The Genetics and Development of an Eyespot Pattern in the Butterfly *Bicyclus anynana*: Response to Selection for Eyespot Shape

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ABSTRACT

The normally circular eyespots on the wing of the butterfly *Bicyclus anynana* were selected to become elliptical in two divergent lines, with antero-posterior elongation of the eyespots in one line and proximo-distal elongation in the other. Selection was continued for nine generations, and symmetrical realized heritabilities of ~15% were achieved initially. The elliptical eyespot shapes characteristic of each line were still produced when the signaling center of the eyespot (the focus) was surgically rotated by 90 or 180° or when an eyespot was induced ectopically by localized damage. We conclude that selection changed general properties of the epidermis that responds to signals emanating from the eyespot focus but did not affect the mechanism of focal signaling.

CELLS in the butterfly wing epidermis differentiate to produce the diverse and spectacular arrangements of colored scales that characterize each wing surface of each species. The underlying developmental mechanisms are not fully understood. Experimental work on wing patterns has mainly concentrated on the specification of the simple eyespot pattern (see NIJHOUT 1980, 1985; FRENCH and BRAKEFIELD 1992, 1995; MONTEIRO *et al.* 1994; BRAKEFIELD and FRENCH 1995). Only by studying the developmental mechanisms involved in the translation of genotype to phenotype can we have a complete understanding of evolutionary change, morphological diversity and its associated limitations and constraints. In previous work, we have examined quantitative genetic variation of the developmental system to understand which of its components controls the *size* and *color composition* of an eyespot pattern (MONTEIRO *et al.* 1994; MONTEIRO *et al.* 1997a). Here we investigate the underlying developmental mechanism controlling eyespot *shape.*

The pigments that make up an eyespot are deposited in precise spatial relation to a central reference point, or "focus", midway between the wing veins. During development, the focus provides "positional information" to the surrounding cells that determines the nature of the differentiation they will undergo and their subsequent production of a specific pigment (NIJHOUT 1978, 1980). Hence removal of the focus at early pupal stage can eliminate the eyespot, whereas grafting it to a different position results in the surrounding host cells responding to the focal signal and forming an ectopic eyespot (NIJHOUT 1980; FRENCH and BRAKEFIELD 1992, 1995; MONTEIRO *et al.* 1994). The focus may signal by producing a diffusible morphogen that forms a gradient in the surrounding epidermis (NIJHOUT 1980, 1991). Progressively lower morphogen concentrations would occur in concentric rings of cells around the focus, and these cells would respond to produce and deposit, later in development, different pigments, forming the concentric eyespot of colored scales. Also, at a particular stage, the epidermis of the pupal wing may respond to local damage (piercing with a fine needle) by producing an ectopic eyespot with the characteristic color rings (see NIJHOUT 1985; MONTEIRO *et al.* 1994; BRAKEFIELD and FRENCH 1995).

The experiments described here are concerned with the genetic and developmental aspects of eyespot formation on the wings of a nymphalid butterfly, *Bicyclus anynana.* Selection experiments were used to estimate genetic variance for shape of the large posterior eyespot on the dorsal forewing. Correlated responses to selection, in the shape of the smaller anterior eyespot on the same wing surface, were also examined. After selection for elliptical eyespots, focal rotation-grafts and wing damage experiments were performed on pupae of the divergent lines to investigate whether the selection produced radial asymmetry in the focal signaling or in the epidermal response component of the developmental process that specifies the (normally circular) eyespot.

MATERIALS AND METHODS

The experimental animals: *B. anynana* were reared at 28°, 12D:12L, 80–90% RH (further details in HOLLOWAY *et al.* 1993). Eggs were obtained from a laboratory stock established in 1988 from ~80 gravid females from Nkhata Bay in Malawi. The stock has been maintained at high (>300) population levels ever since.

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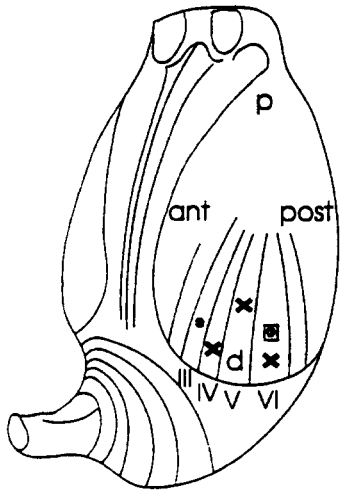

FIGURE 1.—The grafting and damage operation sites on the pupal left forewing. The rotated graft tissue is indicated by a square, including the focus (●) of the large posterior eyespot. Crosses mark the positions of wing damage. Spaces between veins are labeled II-V, and damage sites are designated from anterior to posterior IVd, Vp and VId. ant, anterior; post, posterior; p, proximal; d, distal.

The selection on eyespot shape: In each generation, virgin butterflies were selected on the basis of the shape of the large posterior eyespot on the dorsal surface of the forewing. Using a stereo microscope fitted with an ocular micrometer, eyespot total diameters were measured along two perpendicular axes, crossing the central white pupil: along the wing cell midline (parallel to the wing veins) and orthogonal to it. Parents for the next generation were selected on the basis of the ratio of eyespot diameter along the midline to diameter across the midline. A fat (large ratio—elongated proximal-distally) and a thin (small ratio—elongated anterior-posteriorly) line were selected for nine generations, starting from a single large population from the stock. Truncation selection was applied in both sexes. For the first two generations, 40 females and 80–100 males with the most extreme phenotypes were selected within each line from a total of ~900 (P) or 350 (F_1) individuals. The selection pressure was increased for the remaining generations by reducing the number of females to 25 and that of males to 60–80 (chosen from a total of 300–600 individuals). For the ninth generation, 40 selected females from each line were allowed to lay eggs, and all progeny were measured in an image analysis system, together with 100 butterflies of each sex of the stock population. Measurements of the small anterior as well as the large posterior eyespot were made, to estimate correlated responses to selection. Realized heritabilities were estimated at generation three and eight by regressing all previous generation means against the cumulative selection differential, averaged between the sexes (see FALCONER 1989).

Grafting of a focus: For the surgical experiments, pupae from the fat and thin lines were used, after eight generations of selection. Pupation times were recorded every half hour. Pupae were operated 3–4.5 hours after pupation, when the epidermis of the dorsal surface of the forewing is still attached to the pupal cuticle, which is sufficiently hardened to permit cutting and manipulation. A square piece of cuticle and epidermis was cut around the focus of the posterior eyespot of the left forewing, lifted with fine forceps, rotated either 90 or 180° and lowered back in place (Figure 1). The operated pupae were returned to 28°C and, after emergence, the butterflies were killed by freezing. The sex was scored and the large operated eyespot was measured in the two diameters: along and across the wing cell midline.

Damage experiments: The left forewing of each operated pupa was damaged twice at different time periods. First, the anterior and posterior eyespot foci were pierced with a fine tungsten needle at 6 hr after pupation, to reduce the size of the normal eyespots (see FRENCH and BRAKEFIELD 1992). The pupa was returned to 28°C and then pierced again, either at 12 or at 18 hr, to induce eyespots at two or three nonfocal sites (see Figure 1). Emerged butterflies were frozen and their ectopic patterns measured along and across the wing. All measurements were done blind, with no knowledge of selection line.

RESULTS

The change in eyespot shape: For each selection line and each sex, the white pupil, black disc and gold ring had a different shape within the same eyespot: the outer gold ring was always the "fattest" (with the highest shape ratio; see Table 1), while the white pupil was

TABLE 1

Shape of the posterior and anterior eyespots in fat and thin selected lines after nine generations of selection and in the stock population

	Males					Females					
	Posterior			Anterior			Posterior			Anterior	
Line	Total	Black	White	Total	Black	*n*	Total	Black	White	Total	Black
Fat (n = 103)	1.15	1.10	0.97	1.18	1.12	121	1.11	1.07	0.97	1.18	1.11
Stock (n = 100)	1.04	1.01	0.90	1.12	1.07	100	1.01	0.97	0.94	1.10	1.03
Thin (n = 92)	1.00	0.94	0.86	1.12	1.05	146	0.95	0.91	0.85	1.10	1.05
F	194.7	173.0	15.8	18.9	17.3		328.2	276.2	33.1	61.6	25.1
P	***	***	***	***	***		***	***	***	***	***
Pair-wise comparison	All*	All*	All*	T = S	T = S		All*	All*	F = S	T = S	T = S

Mean values are given of the ratio of proximal-distal to anterior-posterior diameters for the white pupil (White), black disc (Black) and outer gold ring (Total) of the eyespots. *** $P < 0.001$; F and P values are from a one-way ANOVA between the ratios of the three groups (fat, thin and stock). Pair-wise comparisons were done as two sample t-tests. *Significance levels ($P < 0.05$) were corrected for number of comparisons (T, thin; S, stock; F, fat).

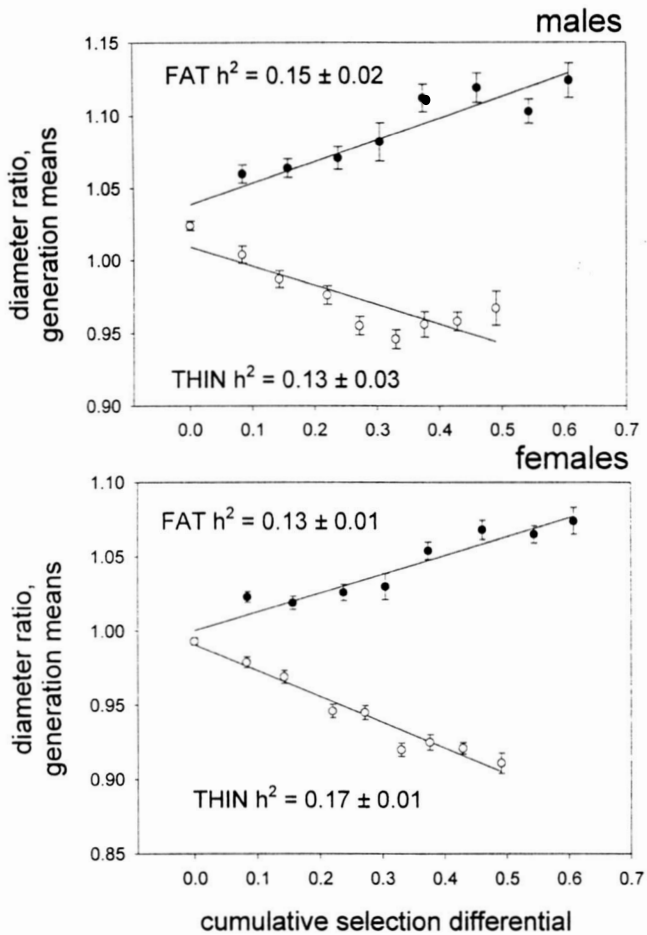

FIGURE 2.—The change in eyespot shape (shown as mean diameter ratio) of fat and thin lines over eight generations of selection. Estimates of realized heritabilities (h^2 ± standard error) were calculated by the slope of the regression line of all generation means on cumulative selection differential. The confidence interval around each generation mean is ±2 standard errors.

always comparatively "thin" in shape. The shape of these components in the selected large posterior eyespot, however, changed to a similar extent in the selected lines relative to stock butterflies (Figure 3a; Table 1). A greater shape change (from stock eyespots) was achieved in the fat line than in the thin line, probably partly due to the larger selection differential applied to fat line butterflies (21% greater; see Figure 2). The anterior eyespot diverged in shape only in the fat line, where it became "fatter" in both the outer gold ring and the black disc. thin anterior eyespots in each sex retained the same shape as stock butterflies.

Realized heritabilities: The two selection lines progressively changed in eyespot shape, over eight generations of selection (Figure 2). The slopes of the regression of shape (mean ratio of diameters) against cumulative selection differential gave estimated realized heritabilities which ranged between 13 and 17% (see Figure 2). Realized heritabilities calculated up until the third generation (with the first four points on the *x*-axis) gave slightly higher estimates (fat males: 0.22 ±0.02; thin males: 0.19 ± 0.06; fat females: 0.12 ± 0.06; thin females: 0.21 ± 0.02). The decrease in heritability, especially in the males, with the continuation of selection was partly due to a plateau being reached from the fifth generation onward, *i.e.*, there was no further divergence between the lines. The eyespots of males (but not females) in both lines appeared to have reached the limit of their circular distortion.

The grafting experiments: The grafting experiment tested for an asymmetry in the focal signaling component of eyespot development in the selected lines. If eyespot shape reflects differences in anterior-posterior *vs.* proximal-distal signaling, it should be changed by a 90°, but not by a 180°, rotation of the focus. Of the 234 operated pupae (from the two lines, with a 90 or 180° rotation), a total of 153 produced adults where the graft had healed, forming appropriately rotated white scales, and was surrounded by a large scorable eyespot pattern (Figure 3b). A general linear model (GLM) analysis was carried out on the data for the ratio of eyespot diameters and three factors were included: selection line (two

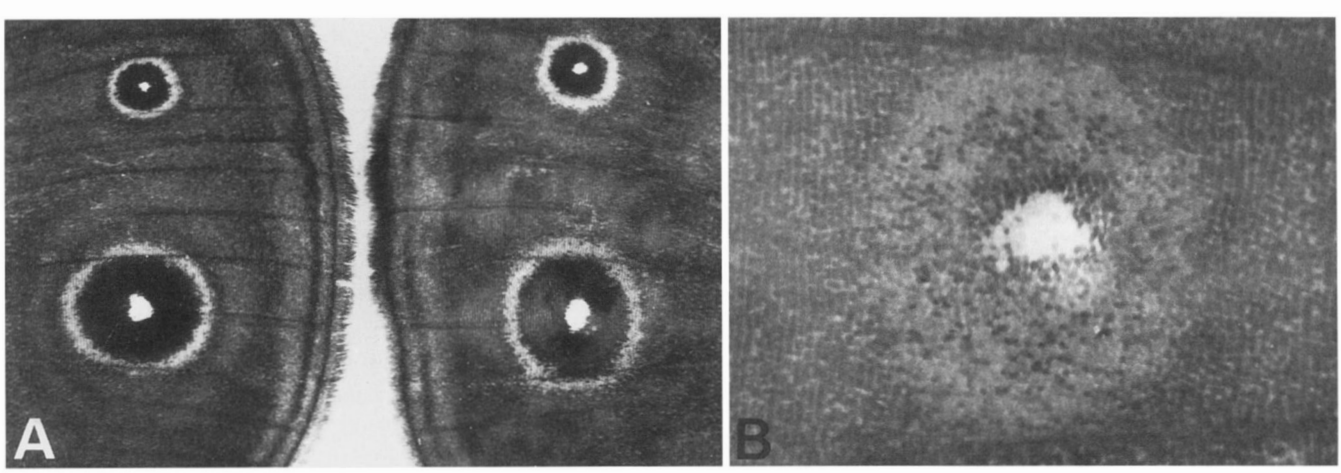

FIGURE 3.—Selected eyespots and focal graft rotation. (a) A fat (left) and a thin wing showing the small anterior and the large selected posterior eyespot. (b) A typical result from a 90° focal rotation on a pupa from the thin line: the resulting adult eyespot is none-the-less "thin" in shape.

TABLE 2

General linear model analysis on eyespot shape after focal rotation with three factors: selection line, rotation of the graft and sex

Source	d.f.	F	P
Line[a]	1	91.39	0.000***
Rotation[b]	1	0.41	0.523
Sex	1	0.09	0.766
Line*rotation	1	0.97	0.327
Line*sex	1	1.61	0.207
Rotation*sex	1	1.21	0.273
Line*rotation*sex	1	2.32	0.130
Error	144		
Total	151		

*** $P < 0.001$.
[a] Fat and thin.
[b] 90 and 180°.

levels: fat and thin), rotation of the graft (two levels: 90 and 180°), and sex. The data were first transformed by raising all values to the power of −0.095 (Taylor's power law, see FRY 1993) to obtain homogeneous variances in the eight groups (Bartlett's test: $\chi^2 = 13.9$, d.f. = 7, P = NS). Analysis showed that no significant part of the eyespot shape variation was explained by graft rotation or sex and there were no significant interaction effects (Table 2). This means that for a butterfly of either sex, focal orientation does not influence the shape of the final eyespot (Figure 4). Selection line was the only significant factor: butterflies from the thin line will have a "thin" eyespot whereas animals from the fat line will have a "fat" eyespot (see Figure 3b).

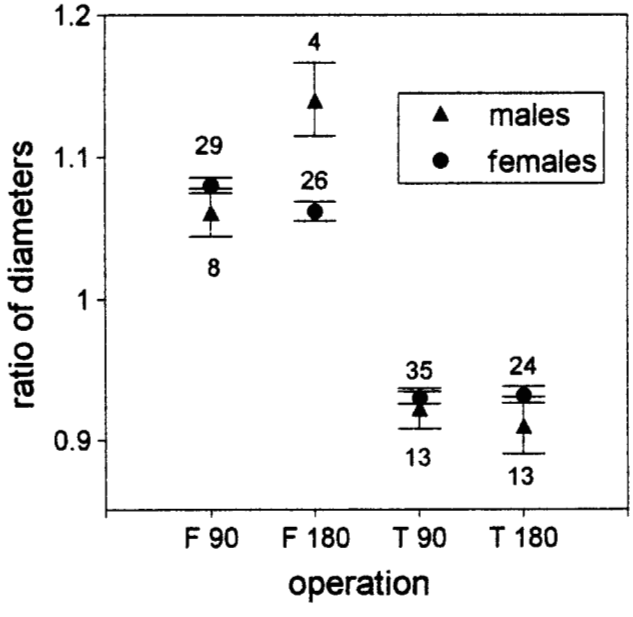

FIGURE 4.—Shape of eyespots from the fat (F) and thin (T) lines after focal rotation by either 90 or 180°. Means with asymmetrical 95% confidence intervals for the ratios in the nontransformed scale. The means have been adjusted by the GLM analysis (Table 2) to account for unbalanced data. Numbers of scorable animals are shown in brackets.

TABLE 3

General linear model analysis on shape of ectopic patterns with three factors: selection line, site of operation and time of operation

Source	d.f.	F	P
Line[a]	1	8.51	0.004**
Site[b]	2	12.14	0.000***
Hour[c]	1	16.04	0.000***
Line*site	2	0.10	0.909
Line*hour	1	0.56	0.454
Site*hour	2	12.25	0.000***
Line*site*hour	2	0.17	0.847
Error	262		
Total	273		

Data based on the ratio of total diameters of all ectopic patterns. ** $P < 0.01$; *** $P < 0.001$.
[a] Fat and thin.
[b] IVd, Vp, and VId.
[c] 12 and 18 hr.

The damage experiments: These experiments tested whether fat and thin lines differed in the shape of eyespots induced by piercing the pupal wing epidermis. As in previous experiments (BRAKEFIELD and FRENCH 1995), such damage at 12–18 hr after pupation induced ectopic patterns consisting of scattered gold scales (not analyzed further), gold patches (GP), gold and black patches (GBP) or ectopic eyespots (EE) with a black disc and outer gold ring. GP, GBP and EE were measured in their total diameters, along and across the wing cell midline. EEs were also measured in their black disc diameter along the same two axes. To test whether the shape of ectopic patterns differed between the fat and thin lines, a GLM analysis was done on the ratio of total diameters of these ectopic patterns. Three factors were included in the analysis: line (with two levels: fat and thin), site (three levels: Vp, IVd and VId; see Figure 1) and hour of cautery (two levels: 12 and 18 hr). Sex was initially included as one of the factors but, due to two empty cells (the males were missing in both the fat and thin line for the IVd site and the 12-hr operation), the full GLM design with interactions could not be calculated. The GLM performed without the interaction terms showed that sex did not account for a significant difference between the ratios, and it was thus removed from the subsequent analysis. The 12 remaining groups of data showed homogeneous variances (Bartlett's test: $\chi^2 = 15.3$, d.f. = 11, P = NS).

The shape of ectopic patterns differed significantly between the lines, the sites and the times of operation (Table 3), with a significant interaction term between time and site of operation. There was a consistent difference between selected lines: fat butterflies produced "fatter" ectopic patterns than thin butterflies at the three sites and the two times of operation (Figure 5, a and b). Also, patterns occurring at the proximal site (Vp) were "fatter" than those at the distal sites, and patterns induced at 12 hr were "thinner" than those

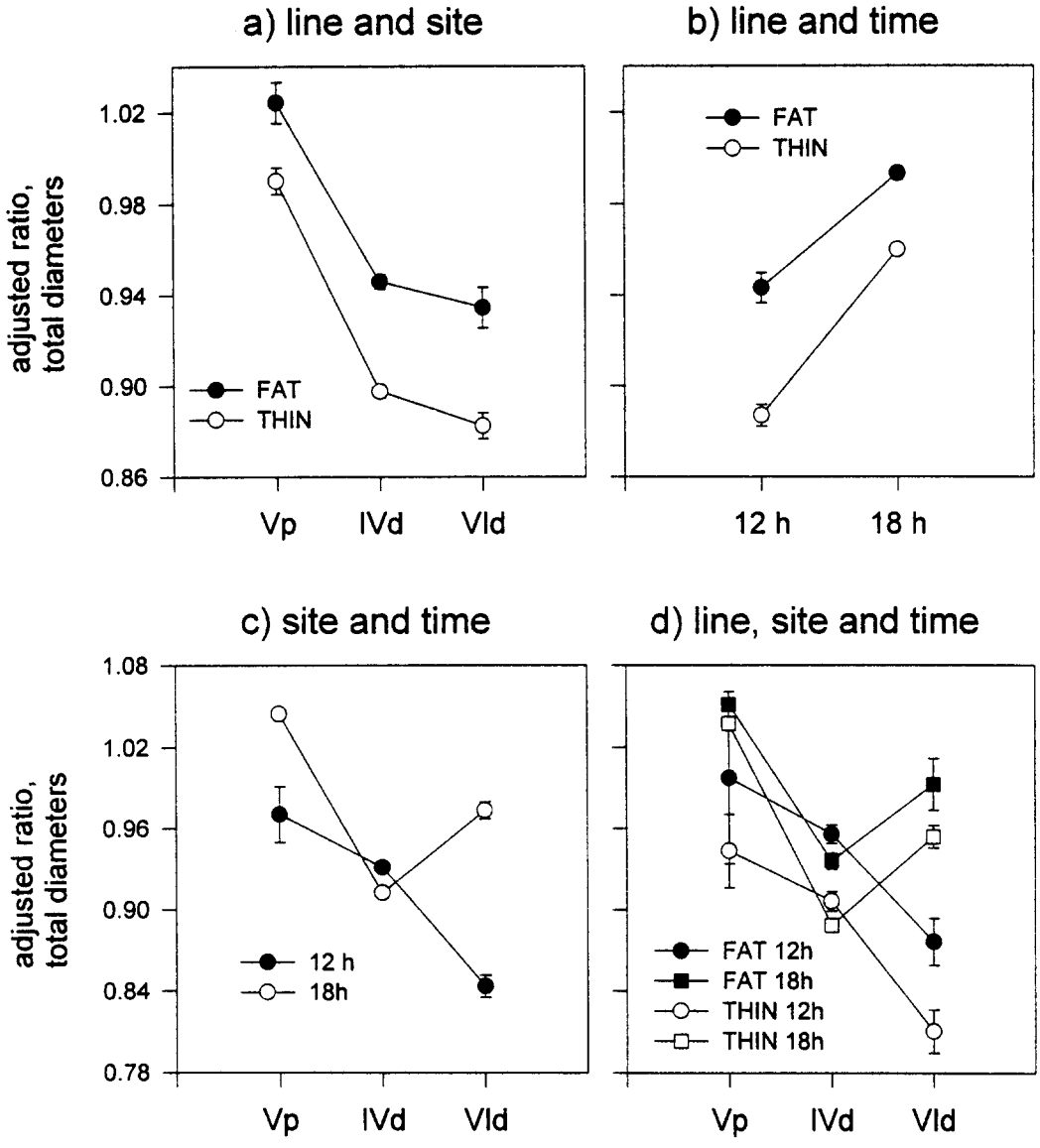

FIGURE 5.—Graphic representation of the GLM analysis of shape of ectopic patterns produced by damage (Table 3). These are the adjusted means (calculated through the GLM analysis) ± 95% confidence intervals of diameter ratios. (a–c) Plots of only the relationship between two factors (the third factor is confounded in the data) whereas d shows the means from all the groups used in the analysis, separated by the three factors.

produced at 18 hr, except at one of the sites (Figure 5, b and c). A summary of the results, with all the interacting factors, is shown in Figure 5d.

A similar analysis was then performed on the measurements made on the black discs of the ectopic eyespots (Table 4). All groups showed equal variances (Bartlett's test: $\chi^2 = 15.9$, d.f. = 11, P = NS). The same pattern was found as in the previous analysis on total ectopic diameters, but the difference between the lines was no longer formally significant and a new significant interaction effect appeared between line and site. Figure 6a shows that the black disc patterns of the fat line were only distinct and "fatter" than those from thin at one site (VId); at the other sites the line means were similar. fat line ectopics were "fatter" than those in the thin line at each of the time periods. As for the total-diameter ratios, the black discs were "fatter" at the proximal site and when induced at 18 hr (Figure 6, b and c). The overall picture is given in Figure 6d.

TABLE 4

General linear model analysis on shape of ectopic eyespots with three factors: selection line, site of operation and time of operation

Source	d.f.	F	P
Line[a]	1	3.71	0.056
Site[b]	2	14.30	0.000***
Hour[c]	1	11.13	0.001**
Line*site	2	6.82	0.001***
Line*hour	1	0.32	0.572
Site*hour	2	1.26	0.287
Line*site*hour	2	2.90	0.058
Error	170		
Total	181		

Based on the diameter ratio for black discs of ectopic eyespots. ** $P < 0.01$; *** $P < 0.001$.

[a] Fat and thin.

[b] IVd, Vp, and VId.

[c] 12 and 18 hr.

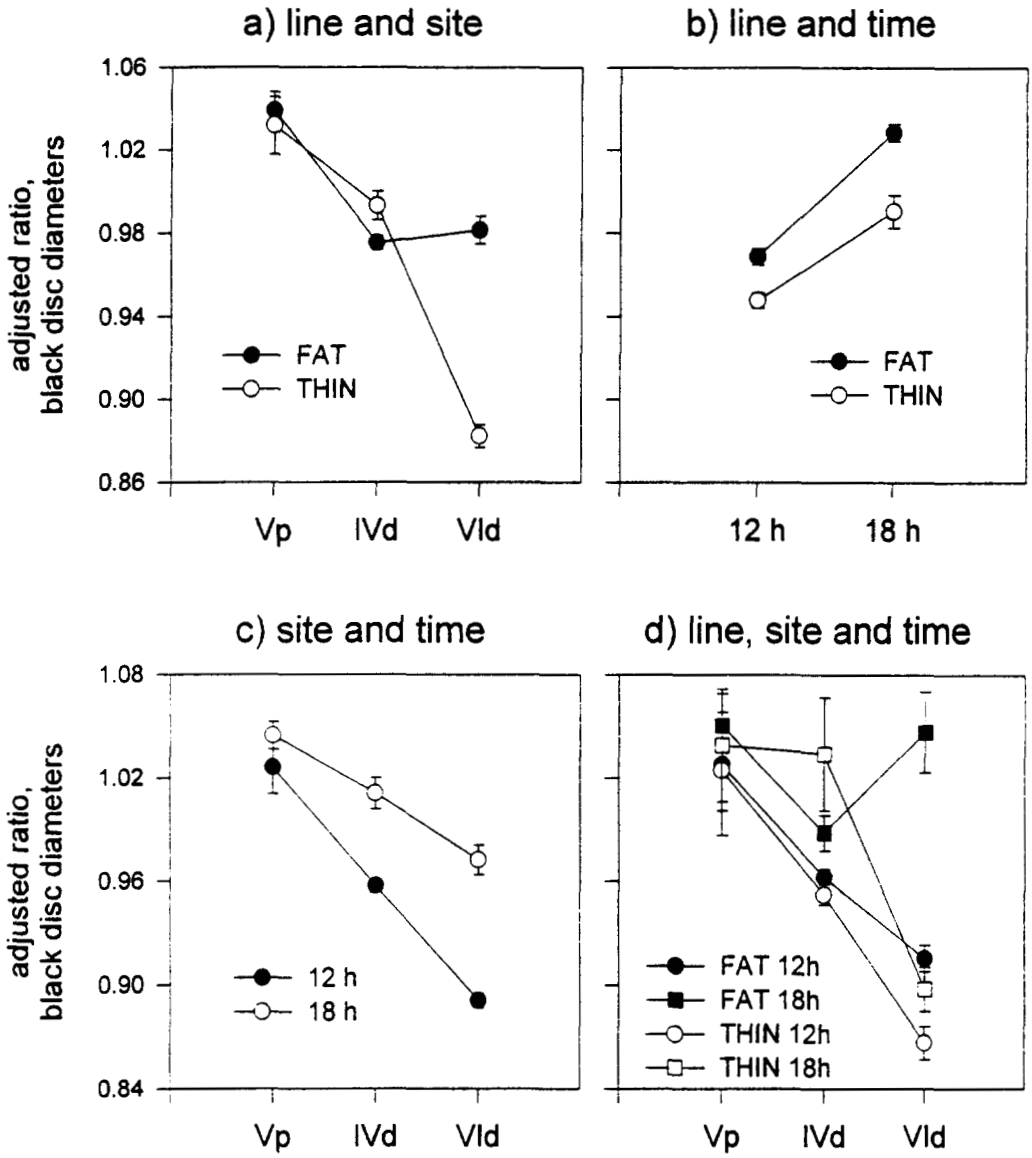

FIGURE 6.—Graphic representation of the GLM results of Table 4. These are the adjusted means (calculated through the GLM analysis) ± 95% confidence intervals of shape of black discs of ectopic eyespots. As in Figure 5, a–c plot only two factors (the third factor is confounded in the data) and d shows the means from all the groups used in the analysis, separated by the three factors.

The area of all ectopic patterns (calculated by multiplying the product of the two radii by Pi) varied between sites (F = 35.8, d.f. = 2, 264, $P < 0.001$; in a GLM with site, line and time of operation as factors), with the largest patterns produced at site Vp and the smallest at site IVd. Areas also varied with the time of operation (F = 106.0, d.f. = 1, 264, $P < 0.001$), the largest ectopics being produced at 12 hr. There was, however, no difference in size of ectopic patterns induced in the fat and thin selected lines (F = 0.4, d.f. = 1, 264, P = 0.51). The consistent difference in shape of the ectopic patterns (at all three sites) and in the central black regions (at one site) support the conclusion, drawn from the grafting experiments, that selection had altered eyespot shape through modifying epidermal response to the underlying signal.

DISCUSSION

Selection succeeded in generating two forms of elliptical eyespots, but the realized heritabilities for shape were low and decreased over the generations. This phenomenon, corresponding to a declining response, indicated that selection either reduced or exhausted the available genetic variation for eyespot shape present in the stock. Selection for shape of the large posterior eyespot led to correlated changes in the shape of the small anterior eyespot on the same wing surface. Correlated changes in eyespots not directly targeted by selection were observed in previous selection experiments for eyespot size (HOLLOWAY *et al.* 1993; MONTEIRO *et al.* 1994) and eyespot color composition (MONTEIRO *et al.* 1997a). These correlated responses were always in the same direction as that in the directly selected eyespot, but were usually of a smaller magnitude. They indicate a common developmental mechanism for all eyespots, that is regulated or fine-tuned in each wing region by a partially independent set of genes. Heritabilities for eyespot shape are lower than those estimated for either size or color composition in the same stock reared at 28° (HOLLOWAY *et al.* 1993; MONTEIRO *et al.* 1994; MONTEIRO *et*

al. 1997a). This lower heritability may suggest a more restricted developmental repertoire and comparatively few alleles influencing eyespot shape.

Formation of the eyespot pattern can be analyzed in terms of the signal from the central focus and the response to it of the surrounding epidermis (NIJHOUT 1980, 1991). In relation to eyespot shape, the genetic variation present in the stock influences only the response component of the developmental mechanism. This is clear from the grafting results that provide no evidence that the orientation of the focus influences eyespot shape. Furthermore, the results of local damage demonstrate that ectopic eyespot shape is influenced by the properties of the nonfocal epidermis of the wing, particularly in the site adjacent to the selected eyespot (VId). It is noteworthy that there appears to be no genetic variation present for the symmetry of focal signaling. A similar lack of genetic variation in the focal signal was obtained in selection for eyespot color composition (MONTEIRO *et al.* 1997a), but contrasts with the strong influence of the focal signal in controlling eyespot size (MONTEIRO *et al.* 1994).

It is likely that eyespot shape would be influenced by the shape of the central focus, which must be established midway between wing veins, earlier in wing development. NIJHOUT (1990, 1991) proposed that focus formation is based on reaction-diffusion processes (see MEINHARDT 1982) spreading from the veins and distal wing margin. This model suggests that the focus resolves from an elongated to a small circular region. It is notable that a similar change is seen in the expression pattern of the regulatory gene, *Distal-less*, that marks the position of the focus in the larval wing imaginal disc (CARROLL *et al.* 1994; NIJHOUT 1994; BRAKEFIELD *et al.* 1996). NIJHOUT (1990) suggests that variation in timing of focus formation could yield foci of different shapes, but the present results give no indication of relevant genetic variation affecting this process.

The genetic variation for eyespot shape that was available for selection in the stock population influenced the nonfocal epidermis. Ectopic eyespots were "fat" in the fat line and "thin" in the thin line. The shape differences between lines were especially evident in ectopics produced just distal to the selected eyespot. Due to selection, properties of the epidermal cells changed, affecting the response to a focal signal or to local damage. One possible basis for effects on eyespot shape could be that a circular region of cells is initially specified around the focus or site of damage, and then a deformation of this region occurs as the pupal wing epidermis expands, eventually to form the cuticle of the larger and differently shaped adult wing. Expansion is likely to involve both cell divisions and cell elongation and may differ in extent, orientation and timing in different parts of the wing surface. It is interesting to note that, for each line, ectopic patterns were "thinner" and larger if induced at 12 than at 18 hr. After 18 hr damage, the pattern was small but closer in shape to the outer border of a normal eyespot. According to the diffusion gradient model (NIJHOUT 1990), the outer edge will be the last region of the eyespot to be specified. Just as the time at which an ectopic eyespot is initiated may influence its final shape, there may be a proximal-distal difference in the properties of the epidermis which underlies the position effect on the shape of ectopic eyespots. Morphometric analysis (MONTEIRO *et al.* 1997b) suggest that the shape differences between eyespots of the fat and thin lines do seem to result from differences in epidermal morphogenesis during the pupal stage, heading to differences in scale cell arrangements and also to different adult wing shapes.

In a preliminary attempt to evaluate eyespot shape differences across the genus Bicyclus, the shape of the posterior dorsal eyespot was measured from photographs of 21 other species. The average shape of this eyespot across species (diameter ratio of 1.04; sexes combined) was similar to that of the stock in *B. anynana.* Some species had more extreme elongation of their eyespots with diameter ratios of 1.20 and 0.82. These shapes fell within the range of the fat and thin selected eyespots, which by the eighth generation had individuals with eyespot ratios of 1.33 and 0.81, respectively. We conclude that, if eyespot shape does not reflect the shape of the signaling focus in these other species, the genetic variation in epidermal response over the genus Bicyclus may be roughly similar to that present in the studied species *B. anynana.*

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