *The Development and Evolution of Butterfly Wing Patterns* (Smithsonian Inst. Press, Washington, 1991).
4. Brakefield, P. M. & Larsen, T. B. *Biol. J. Linn. Soc.* **22,** 1–12 (1984).
5. Brakefield, P. M. & Reitsma, N. *Ecol. Entomol.* **16,** 291–303 (1991).
6. Shapiro, A. M. *Evol. Biol.* **9,** 259–333 (1976).
7. Kingsolver, J. G. *Evolution* **49,** 932–941 (1995).
8. Kingsolver, J. G. *Evolution* **49,** 942–954 (1995).
9. Rountree, D. B. & Nijhout, H. F. *J. Insect Physiol.* **41,** 987–992 (1995).
10. Rountree, D. B. & Nijhout, H. F. *J. Insect Physiol.* **41,** 1141–1145 (1995).
11. Stearns, S. C. *Bioscience* **39,** 436–445 (1989).
12. Via, S. *et al. Trends Ecol. Evol.* **10,** 212–217 (1995).
13. Schlichting, C. D. & Pigliucci, M. *Evol. Ecol.* **9,** 154–168 (1995).
14. Pigliucci, M. *Trends Ecol. Evol.* **11,** 168–173 (1996).
15. Nijhout, H. F. *Dev. Biol.* **80,** 276–274 (1980).
16. French, V. & Brakefield, P. M. *Dev. Biol.* **168,** 112–123 (1995).
17. Carroll, S. B. *et al. Sciences* **265,** 109–114 (1994).
18. Brakefield, P. M. & French, V. *Acta Biotheor.* **41,** 447–468 (1993).
19. Windig, J. J.. Brakefield, P. M., Reitsma, N. & Wilson, J. G. M. *Ecol. Entomol.* **19,** 285–298 (1994).
20. Brakefield, P. M. & Mazzotta, V. *J. Evol. Biol.* **8,** 559–573 (1995).
21. van Noordwijk, A. J. *Bioscience* **39,** 453–458 (1989).
22. Kooi, R. E., Brakefield, P. M. & Schlatman, E. G. M. *Proc. Exp. Appl. Entomol.* **5,** 47–52 (1994).
23. Holloway, G. J., Brakefield, P. M. & Kofman, S. *Heredity* **70,** 179–186 (1992).
24. Monteiro, A. F., Brakefield, P. M. & French, V. *Evolution* **48,** 1147–1157 (1994).
25. Wright, S. *Evolution and the Genetics of Populations* Vol. 1, *Genetics and Biometrical Foundations* (Univ. Chicago Press, 1968).
26. Lande, R. *Genetics* **99,** 541–553 (1981).
27. Cockerham, C. C. *Genetics* **114,** 659–664 (1986).
28. Zeng, Z. -B., Houle, D. & Cockerham, C. C. *Genetics* **126,** 235–247 (1990).
29. Koch, P. B., Brakefield, P. M. & Kesbeke, F. *J. Insect Physiol.* **42,** 223–230 (1996).
30. Nijhout, H. F. *Insect Hormones* (Princeton Univ. Press, 1994).
31. Zweifel, R. G. *J. Heredity* **72,** 238–244 (1981).
32. King, R. B. *Can. J. Zool.* **71,** 1985–1990 (1981).
33. Sheppard, P. M. *Natural Selection and Heredity* (Harper, New York, 1960).
34. Endler, J. A. *Evol. Biol.* **11,** 319–364 (1978).

SUPPLEMENTARY INFORMATION is available on *Nature*'s World-Wide Web site (http://www.nature.com) or as paper copy from Mary Sheehan at the London editorial office of *Nature*.

ACKNOWLEDGEMENTS. We thank E. Schlatmann and B. de Winter for supplying maize for butterfly husbandry; H. J. Tanke and colleagues for access to their confocal microscope; A. Sebring for the recombinant *Dll* protein; S. Paddock, L. Olds and A. t'Hooft for help with the fitures; J. Wilson for preparation of the manuscript; D. Lewis, G. Panganiban, J. Selegue, M. Grbic, J. Fallon, R. ffrench-Constant and S. Paddock for comments on the manuscript; and L. Brakefield for nomenclature. This work was suported by the IEES (Leiden) (P.M.B.), a grant from the National Science Foundation (S.B.C.), and the Howard Hughes Medical Institute (S.B.C.).

CORRESPONDENCE and requests for butterfly materials should be addressed to P.M.B. (e-mail: brakefield@rulsfb.leidenuniv.nl), and requests for molecular reagents should be addressed to S.B.C. (e-mail: sbcarrol@facstaff.wisc.edu).