Short Review

# The evolution–development interface and advances with the eyespot patterns of *Bicyclus* butterflies

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Studies of the evolution of diversity in colour pattern or morphology have seldom included any analysis of the developmental processes involved in translating genetic variation into the phenotypes influenced by natural selection. This gap is being filled by research on eyespot patterns in the butterfly *Bicyclus anynana*. This review discusses how data on the developmental genetics of eyespot traits can help in the description and analysis of evolutionary constraints.

**Keywords:** butterfly, development, evolution, eyespot pattern, heritability, mutants.

## Introduction

Studies of animal colour patterns have provided some of the most successful analyses of the evolution of diversity. However, there are very few examples in which each level of organization from gene through developmental pathway to phenotype, function and fitness have received attention. While for many case studies the genetical basis of the phenotypic variation and the influence of natural selection are well documented (Endler, 1986), little or nothing is usually known about how genes regulate development to produce divergence in adaptive design. This review will show how this gap is beginning to be filled by studies on butterfly wing patterns which have built on Fred Nijhout's classic experiments on the development of an eyespot pattern in *Precis coenia* (Nijhout, 1980).

We can fill the gap between genes and phenotypes because of the particular features of butterfly eyespots. They are elements of functional design which show great diversity and their development can be studied using manipulative experiments and molecular approaches (Nijhout, 1991; Carroll *et al*., 1994; French & Brakefield, 1995). We are also able to genetically probe the eyespot developmental pathway in the African butterfly, *Bicyclus anynana*, enabling a fully integrated approach to the study of diversity in colour pattern (Brakefield & French, 1993; Brakefield *et al*., 1996).

## Genetics and natural selection on butterfly wing patterns

Butterfly wing patterns offer a spectacular example of diversity which has long fascinated biologists. Most of the 12000 or so species can be distinguished on the basis of their colour patterns. Striking variation is also found within some species which exhibit genetic polymorphism or seasonal polyphenism. Industrial melanism in the peppered moth *Biston betularia* provides one of the most thoroughly researched examples of evolution by natural selection (reviewed in Brakefield, 1987). The polymorphism for black, melanic forms and the pale, nonmelanic wild type is specified by an allelic series at the *carbonaria* gene. However, even in this particularly well known example of evolutionary change, we do not know how the biochemical process of melanization is regulated by the *carbonaria* gene.

Genes controlling mimetic colour patterns in species of *Heliconius* and *Papilio* butterfly are well documented (e.g. Clarke & Sheppard, 1960, 1971, 1972; Sheppard *et al*., 1985; Jiggins & McMillan, 1997). Studies across hybrid zones between the geographical races of *Heliconius* have also examined how birds can exert stabilizing selection on their colour patterns (Mallet & Barton, 1989).

Eyespot patterns on butterfly wings can function in various ways. Large, conspicuous eyespots often co-occur with patches of bright colour. They may be exposed only when a resting individual is disturbed by a potential predator. Such exposure is frequently associated with a ritualized display (Blest, 1957). Other eyespots are positioned close to the edge of the wings and are exposed continuously in feeding or resting individuals. Such patterns can act as deflection devices which tend to direct any attacks on the individual away from the vulnerable body (Wourms & Wasserman, 1985). In other species, eyespot patterns may be fully integrated within a camouflaged wing pattern (Brakefield, 1984; Brakefield & Larsen, 1984; Brakefield & Reitsma, 1991).

There have been extensive studies of the ecological genetics of variation in the eyespot pattern of the butterfly *Maniola jurtina* (Brakefield, 1984, 1990). Some estimates of heritability have also been obtained for eyespot traits in *M. jurtina* (Brakefield, 1984; Brakefield & van Noordwijk, 1985). The

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developmental studies of Nijhout (1978, 1980) on *P. coenia* showed that each eyespot is formed around a developmental organiser known as a focus. Models about how particular genes may specify development of mimetic colour patterns in species *Heliconius* or *Papilio* (Nijhout & Wray 1988; Nijhout *et al*., 1990; Nijhout, 1991, 1994) are based broadly on the same principles as underlie eyespot patterns but as yet lack empirical support. It will be exciting if future work can provide experimental confirmation of such models but at present robust data on developmental processes only exist for eyespot patterns in *Precis* and *Bicyclus* butterflies (Nijhout, 1980; French & Brakefield, 1995; Brakefield *et al*., 1996).

## The developmental pathway of eyespot patterns

We have used a combination of genetical, developmental and molecular studies to analyse eyespot development in *B. anynana*. Butterfly wings develop in the larva as paired internal epidermal pouches, the imaginal discs. There are two discs on each side, that protrude at metamorphosis to form the immobile pupal wings, the forewing lying above the hindwing. The effect of experimental manipulations on one wing can be examined relative to the opposing, control wing. The wings are strengthened by tracheae or veins, most of which run proximal to distal. Each area bounded by wing veins is referred to as a wing cell. A wing has dorsal and ventral surfaces, each with a layer of pigmented scale cells. Shortly after pupation the layer of cells which will form the dorsal surface of the forewing is attached to the overlying pupal cuticle enabling transplantation or grafting experiments

(Nijhout, 1980). Furthermore, the position of each wing cell and of the putative centres of forewing eyespots are visible as a sort of map on the outer surface of the pupal wing case.

The colour patterns of butterfly wings result from the 2-D matrix of scale cells arranged like tiles on a roof. Each scale cell contains a single colour pigment. Comparative analyses of the wings of butterflies have shown that their pattern elements - bands, eyespots or chevrons - form a system of homologies known as the nymphalid groundplan (see Nijhout, 1991, 1994). According to this groundplan, the pattern elements are arranged in parallel columns running anterior to posterior over each wing. One such column consists of a series of eyespots, each of which is made up of concentric rings of scale cells with different colour pigments.

The wild type of *B. anynana* has a series of eyespots towards the wing margins, each of which has a central white pupil, an inner black disc and an outer gold ring (Fig. 1a). The forewing has a small anterior eyespot and a large posterior eyespot. The hindwing has a full series of seven or eight ventral eyespots which also have a characteristic pattern of relative size (Fig. 1d). The L–R pairs of eyespots in *Bicyclus* and other butterflies represent serial developmental homologues with each eyespot based on a common developmental mechanism (Monteiro *et al*., 1994).

The critical phases in eyespot formation occur in the late larva and early pupa (Nijhout, 1980; French & Brakefield, 1992, 1995; Brakefield *et al*., 1996). Initially, a prepattern is established before pupation in the growing wing discs. This includes the location of potential eyespot 'organizers' known as foci in each wing cell. Shortly before pupation, this prepat-
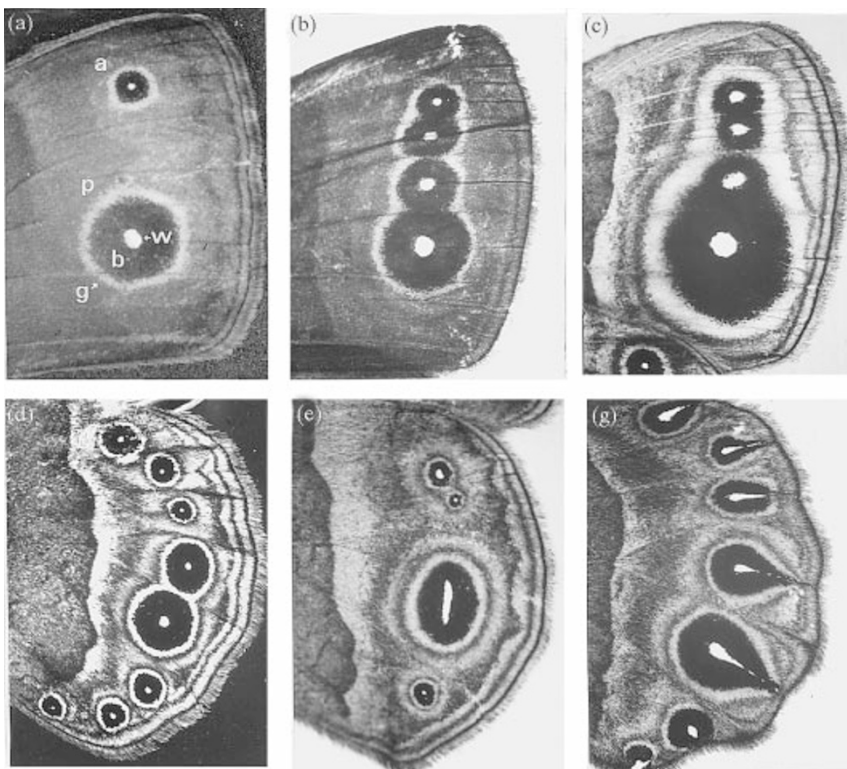


**Fig. 1** Single gene effects on the wing pattern of *Bicyclus anynana*. Top row shows the forewing, and bottom row, the hindwing. The left hand column with dark background shows the wild type wing pattern of the ventral hindwing (d) and dorsal forewing (a): a, anterior eyespot; p, posterior eyespot with g, outer gold ring; b, inner black disc and w, central white pupil. The four panels with light background are all mutant phenotypes: (b) *Spotty* (dorsal); (c) *Bigeye* and *Spotty* double mutant (ventral); (e) *Cyclops* (ventral; the central two eyespots of the wildtype have coalesced); (f) *comet* (ventral).

tern becomes a series of specified foci corresponding to the centres of each adult eyespot. According to the simple and plausible model of Nijhout (1978, 1980, 1991), this is followed after pupation, by each focus inducing a signal to surrounding cells, apparently by diffusion of an unstable chemical morphogen to form a radial concentration gradient. The surrounding cells — the scale cells to be — then interpret this signal by 'reading' its concentration to gain positional information and become differentiated. However, the synthesis of the colour pigments in the scale cells does not occur until some time later, just before adult eclosion (the pupal stage lasts about one week in *Bicyclus* at our standard rearing temperature).

Strong support for this developmental pathway and Nijhout's original model comes from ectopic eyespots produced by grafting the focal cells of dorsal forewing eyespots shortly after pupation to different positions on a developing wing (Nijhout, 1980; French & Brakefield, 1995). Furthermore, damage of focal cells in the early pupae by piercing the pupal cuticle with a finely pointed needle ('sham-cautery') leads to smaller eyespots on the adult wing (Nijhout, 1980; French & Brakefield, 1992; Brakefield & French, 1995). Additional evidence comes from more recent work on single gene mutants, selected lines and different species using a molecular probe for the protein product of the developmental gene *Distal-less* (Carroll *et al.*, 1994; Brakefield *et al.*, 1996). There is a close matching between the eyespot phenotypes on the adult wing, especially the distribution and shape of their white pupils, and the expression of the *Distal-less* gene product in cells of the presumed foci in imaginal discs dissected from late larvae or early pupae. *Distal-less* protein acts as a marker for the organising focus of each eyespot.

To date we cannot distinguish between Nijhout's 'source' model for an organizing focus and an alternative involving the focus acting as a local sink for a morphogen present at high concentration throughout the wing epidermis (see French & Brakefield, 1992). Further research is needed to directly confirm other aspects of the signal-response model, especially the involvement of a long-distance morphogen gradient. Nijhout & Paulsen (1997) show that mathematical models based on how genes might influence different parameters of this developmental model may help to understand the potential effects of interactions among the different components.

### Genetic variation and eyespot development

We have used a dual experimental approach to analyse the developmental genetics of variation in the eyespot pattern using an outcrossed laboratory stock of *B. anynana*. First, in the course of rearing very large numbers of insects, four spontaneous single gene mutants with major effects on wing pattern have been isolated, and their modes of inheritance determined. We have begun to examine how these genes may perturb the different stages of eyespot formation (Brakefield *et al.*, 1996). Secondly, we have used artificial selection to produce stocks with different eyespot phenotypes and then examined how the developmental pathway has diverged

(Monteiro *et al.*, 1994, 1997a,b,c). For particular eyespot traits (e.g. size or shape), the responses to selection provide estimates of the realized heritability. Direct responses to selection are usually rapid and progressive indicating high levels of additive genetic variance and the influence of numerous genes of small phenotypic effect. Some inferences about how the differences in allele frequency between upward- and downward-selected lines regulate the developmental pathway have been made by application of sham-cautery or grafting experiments to the selected lines. This has enabled us to move one step further than comparable artificial selection experiments on morphological traits of *Drosophila* wings (Scharloo, 1990). While some of these latter experiments have led to plausible interpretations of the change in phenotype in terms of deformations of information gradients across the wing blade, they lack direct experimental support.

Figure 1 illustrates the four spontaneous mutants of *B. anynana*. *Spotty* is a semidominant mutation which leads to additional foci, and thus extra eyespots, arising on each surface of the forewing (Fig. 1b). This has been revealed by both grafting experiments and examination of the *Distal-less* expression pattern (Brakefield & French, 1993; Brakefield *et al.*, 1996). *Cyclops* is dominant with homozygous lethality. Although there is some variability in expression, the most extreme phenotype has a single large, ellipsoidal eyespot with an elongated white pupil on each ventral wing surface (Fig. 1e). In this mutant, the pattern of *Distal-less* expression clearly reveals that the prepattern and establishment of the foci is highly perturbed. This is probably due to an early disruption of the venation pattern which leads to some coalescence of two adjacent wing cells on each wing within which the large, modified eyespot is formed (Brakefield *et al.*, 1996). In the dominant mutant, *Bigeye*, the eyespots, especially the large ventral forewing eyespot, are enlarged in size (Fig. 1c). *Distal-less* expression in the eyespot foci does not differ between this mutant and the wild-type. This strongly supports an influence of the *Bigeye* gene on aspects of the response to the focal signal rather than the signal itself (Brakefield *et al.*, 1996). Finally, the previously undescribed recessive mutant, *comet*, produces dramatically pear-shaped eyespots, each component being elongated towards the wing margins (Fig. 1f).

Figure 2 illustrates phenotypes produced by artificial selection on different features of the posterior eyespot of the dorsal forewing. Selection usually produced lines which were completely divergent for the eyespot trait within five to 10 generations. Our first pair of lines were for eyespot size: low and high lines with small and large eyespots, respectively (Monteiro *et al.*, 1994). The realized heritability was close to 50 per cent. Grafting of the eyespot foci between pairs of individual pupae from the same or different line showed that additive genetic variance in eyespot size exists for both the response and, in particular, the signaling components of the developmental pathway. Thus, foci from high-line butterflies consistently produce larger ectopic eyespots than those from low-line donors but high-line foci transplanted into high-line hosts tend to produce larger ectopics than those grafted into
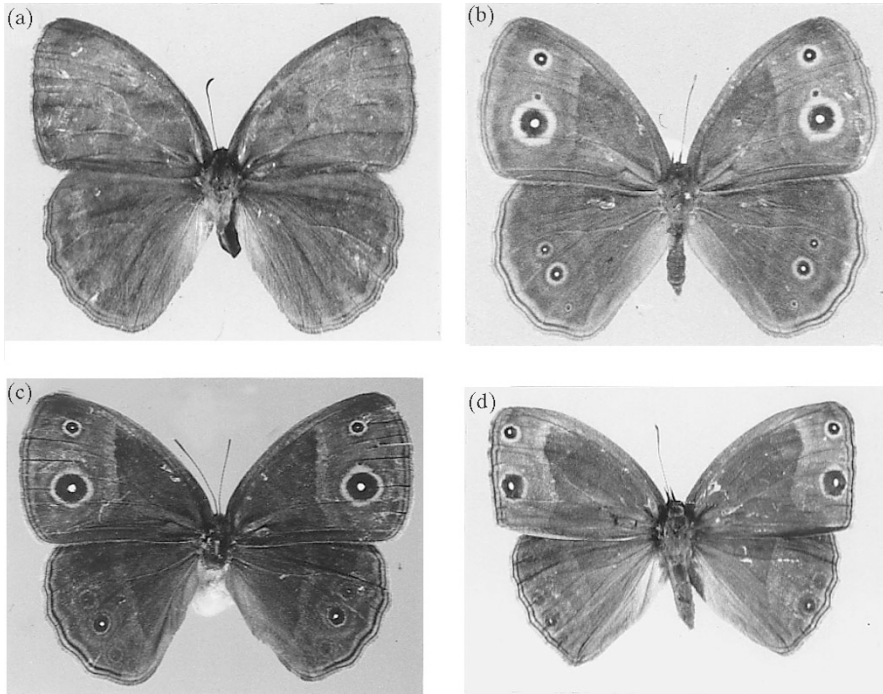
**Fig. 2** Butterflies illustrating the responses to artificial selection on eyespot size, colour, shape and position for the dorsal wing pattern of *Bicyclus anynana*. Figure 1a shows the forewing pattern of the unselected stock. (a) a female with no eyespots; (b) a female with large outer gold rings; (c) a female with eyespots flattened in the proximal-distal axis; (d) a female with eyespots in a more distal position. See text for further details.

low-line hosts. More prolonged selection of the low line has yielded butterflies without any dorsal eyespots (Fig. 2a).

A similarly rapid response with only slightly lower heritabilities was obtained for eyespot colour composition when 'gold' (Fig. 2b) and 'black' lines with large gold or black rings were selected, (Monteiro *et al.*, 1997a). In contrast to the size lines, grafting experiments showed that the divergence in phenotype between the gold and black lines could be completely accounted for by genes influencing responses to the focal signals; the colour of ectopic eyespots depended only on the type of host. There was no evidence of any variation in the relative size of the colour rings within an eyespot due to genes influencing the activity of the focal signal: a focus, irrespective of the donor line, always produced an ectopic eyespot typical of the host tissue. In contrast to both size and colour, there is substantially less additive genetic variance for eyespot shape, either in the anterior-posterior or proximo-distal axis (Fig. 2c). The limited response to selection is, like that for colour, not due to any change in focal activity. However, eyespot shape was correlated not only with localized aspects of response in the wing cell, but also with changes in scale cell distribution and the overall shape of the wing (Monteiro *et al.*, 1997b,c). Finally, selection in a single line on the position of the eyespots has yielded individuals with eyespots aligned along the extreme distal edge of the wing (Fig. 2d; selection was by eye on the ventral hindwing eyespots).

We have thus performed direct artificial selection on each eyespot trait except number. However, the lines selected for large eyespots also show an increased frequency of small, additional eyespots in wing cells where they are normally absent, suggesting substantial additive genetic variance for eyespot number (Holloway *et al.*, 1993; Monteiro *et al.*, 1994).

This represents an interesting difference with the gene *Bigeye* which does not appear to influence the number of spots (P.M. Brakefield, unpublished data). This can be accounted for by different underlying changes in the developmental pathway. As revealed by the pattern of *Distal-less* expression, *Bigeye* probably does not influence the determination of eyespot foci; only where foci are present do larger eyespots result. The additional eyespots specified by the *Spotty* allele are larger in double homozygotes of *Bigeye* and *Spotty* (see Fig. 1).

These observations for both the single genes of major effect and the selected lines are summarized in Table 1. Our initial results show that in terms of quantitative genetics there is moderate or high additive genetic variance for each eyespot trait, except shape. However, one of our single gene mutants, *comet*, has dramatic effects on eyespot shape. The *comet* allele probably regulates the prepattern or specification of the eyespot foci themselves. *Cyclops* can also be considered to influence eyespot shape, again by effects early in the developmental pathway (as revealed by *Distal-less* expression). In contrast, the variation which led to the limited response of shape to artificial selection was restricted to genes influencing the response to the signals induced by eyespot foci (or to less-localised effects). Eyespot shape is probably more highly conserved within *Bicyclus* than other traits (Monteiro *et al.*, 1997c). Perhaps there are indeed comparatively few options in terms of genetics and development for any marked changes in eyespot shape. Alternatively, eyespot shape may be conserved because circular eyespots are favoured functionally and there is stronger stabilizing selection.

There is an interesting contrast between eyespot size and colour composition. While additive genetic variances are

**Table 1** A simplifying summary of the known genetical and developmental basis of eyespot traits in *Bicyclus anynana*. 'Signal' and 'response' are broad terms indicating evidence for trackable effects up to or after the period of establishment of the focal signals, respectively. See text for further details.?, indicates an interpretation made without robust data.

| Eyespot trait | Heritability | Dev. effect | Single gene | Dev. effect |
|---|---|---|---|---|
| Size | high | signal (+response) | *Bigeye* | response |
| Colour | high | response | no data | |
| Shape | low | response | *comet* & *Cyclops* | signal |
| Number | ? high | ? signal | *Spotty* | signal |
| Position | high | ? signal | (*Cyclops*) | signal .... |

similar, the effects on the developmental pathway appear to be very different. Thus the additive genetic variance influencing eyespot size in our stock appears to involve primarily genes that influence the strength of the eyespot foci. There are, however, also some genes which produce their phenotypic effect by changing responses to the focal signals. For eyespot colour composition, the complete response to selection can be accounted for by the genes with the latter mode of developmental action.

Selection for a more distal position of the eyespots has produced a remarkable phenotype extremely divergent from anything present in the outcrossed stock (Figs 2 and 4). Although quantitative data are not yet available, there are several differences apparent between this response to selection and those for the other three eyespot traits. First, the response was probably not a smooth one and phenotypes close to the wild type still appear in the selected line suggesting that genes of major effect may be segregating in the line. Secondly, the response is at least in part due to eroding of the edge of the wing (this is also visible in the pupal stage). Thirdly, the expression is extremely temperature sensitive; rearing material at a low temperature produces only butterflies with the unselected phenotype. The last two points indicate genetic effects comparable to those of *Notch* in *Drosophila*.

## Predictions and future directions

The series of eyespots in *Bicyclus* can be considered as a single module within which each eyespot is based on a common developmental mechanism (Monteiro *et al*., 1994; see Wagner, 1996). While Nijhout (1991, 1994; see also Paulsen & Nijhout, 1993; Paulsen, 1994, 1996) has emphasized the contribution made by the developmental and genetical independence of the different series of pattern elements within the nymphalid groundplan to the spectacular diversity of butterfly wing patterns, evolution of a particular trait within a single one of these series may be more constrained.

Although we have not quantified the responses very carefully, it appears that with the exception of eyespot size and number there are low genetic covariances across eyespot traits in *B. anynana*. For example, selection on colour composition has very little or no effect on eyespot size (Monteiro

*et al*., 1997a; note also that *Spotty* adds two eyespots with no effect on the size of other eyespots). This suggests that in a similar manner to the proposed independence among series of pattern elements, the different eyespot traits may also be rather free to follow their own evolutionary trajectories.

There are about 80 species of *Bicyclus* in Africa (Condamin, 1973). Future progress will enable the insights about genetical and developmental processes in *B. anynana* to be mapped onto patterns of morphological diversity and phylogenetic relationships across species (see Brakefield & French, 1993; Roskam & Brakefield, 1996; Monteiro *et al*., 1997a). At the same time, we will continue our work on the developmental genetics in *B. anynana* concentrating on further genetic probing of the developmental pathway and on an analysis of potential evolutionary constraints within the eyespot module. The latter approach will focus on the extent to which individual eyespots or subsets of eyespots are free to follow independent evolutionary paths when a particular trait, such as size or colour, is considered.

The eyespots of *B. anynana* are characterized by a conserved pattern in relative size; the anterior forewing eyespot is always much smaller than the posterior eyespot (Figs 1 and 2). On any one wing surface, the eyespots also show similar colour-composition, shape and proximo-distal position. Furthermore, responses to artificial selection targeted on a single eyespot are characterised by strongly positive correlated responses for the same trait in other eyespots, especially those on the same wing surface (see Holloway *et al*., 1993; Monteiro *et al*., 1994, 1997a; see also the 'gold' specimen in Fig. 2b).

Single genes which influence only a subset of the eyespots may be critical in decomposing or changing the pattern of modularity within the eyespot serial homologues (cf. Wagner, 1996). *Spotty* appears to provide an example of a gene which affects only a subset of the eyespots (Fig. 1). Genes with this type of effect may be of evolutionary importance in their potential to facilitate novel patterns which would be very difficult or slow to occur under selection on quantitative trait loci of small phenotypic effect. Such genes may thus be instrumental in enabling a decoupling among the eyespots, conferring some decomposition of the eyespot module or at least a shift in its state. The importance of such a mechanism involving single genes of strong phenotypic effect relative to

changes to the pattern of genetic covariances through selection needs experimental testing.

We will use artificial selection experiments to explore this issue for both eyespot size and colour. Figure 3 illustrates planned experiments to compare responses to selection on the same trait in different directions for the pair of forewing eyespots. The highly positive correlated responses to artificial selection observed when only the posterior eyespot is the direct target of selection show that responses to natural selection in favour of concerted evolution of these eyespots will be rapid and unconstrained (the rising diagonal axis in Fig. 3). In contrast, we predict that responses to selection favouring opposing directions for the same trait in different eyespots

### (a) Eyespot size



### (b) Eyespot colour



**Fig. 3** Diagram of proposed artificial selection experiments on *B. anynana* to examine responses in different directions within phenotypic space for two different eyespot traits. Solid lines illustrate responses in directions for which at least partial responses have already been obtained. Dashed lines indicate responses in other directions which are predicted to be more difficult to obtain.

will be much slower and more constrained, at least where short-term adaptive responses are required. They may indeed need the facilitation of 'decoupling' genes although we do not know to what extent the latter role can be filled by genes of relatively small phenotypic effect and whose occurrence is reflected in departures of genetic correlations among eyespots from unity.
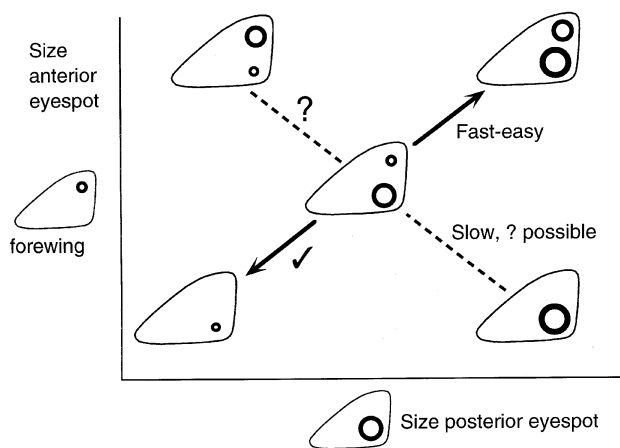
As Nijhout (1994) points out, we also do not yet know how phenotypic change maps onto any measure of change in development. Do the single genes of major phenotypic effect (Fig. 1) reflect a more fundamental or a different mode of change in the developmental pathway than the genes involved in the responses of an eyespot trait to artificial selection (Fig. 2)?

The results of selection on spot position are especially illustrative of several of these issues (Fig. 4). Selection has produced a concerted change in which all eyespots are more distal in position (Fig. 4b). This phenotype is remarkably similar to that of a species group typified by *B. buea* (Fig. 4d) which occurs in the tropical forests of West Africa. Whether this similarity is matched by any underlying homology in genetical and developmental mechanisms is unknown. In contrast, the wing pattern of the species group within which *B. ignobilis* occurs may exemplify the involvement of single genes of the 'decoupling' type which lead to a very disjointed positioning of the series of hindwing eyespots in which particular pairs are shifted distally (Fig. 4c).
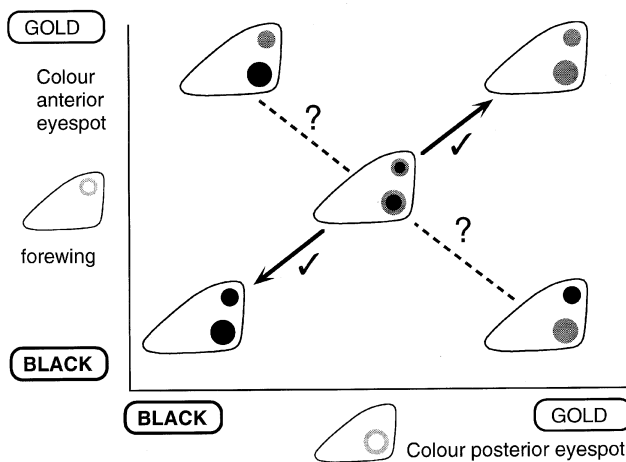
In other examples of changes in pattern it is clear that a given type of pattern shift can be produced in very different ways with respect to both genetical and developmental mechanisms (Table 1). This is exemplified by the larger eyespots of both the *Bigeye* mutant phenotype and the upward selected line for eyespot size (although the former may have smaller white pupils). Such examples beg the question of whether predictions are possible about which type of change will actually be involved in a particular evolutionary change leading to eyespot divergence in these butterflies. For the two selection experiments depicted in Fig. 3 we predict that the responses will be slower or more limited for colour-composition since the developmental options for independent evolution of this trait among eyespots are probably more limited and require localized changes over the wing blade in the responses to focal signals. We know, however, that species of butterfly exist with extremely divergent eyespots in adjoining wing cells (Nijhout, 1991; plate 4A). Such marked divergence in the colour composition of eyespots on the same wing surface may only be possible through the involvement of one or more 'decoupling' genes which yield discrete shifts in phenotype. A challenge for the future will be to determine the validity of such predictions and also to examine them in the context of phylogenetic and functional aspects of the eyespot diversity.

This review has shown the potential of studies of how genes regulate the eyespot developmental pathway in *Bicyclus* butterflies to lead to more precise predictions about the evolvability of the eyespot pattern than a description of genetic variances alone. It has shown how an understanding
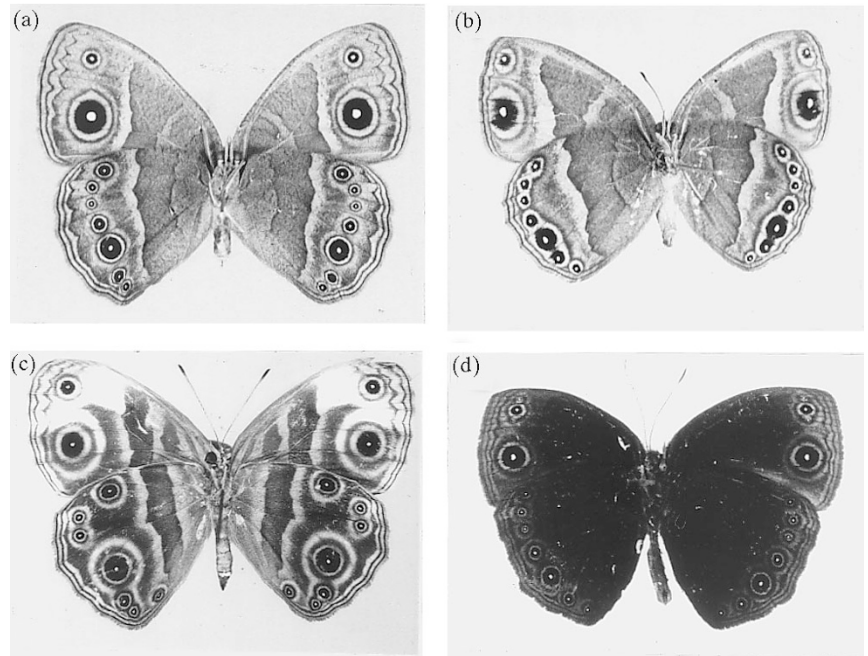
**Fig. 4** *Bicyclus* butterflies illustrating divergence in the positioning of the ventral eyespots. (a) *B. anynana* wild type; (b) *B. anynana* from the line selected for more distal eyespots; (c) *B. ignobilis*; (d) *B. buea*.

of the development of a particular trait, as well as its genetics, may provide a better basis for predicting biases in, or constraints on evolutionary trajectories.

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