

BUTTERFLY EYESPOTS: THE GENETICS AND DEVELOPMENT OF THE COLOR RINGS

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Abstract.—The butterfly *Bicyclus anynana* has a series of distal eyespots on its wings. Each eyespot is composed of a white pupil, a black disc, and a gold outer ring. We applied artificial selection to the large dorsal eyespot on the forewing to produce a line with the gold ring reduced or absent (BLACK) and another line with a reduced black disc and a broad gold ring (GOLD). High heritabilities, coupled with a rapid response to selection, produced two lines of butterflies with very different phenotypes. Other eyespots showed a correlated change in the proportion of their color rings. Surgical experiments were performed on pupal wings from the different lines at the time of eyespot pattern specification. They showed that the additive genetic variance for this trait was in the response of the wing epidermis to signaling from the organizing cells at the eyespot center (the focus). This response was found to vary across different regions of the wing and also between the sexes. The particular eyespot color composition found for each sex, as well as the maintenance of the high genetic variation, are discussed with reference to the ecology of the butterfly, sexual selection, and visual selection by predators.

Key words.—*Bicyclus anynana*, butterfly, color pattern, correlated responses, development, eyespot, gradient, pattern formation, selection, thresholds, wing pattern.

Received September 6, 1996. Accepted April 23, 1997.

To understand diversity, it is critical to appreciate the differences in developmental mechanisms that underlie phenotypic variation. The nature of these mechanisms may provide an insight into which alterations in phenotype can result from genetic change. Thus, such an understanding can help to determine the limits and constraints on the phenotypes upon which selection can operate in the short term (Cheverud 1984; Maynard Smith et al. 1985; Scharloo 1990).

We are interested in the potential for evolutionary change of butterfly wing patterns and, more specifically, in morphological variation of the eyespot patterns of the nymphalid butterfly, *Bicyclus anynana* (Brakefield and French 1993; Monteiro et al. 1994). This species, and others of the genus, have a series of distal eyespots on the wings. All eyespots are considered homologous pattern elements and show high genetic correlations for several characters, presumably due to a common developmental mechanism (Nijhout 1991; Paulsen and Nijhout 1993; Paulsen 1994).

All eyespots in *B. anynana* have a white pupil, a black disc, and a gold outer ring. These pigments are deposited in the scales just before adult emergence, but the future pattern is specified in the wing epidermis much earlier, during the first few hours of pupal development. Through transplantation and damage experiments in *Precis coenia*, Nijhout (1980) has shown that the cells at the center of a future eyespot pattern, the focus, are responsible for organizing the pattern. Hence, early focal damage can abolish the eyespot, while an ectopic eyespot will form around a focus grafted into a dif-

ferent location. The mechanism of focal signaling is still not understood, but a simple gradient model (Nijhout 1978, 1990) has been helpful in interpreting many experimental results. The focal cells may produce a “morphogen” that diffuses away through gap-junctions, to form a radial concentration gradient. Cells at different distances from the focus would experience different morphogen concentrations at the time of pattern determination, leading them later to produce different pigments. The threshold responses to morphogen concentration would determine the extent of the different color rings and hence the total size and proportions of the eyespot. Alternatively, the focus may produce a gradient by functioning as a local sink for a morphogen present at high concentration throughout the wing epidermis (see Nijhout 1985; French and Brakefield 1992).

By selecting on features of eyespot morphology in *B. anynana*, we have shown that there is substantial genetic variation present for eyespot size (Holloway et al. 1993; Monteiro et al. 1994), eyespot shape (Monteiro et al. 1997) and position of eyespots on the wing (P. M. Brakefield, unpubl. data). In this study, we use selection experiments to estimate genetic variances for proportion (color composition) of the large posterior eyespot on the dorsal forewing of *B. anynana*. Correlated responses to selection, in the color composition of other eyespots, are also examined. Eyespot foci were then grafted between the divergent selected lines, to investigate whether the response to selection produced a change in (a) signaling from the eyespot focus; or (b) the interpretation (or response) of the surrounding cells. Also, as local damage can induce ectopic eyespots (French and Brakefield 1992; Brakefield and French 1995), we tested for a difference between selected lines in the composition of eyespots formed in response to wing damage.

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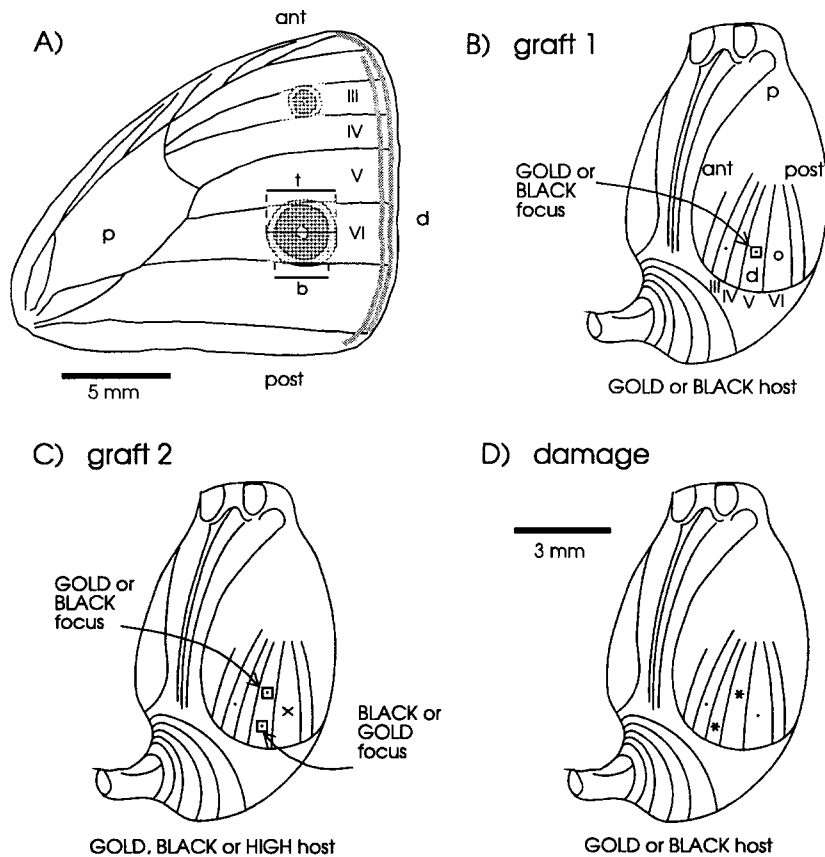


FIG. 1. (A) Dorsal surface of the adult forewing of *Bicyclus anynana* showing the small anterior and large posterior eyespots and the measurements of black (b) and total (t) eyespot diameters. III-VI, spaces between veins (wing cells); ant, anterior; post, posterior; p, proximal; d, distal. (B-D) Pupae showing grafting and wing damage operations. (B) Graft experiment 1: the posterior focus from a GOLD or BLACK pupa (square with dot) was grafted into the Vth wing cell. The posterior focus of the host pupa was removed (circle) to be grafted into another pupa. (C) Graft experiment 2: two foci from different lines were grafted into proximal and distal positions on the Vth wing cell. The posterior focus (cross) was also pierced to reduce eyespot size and prevent merging with the ectopic patterns. (D) Wing damage experiment: the sites marked with asterisks were pierced at time periods 6, 12, and 18 h after pupation.

MATERIAL AND METHODS

Experimental Animals

For selection, several hundred eggs were collected on the larval food plant (maize) from the stock population, and reared through to adult stage in a climate room, at 28°C, high humidity, and 12h light:12h dark photoperiod. The laboratory stock was established about five years earlier from about 80 gravid females from Nkhata Bay in Malawi, and has been maintained at high adult numbers (> 500) at 23°C and produced around six generations per year.

Selection Procedure

Adult males and females were separated on the day of emergence and placed apart in hanging cages. The large posterior eyespot on the dorsal surface of the forewing was measured in the proximal-distal axis (parallel to the wing veins) for total diameter and for diameter of the black disc (Fig. 1A) using a micrometer eyepiece in a binocular microscope.

The butterflies were numbered with a black felt pen on the ventral surface of the hindwing. After roughly the first 100 males and females had emerged and were measured, the mean ratio (and standard deviation) of black/total diameter was

estimated for the whole generation. Assuming a normal distribution of eyespot ratios, two thresholds were determined for each of the sexes, which would yield approximately 100 high-ratio and 100 low-ratio selected males and females, by the time the last butterfly had been measured. Following choice of thresholds, the butterflies were selected and introduced into mating cages. A GOLD and a BLACK line were established from butterflies with low and high ratios, respectively.

Thereafter, if the eyespot ratio of newly emerged butterflies fell either above or below the established thresholds, they were marked and immediately released into these cages. Selection, thus, started before all individuals had emerged. The young age of the selected butterflies at mating, as well as the chosen density of butterflies, maximizes the proportion of individuals that successfully pair.

After mating had taken place, and to increase the selection intensity, only the 40 most extreme females from each line were allowed to lay eggs for the F₁ generation. These females were removed from the mating cage and introduced into a cage with young maize plants. Selection was performed for nine generations. The average number of butterflies emerging in the F₁ and subsequent generations was ~ 600 in each line

(~ 900 in parent population). Realized heritabilities for eyespot composition were estimated by regressing all generation means (up to generation 8), against the cumulative selection differential averaged between the sexes. The slope of the regression line estimates the realized heritabilities (Falconer 1989).

Correlated Responses

To estimate correlated responses to selection, both the large posterior and the small anterior eyespot on the dorsal surface of the forewing were measured in generation 9 butterflies. Both eyespots from a group of STOCK butterflies, raised in the same conditions, were also measured (100 from each sex). All measurements were made using an image analysis system (see Windig 1991).

Focal Grafting Experiments

Grafts were performed three to five hours after pupation, by moving the focus of the posterior dorsal eyespot to a different position on the wing of another pupa. A small square of focal epidermis plus cuticle was cut with a razor-blade knife, rotated 180°, and transplanted into a similar square hole opened previously in the host wing. After adult emergence (at 28°C), the ectopic eyespots formed around grafted foci were measured in their total and black disc diameters and the ratio of black diameter/total diameter was calculated for each eyespot. Ectopic eyespots were included in the analysis only where a white pupil was present and the eyespot extended beyond the rotated scales of the grafted tissue.

Graft Experiment 1

Reciprocal grafts were performed, after four generations of selection, between GOLD and BLACK line pupae and also between pairs of pupae from the same line (Fig. 1B). In each case, the posterior focus from the left wing was grafted to a more anterior position on the left wing of the other pupa. For analysis, data were grouped into four categories: GOLD foci grafted into GOLD hosts, GOLD foci grafted into BLACK hosts, BLACK foci grafted into BLACK hosts, and BLACK foci grafted into GOLD hosts. The medians of the eyespot ratios were compared using the nonparametric Mann-Whitney test.

Graft Experiment 2

In this experiment, eyespot foci from different lines were compared directly on a common host wing (Fig. 1C). Pupae were used after five generations of selection and the posterior (left wing) foci from one GOLD and one BLACK pupa were grafted together into three different types of host: pupae from the GOLD, BLACK, or HIGH line (a line selected for an enlarged posterior dorsal eyespot, showing enhanced epidermal response to a focus; Monteiro et al. 1994). Each experiment was replicated by using the right wing foci of the donor pupae and grafting them into the same positions on a second host pupa. The host sites to which the GOLD or BLACK foci were grafted remained the same between the replicates, but alternated with each experiment. For the following analysis, the replicates were treated as independent

data points as there was no consistency in the correlation coefficients (Spearman rank) between the two eyespot ratios, calculated for each type of host (a test of homogeneity among coefficients showed significant heterogeneity; Sokal and Rohlf 1995).

Wing Damage Experiments

Ectopic eyespots were induced by wing damage to pupae of the generation 6. At 6 h, 12 h, or 18 h (± 15 min) after pupation, the left pupal wing was pierced with an unheated, finely sharpened, tungsten needle, at two sites (see Fig. 1D). Operated pupae were returned to 28°C, the emerged butterflies were frozen and then ectopic eyespots were measured. All analyses, except when indicated otherwise, were done using parametric tests on square-root transformed data, to make the variances homogeneous. Analyses of covariance (ANCOVAs) were performed on black disc diameters of ectopic eyespots, taking total diameter as a covariate.

RESULTS

Response to Selection on Eyespot Color Composition

Both males and females responded to selection for a decrease (GOLD line) and an increase (BLACK line) in the relative size of the central black disc of the posterior dorsal eyespot (Fig. 2A). The butterflies from the GOLD and BLACK lines first showed nonoverlapping distributions in the ratio of black diameter/total eyespot diameter in the generation 5 (data not shown). Figure 3 shows the changes in mean ratio through eight generations of selection. For females of both lines, the estimates of realized heritability are similar: around 38%. Males show some asymmetry in the heritability estimate that varies between 32% and 53%. Selection was stopped in the BLACK males after generation 5, since most eyespots were composed only of the white pupil and surrounding black scales, with no outer gold ring. By generation 8, some BLACK females lacked the outer gold ring, so it is likely that further selection would also produce completely black female eyespots. It is not clear whether prolonged selection of the GOLD line would eventually produce eyespots lacking the central black disc.

Correlated Responses

The proportions of the small anterior dorsal eyespot diverged almost to the same extent as those of the selected eyespot (Table 1). Strong correlated responses to selection were also apparent in other eyespots (see Fig. 2B), indicating that a common developmental mechanism underlies eyespot color composition on all wing surfaces.

There was no significant difference in posterior eyespot size between GOLD and BLACK lines measured at the end of selection (males: mean GOLD = 64.1, SD = 10.1, mean BLACK = 63.5, SD = 8.38, with $F = 0.21$, $P = \text{ns}$, $df = 1, 201$; females: mean GOLD = 91.9, SD = 12.0, mean BLACK = 91.1, SD = 11.2, with $F = 0.26$, $P = \text{ns}$, $df = 1, 222$). Correcting these data for wing length in an ANCOVA did not change the result.

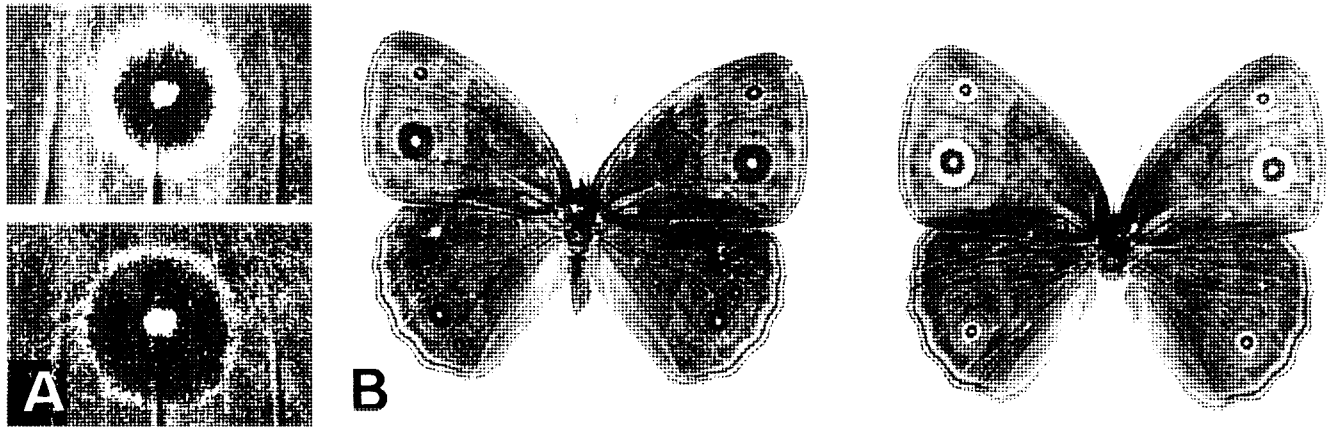


FIG. 2. (A) The target of selection for eyespot composition: GOLD (top) and BLACK (bottom) posterior eyespots on the dorsal forewing. (B) Dorsal view of butterflies from the GOLD and BLACK selection lines.

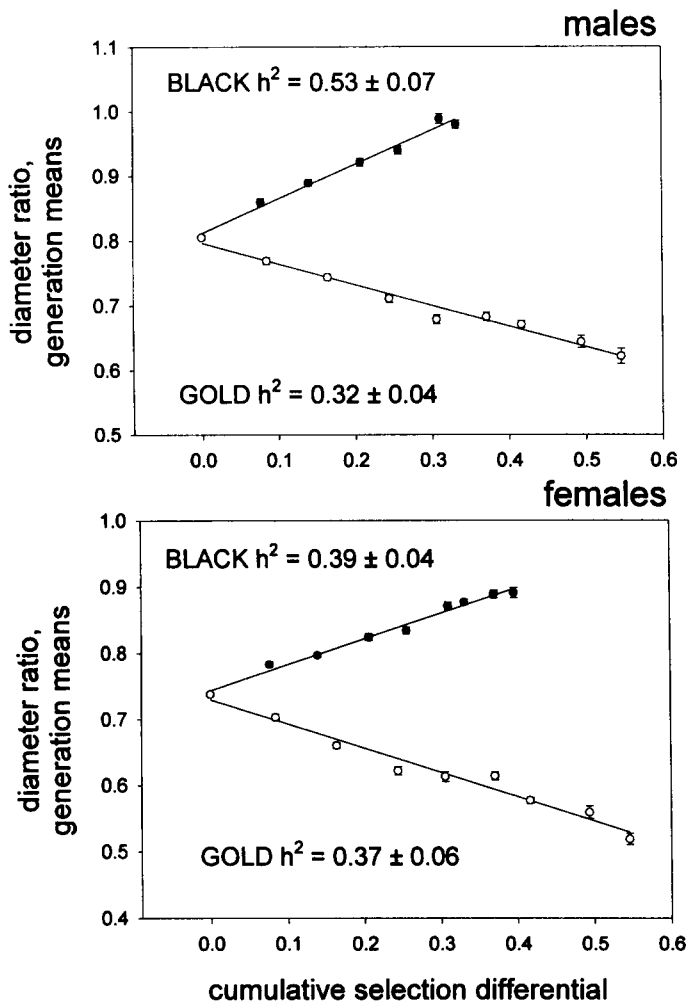


FIG. 3. The change in eyespot composition (shown as mean ratio of black disc/total eyespot diameter) of BLACK and GOLD lines over eight generations of selection. Estimates of realized heritabilities ($h^2 \pm 95\%$ CI) were calculated by the slope of the regression line of the diameter ratio generation means on cumulative selection differential. Bars around each generation mean correspond to 95% confidence intervals.

Eyespot Color Ring Allometry

It is clear from Table 1 that eyespots in males have relatively larger black discs than in females, but it is also the case that the male eyespots are smaller in absolute size (see text above). Within the same sex, however, the smaller anterior eyespots are "golfer," that is, have a broader gold ring, than the posterior ones (Table 1). Since color composition may vary with the size of any particular eyespot, we performed ANCOVAs on the black disc diameters, using total diameters as the covariate, within each of the lines to examine whether: (a) anterior and posterior dorsal eyespots; or (b) eyespots from males and females differed in their color composition when they were of comparable size. We found that both anterior and posterior eyespots from males have larger black discs than the corresponding female eyespots (Table 2A). There was one exception: male and female posterior BLACK eyespots had comparable black disc diameters (explained by both male and female posterior eyespots being almost entirely black in this line). For most ANCOVAs, the slopes of the regression lines of black diameter on total diameter were homogeneous between the sexes (i.e., the interaction term was not significant). In two cases, however, there was significant heterogeneity (Table 2A). Calculation of the adjusted black disc diameters (for total eyespot diameter) and significance testing was still performed, using a common regression line computed with a pooled regression coefficient within the groups. The high F -values for differences in the elevation of the slopes (F -value for sex; especially in the STOCK posterior eyespot) indicated that, although the slopes were not parallel, their intercepts were sufficiently different for the analysis to be meaningful.

ANCOVAs performed within each sex (Table 2B) showed that when anterior and posterior eyespots were of a comparable size, the former were "golfer" than the latter. For GOLD females, however, the difference was not significant. The relationship between black center and total diameter was collinear in most analyses, for both eyespots, even though there was little overlap in eyespot size between the two groups. In two cases there was a significant interaction effect:

TABLE 1. Mean ratio (\pm SD) of black disc/total eyespot diameter of the selected posterior eyespot on the dorsal forewing, and correlated responses measured on the anterior eyespot on the same wing surface, after nine generations of selection in GOLD and BLACK lines. STOCK (unselected) butterflies were measured for comparison. Numbers in parentheses are sample sizes.

Line	Males		Females	
	Anterior	Posterior	Anterior	Posterior
GOLD	0.55 \pm 0.06 (137)	0.69 \pm 0.05 (137)	0.52 \pm 0.06 (114)	0.57 \pm 0.05 (114)
STOCK	0.71 \pm 0.05 (100)	0.85 \pm 0.05 (98)	0.66 \pm 0.04 (100)	0.76 \pm 0.04 (99)
BLACK	0.86 \pm 0.09 (78)	0.97 \pm 0.06 (68)	0.79 \pm 0.06 (110)	0.88 \pm 0.06 (110)

the slope for posterior GOLD eyespots, for each sex, was steeper than for anterior eyespots. In all cases, however, with the exception of the GOLD females, the adjusted means were very different between the eyespots (see *F*-values in Table 2B). For further evidence that anterior eyespots, independent of their size, are really "golder" than posterior eyespots, an ANCOVA was performed between the black centers of posterior eyespots in a line of butterflies selected for small posterior eyespots (LOW line, see Monteiro et al. 1994) and the black centers of STOCK anterior eyespots. These two eyespots were of comparable sizes in males (mean LOW posterior = 40.3, SD = 8.8; mean STOCK anterior = 40.8, SD = 7.6; $T = -0.43$, $P = \text{ns}$, $df = 154$). The ANCOVA showed that STOCK anterior eyespots were indeed "golder" than LOW posterior eyespots (adjusted means of black centers: STOCK anterior = 28.9, SD = 0.3, LOW posterior = 33.6, SD = 0.3; $F = 137.2$, $P < 0.001$, $df = 1, 176$). There was no interaction effect between the two regression lines ($F = 0.13$, $P = \text{ns}$, $df = 1, 175$). Thus, in summary, anterior eyespots are "golder" than posterior eyespots even when, through selection, both eyespots achieve a comparable size, and females produce "golder" eyespots than males, throughout their overlapping range of sizes.

Ectopic Eyespots Induced by Grafted Foci

The ectopic eyespots induced by grafted foci were analyzed to determine whether differences in eyespot composition between GOLD and BLACK selected lines resulted from differences in focal signal or in epidermal response.

Graft Experiment 1

The reciprocal grafts between pupae (Fig. 1B) showed that GOLD or BLACK foci in a BLACK host induced eyespots with a very narrow gold ring (large black/total diameter ratio) while, when grafted into a GOLD host, they each produced eyespots with a reduced black disc and a broad gold ring (small ratio). The four different categories of graft were arranged in pairwise comparisons, and the eyespot ratio medians were compared using the nonparametric Mann-Whitney test (Table 3). It is clear that there is a major effect of host "environment" on eyespot color composition (Table 3a,d,e) while the origin of the focus has little effect (Table 3b,c). In grafts to GOLD hosts, however, BLACK foci produce somewhat "golder" eyespots than GOLD foci (Table 3b). This is explained by a positive correlation between size (total diameter) of an induced eyespot and its ratio ($r_s = 0.331$, $P < 0.01$; for all eyespots induced on a GOLD host), coupled with the finding that eyespots induced by the BLACK foci were smaller than those induced by GOLD foci. Other significant size differences (e.g., Table 3a,d) do not affect the results of the ratio analysis, since there the smaller eyespot is also the "blacker" (with the broadest black disc relative to total diameter).

Graft Experiment 2

The double graft of foci from BLACK and GOLD pupae (Fig. 1C) resulted in formation of two ectopic eyespots on the host wing (Fig. 4A). The data from the three different

TABLE 2. Results of ANCOVAs performed on black disc diameters of (A) male and female eyespots; and (B) anterior and posterior eyespots, taking total eyespot diameter (size) as the covariate. The adjusted means for each test were always higher for males than for females (A) and higher for the posterior selected eyespot (B). The covariate (total diameter) was highly significant ($P < 0.000$) in all analyses with *F*-values ranging from 487 to 1540 (for sample sizes see Table 1).

(A) Eyespot	Line	Factors in ANCOVA	<i>F</i>	(B) Sex	Line	Factors in ANCOVA	<i>F</i>
Anterior	GOLD	sex	13.8***	Males	GOLD	eyespot	149.7***
		sex \times size	3.0			eye \times size	6.2*
	BLACK	sex	4.6*		BLACK	eyespot	158.8***
		sex \times size	5.1*			eye \times size	2.6
	STOCK	sex	77.1***		STOCK	eyespot	77.0***
		sex \times size	0.5			eye \times size	2.8
Posterior	GOLD	sex	153.4***	Females	GOLD	eyespot	3.7
		sex \times size	0.7			eye \times size	6.9**
	BLACK	sex	0.7		BLACK	eyespot	68.0***
		sex \times size	1.2			eye \times size	2.5
	STOCK	sex	40.4***		STOCK	eyespot	26.2***
		sex \times size	3.9*			eye \times size	0.0

*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.

TABLE 3. The "blackness" (ratio of black disc/total diameter) of eyespots induced by a grafted focus in experiment 1. The comparison "GOLD into BLACK" indicates a GOLD line focus grafted into a BLACK line pupa (a ratio of zero means that only gold scales were present outside the grafted tissue); n = number of scorable results from each category of graft operation. The Mann-Whitney (M-W) test statistics are given.

Comparison (focus into host)	n	Ratio (median)	M-W (for ratio)	Size of ectopic eyespot (median)	M-W (for size)
(a) GOLD into BLACK	27	1.00	$W = 1207.5$	37.0	$W = 628.5$
BLACK into GOLD	33	0.00	***	52.0	***
(b) GOLD into GOLD	36	0.35	$W = 1411.5$	61.5	$W = 1954.5$
BLACK into GOLD	33	0.00	*	52.0	*
(c) BLACK into BLACK	13	1.00	$W = 285$	43.0	$W = 301.0$
GOLD into BLACK	27	1.00	ns	37.0	ns
(d) GOLD into GOLD	36	0.35	$W = 750.5$	61.5	$W = 1447.0$
GOLD into BLACK	27	1.00	***	37.0	***
(e) BLACK into BLACK	13	1.00	$W = 520$	43.0	$W = 244.0$
BLACK into GOLD	33	0.00	***	52.0	ns

*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; ns = nonsignificant.

hosts were pooled and then split into two categories: all hosts where the GOLD focus was grafted to the proximal site (and BLACK to distal) and those with the GOLD focus grafted distally (and BLACK proximally). The data were analyzed with a Wilcoxon paired-comparison test where the eyespots induced by the GOLD and BLACK foci in the same host made up a data pair, with the null hypothesis of no difference in the composition of the eyespots. The ratio of black/total diameter for the BLACK focus-induced eyespot was subtracted from that of the GOLD focus-induced eyespot, a negative value indicating that BLACK foci induced a "blacker" eyespot. The differences were then ranked from smallest to largest, irrespective of their sign. The original sign of the differences was assigned to the ranks and the sum of the positive and negative ranks calculated. The smallest sum in absolute value, T_s , is the test statistic (Sokal and Rohlf 1995). There was a strong position effect (Table 4): whichever focus was grafted into the proximal position yielded the ectopic eyespot with the largest proportion of gold. The effect of host site was much stronger than any effect of the foci in controlling the color composition of induced eyespots, thus the data could not be examined in a pairwise manner as had been

initially planned. The enlarged gold ring in the proximal eyespots may be related to their smaller size: a GLM analysis of square-root transformed total diameter data (to produce homogeneous variances; chi-square = 9.86, $df = 11$, $P = ns$) with four factors (site of operation, type of focus, type of host, and sex) showed that proximal eyespots were smaller than distal ones, GOLD foci induced smaller eyespots than BLACK foci, and BLACK hosts as well as male hosts formed the smaller eyespots (Table 5).

No parametric test could be performed on the ratio of black/total diameters since the variances for the different groups were heterogeneous and the data highly skewed. A series of nonparametric comparisons were then performed, for each grafting site, to compare the effect of the GOLD and BLACK foci, both within each host line (GOLD, BLACK, and HIGH) and with all hosts combined (Table 6). This analysis did not detect any effect of line of origin of focus on eyespot color proportions, but it was evident that a BLACK host produced "blacker" eyespots than a GOLD host.

We conclude from graft experiments 1 and 2 that it is mainly the epidermal response to a focal signal that has been changed in the GOLD and BLACK lines as a result of ar-

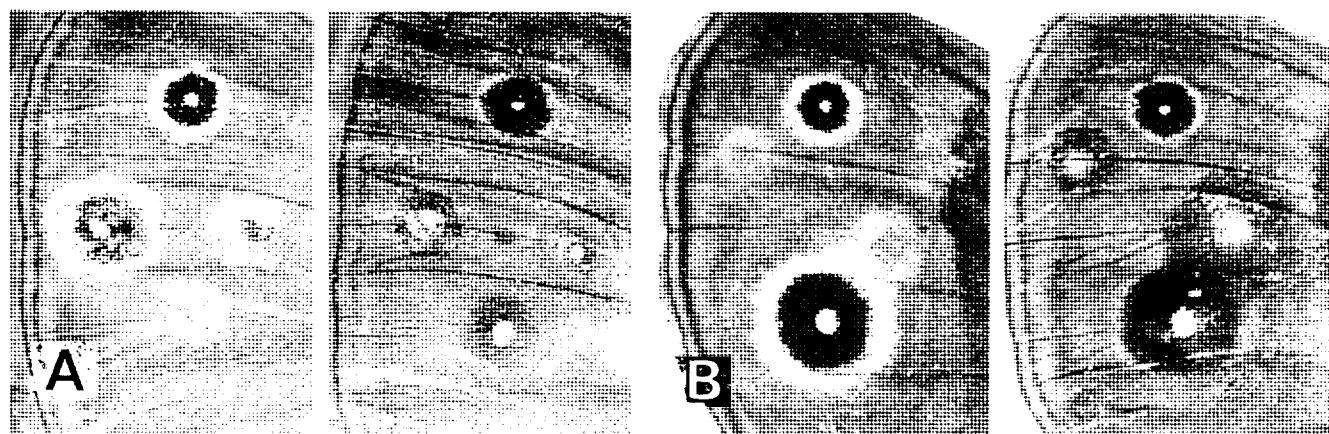


FIG. 4. (A) Graft experiment 2: a GOLD and a BLACK focus were grafted into a proximal and a distal position, respectively, on a GOLD host (left) and BLACK host (right). (B) Ectopic eyespots induced by damage during the pupal stage on GOLD (left) and BLACK (right) line butterflies.

TABLE 4. Analysis of the two ectopic eyespots induced in graft experiment 2 by grafting foci into proximal and distal positions: Wilcoxon sign-ranked test for paired data to compare differences in color composition of eyespots induced by the GOLD and BLACK foci (see results for details); n = number of host wings bearing two scorable ectopic eyespots.

Focus position	n	Sum of positive ranks	Sum of negative ranks	Wilcoxon sign-ranked tabled Ts
GOLD-proximal BLACK-distal	38	108	633	194 (> 108) $P = 0.005$
BLACK-proximal GOLD-distal	31	411	85	118 (> 85) $P = 0.005$

tificial selection. The source of the focus seems to have no influence on the relative extent of black and gold scales.

Ectopic Patterns Induced by Wing Damage

Wings of BLACK and GOLD line pupae were damaged in two positions (Fig. 1D) and the resulting patterns ranged from a few scattered gold or black scales, to a more compact patch of gold or gold and black scales, to a fully differentiated ectopic eyespot with a black center and an outer gold ring (Fig. 4B). Damage at 6 h produced mostly scattered scales or patches, whereas operations at both 12 h and 18 h induced mainly eyespots (Fig. 5). We have analyzed the proportions of gold and black in ectopic eyespots only, and compared them between selection lines.

A GLM analysis on ectopic eyespot size (not shown) demonstrated that ectopics produced at 12 h were larger than at 18 h; proximal ectopics were larger than distal ones; females produced larger ectopics than males at 18 h; and GOLD line ectopics were larger than BLACK line ectopics. The color composition of eyespots induced in the BLACK and GOLD lines could not be compared using an analysis of covariance (because the slopes of the regressions of black diameters against total ectopic eyespot diameters were not parallel over the lines), so the ratios of black/total eyespot diameters were compared by means of two-sample Mann-Whitney nonparametric tests. In all comparisons of median ratios (per time of operation, sex and site of operation; Table 7), induced eyespots on the GOLD were "golder" than those on the BLACK butterflies. These results support the hypothesis that the response properties of the epidermis have been changed by selection for GOLD and BLACK eyespots.

The effects of sex and site and time of operation on color composition of the ectopic eyespots were analyzed within each line by an ANCOVA of black disc diameters, using total diameter as a covariate (not shown). For both lines, 18-h ectopics were "golder" than 12-h ones. In addition, distal ectopics were "golder" than proximal ones in the GOLD line and, in the BLACK line, males produced "blacker" ectopics than females.

The color composition of ectopic eyespots and control posterior eyespots of the same butterflies were compared by an ANCOVA (not shown). The analysis showed that GOLD line 12-h ectopics (both proximal and distal) showed a larger black disc than control eyespots, whereas 18-h ectopics were on average "golder" than their control eyespots. For the

TABLE 5. GLM analysis on size of ectopic eyespots resulting from graft experiment 2. The analysis was performed with four factors (site of operation, line of grafted focus, line of host, and sex). The eyespot diameters were square-root transformed. Analysis of interaction terms (not shown) showed a significant ($P < 0.05$) interaction between host line and sex.

Source	df	F	P	Levels within factors	Means	SD
Site	1	7.53	**	Proximal	6.87	0.09
				Distal	7.22	0.09
Focus	1	5.50	*	GOLD	6.90	0.10
				BLACK	7.19	0.08
Host	2	29.74	***	GOLD	7.50	0.09
				BLACK	6.23	0.15
				HIGH	7.40	0.06
Sex	1	27.41	***	Males	6.72	0.10
				Females	7.37	0.07
Error	231					

*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.

BLACK line, female 12-h and 18-h ectopics were always "blacker" than control eyespots, whereas male 18-h ectopics were "golder" than the corresponding control eyespots.

DISCUSSION

Substantial additive genetic variation is present for the color composition of the dorsal posterior eyespot, even in our laboratory stock of *B. anynana*. The estimates of heritability are similar to, although a little lower than, those for the size of both the ventral and dorsal posterior eyespots (Holloway et al. 1993; Monteiro et al. 1994). Selection in the BLACK line reached its phenotypic limit, especially in males, by generation 5, producing mostly "black" eyespots. No limit was reached in the GOLD line, as all eyespots still had a (reduced) central area of black scales in generation 8 (Fig. 3). Asymmetries in the realized heritability estimates between males of the BLACK and GOLD lines are likely to have been influenced by a skewed distribution in the BLACK males, caused by the appearance of some individuals with completely "black" eyespots, from the second generation onward. This skewness will lead to a slight raising of the heritability estimate because, although both generation means and selection differentials suffer a decrease in their mean values, the latter are more affected by the skewed distribution, making the regression slope steeper. Further speculation on the causes of this asymmetry, however, is not profitable in the absence of replicate lines.

All other eyespots, on both wing surfaces, changed their color composition in parallel with the selected eyespot (see Fig. 2B for dorsal eyespots only). Butterflies from the GOLD line became much more striking in appearance, whereas BLACK line butterflies became dark and less conspicuous (Fig. 2B). Similar strong correlated responses to selection were found in selection experiments on the size of both the ventral small anterior (Holloway et al. 1993) and dorsal large posterior (Monteiro et al. 1994) eyespots of *B. anynana*. These responses, indicating strong genetic correlations among all eyespots, support the idea that eyespots are de-

TABLE 6. Comparisons between the ratio of black/total eyespot diameters (median) produced in graft experiment 2 by a GOLD or BLACK grafted focus, per site insertion of graft and type of host pupae. Values in parentheses are sample sizes. For each Kruskal-Wallis test, $df = 1$, $P = n.s.$

Site	Focus	Host pupa			
		GOLD	BLACK	HIGH	All hosts
Proximal	GOLD	0.000 (19)	0.789 (5)	0.000 (37)	0.000 (61)
	BLACK	0.000 (10)	0.778 (9)	0.000 (30)	0.000 (49)
	Kruskal-Wallis	$H = 1.69$	$H = 0.02$	$H = 0.26$	$H = 0.16$
Distal	GOLD	0.000 (13)	0.773 (9)	0.536 (35)	0.554 (57)
	BLACK	0.247 (19)	0.837 (14)	0.590 (34)	0.571 (67)
	Kruskal-Wallis	$H = 0.04$	$H = 0.53$	$H = 0.32$	$H = 0.15$

developmental homologues formed by a common developmental mechanism (Nijhout 1991).

The proportions of the different colored rings in an eyespot are mainly determined by the response properties of the wing epidermis. Both focal grafting (Tables 3, 6) and wing damage results (Table 7) support this conclusion. Thus, any focus or local damage in BLACK wing epidermis induces a "black" eyespot, with a very narrow gold ring. When a GOLD pupa is used, the focus or damage will induce an eyespot with a small black disc and a broad gold ring (Fig. 4).

Using the unselected stock, French and Brakefield (1995) grafted anterior and posterior foci into different positions in the wing and found that posterior foci always induced "black-er" ectopic eyespots than anterior foci. They argued that the color ring proportions of the eyespot depended on the identity of the focus and not on the responding epidermis. Their analysis, done with ratios, without attending to the total eyespot size, is only partly correct. It is now clear that the grafting of a posterior focus, into any given site, will induce a "black-er" eyespot only due to the fact that it will also induce a larger eyespot than an anterior focus.

In relation to the gradient model of eyespot formation (see introduction), the present results indicate that genetic variation for color composition affects threshold levels of response of the epidermal cells to the focal signal. Figure 6 illustrates a sink variant of the gradient model, where re-

sponding cells have two thresholds of sensitivity to morphogen concentration. A narrow gold ring can be produced by selection bringing the two thresholds closer, while separating them leads to a "golder" eyespot. Change is shown only in the lower threshold (T1), as there was no difference in eyespot outer diameter between the BLACK and GOLD lines. In previous selection experiments for eyespot size (Monteiro et al. 1994), the major change was in focal signal but changes in response (i.e., in threshold T2) had also occurred. These size-selected lines had a similar color ring composition: no difference for the males and black/total diameter ratios of 0.76 for the HIGH line and 0.70 for the LOW line females (Monteiro et al. 1994). Comparing the results from the two experiments one may speculate that there is additive genetic variance controlling the height of both thresholds (but keeping the distance between them constant) as well as genetic factors involved in regulating the height of T2 independently of T1. The selection procedure described in this paper would have targeted only the second set of genes, since alteration in allele frequencies of the first set would bring the individuals no selection advantage. Selection for size, however, would have targeted the first set of genes.

Apart from differences of response between the selected lines, within individuals there were differences across the wing epidermis. Eyespots induced by a grafted focus at a distal site were "black-er" than eyespots of a comparable size induced more proximally. Response thresholds are, thus, not uniform across the wing epidermis (for equivalent positions, similar results were found in French and Brakefield 1995). We also found a difference in color composition for the normal anterior and posterior eyespots of *B. anynana*: anterior eyespots are "golder" than posterior ones of a comparable size, indicating a difference of the response along this wing axis.

The frequency and size of ectopic eyespots induced by focal damage at different times and sites were similar to the results of previous studies (French and Brakefield 1992; Monteiro et al. 1994; Brakefield and French 1995). Eyespot induction by damage, although not well understood, can be interpreted in terms of the gradient model (Fig. 6; for discussion, see French and Brakefield 1992; Brakefield and French 1995). The large differences in the color pattern of ectopics produced in the BLACK and GOLD lines, mimic the respective eyespot phenotypes. The relationships between eyespot composition and size for these ectopic patterns were, with one exception, comparable to those for the control eye-

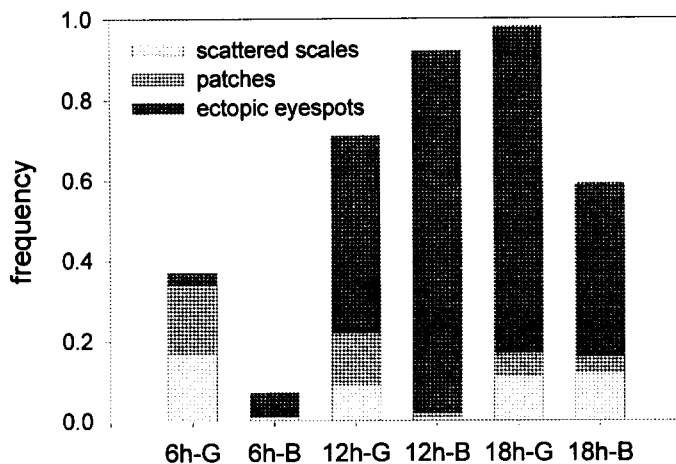


FIG. 5. Frequency of the different types of ectopic pattern induced by wing damage (data from the two sites are combined). Wings of GOLD (G) and BLACK (B) pupae were pierced either at 6, 12, or 18 h after pupation.

TABLE 7. Color composition (ratio of black/total diameter) of ectopic eyespots induced by damage to GOLD and BLACK pupae. A Mann-Whitney test was performed between the medians of the ratio of black/total diameters of the GOLD and BLACK ectopics for each position (see Figs. 1D, 4B), age at cautery (12 h and 18 h), and sex. Numbers in parentheses are sample sizes.

Age	Line	Males		Females	
		Proximal	Distal	Proximal	Distal
12 h	GOLD	0.95 (8)	0.74 (15)	0.65 (22)	0.68 (25)
	BLACK	1.00 (25)	1.00 (32)	1.00 (32)	1.00 (31)
Mann-Whitney		$W = 67.0$ ***	$W = 187.5$ ***	$W = 345.0$ ***	$W = 450.0$ ***
18 h	GOLD	0.50 (58)	0.43 (57)	0.45 (29)	0.42 (21)
	BLACK	1.00 (18)	1.00 (24)	0.77 (6)	1.00 (13)
Mann-Whitney		$W = 1728$ ***	$W = 1676.5$ ***	$W = 474.0$ *	$W = 237.5$ ***

*** $P < 0.001$; * $P < 0.05$.

spots. In terms of the model, this suggests that cells respond according to the same thresholds, and the same morphogen gradient is established around the natural focus and around an injury, presumably by diffusion.

The eyespots of male *B. anynana* normally have narrower gold rings than those of females (also when eyespot size is accounted for). This sexual dimorphism may be influenced by several selective factors. Small, submarginal eyespots can deflect attacks of vertebrate predators away from the vulnerable body parts (Brakefield and Larsen 1984; Wourms and Wasserman 1985; Brakefield and Reitsma 1991). Their effectiveness in this function and their overall conspicuousness are likely to depend on the color composition of the eyespots and the degree to which they contrast with the background wing color. Visual selection by predators may have led to different optimal phenotypes for the sexes because of differences in wing size, behavior and resting background, or environment between males and females. The ventral wing color of male *B. anynana* is generally darker than in females. This is probably because male mating success is dependent on effective thermoregulation through lateral basking behavior in sunspots (see Windig et al. 1994; Van Dyck et al. 1997).

Females may be less constrained by thermal biology and, therefore, have "golder" eyespots, which are more contrasting with the paler background color of their ventral wings. Genetic correlations between dorsal and ventral wing surfaces (Monteiro et al. 1994) may then yield a sexual dimorphism for the dorsal eyespots. On the other hand, the eyespots of *B. anynana*, especially those of the dorsal surface, which remain unexposed to predators, may be involved in mate choice; females may prefer to mate with males with "blacker" eyespots. We are now performing experiments on female choice. Since the developmental mechanisms and levels of additive variance allow for substantial phenotypic responses to short-term artificial selection, complex interactions among mate choice, thermoregulation, and visual selection across the sexes may have determined the particular color composition of eyespots favored in males and females in the wild.

In previous selection experiments for eyespot size (Monteiro et al. 1994), we argued that the high genetic variances observed were likely to be maintained due to differences in selection across seasons (dry and wet season). This cannot apply to eyespot color composition since, in the dry season,

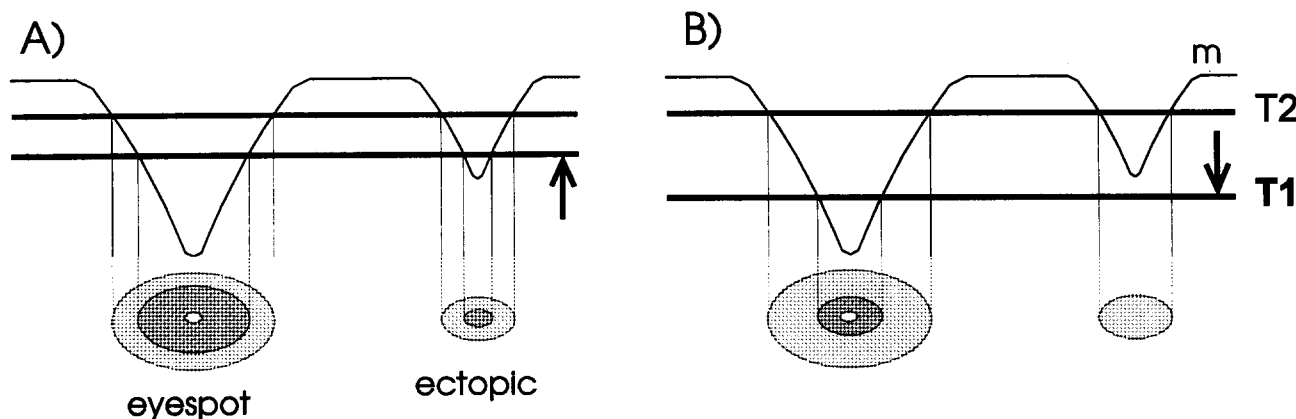


FIG. 6. Gradient model of eyespot specification involving two thresholds of response to concentration of morphogen (m). Cells experiencing morphogen concentration below $T1$ later form black scales; between $T1$ and $T2$, gold scales (stippled); and above $T2$, the background brown scales. In this version of the model, the focus acts as a local sink, producing a pit in morphogen concentration, and hence an eyespot pattern. Appropriately timed local damage also depletes morphogen (see text), leading to an ectopic pattern. Selection in (A) the BLACK line and (B) the GOLD line may raise or lower the $T1$ threshold and hence change color composition (but not size) of the normal and ectopic eyespots.

the eyespots are absent or very reduced and differences in color ring proportions would not be easily detectable.

Between species in the genus *Bicyclus*, there is substantial variability in the proportions of gold and black on the eyespots (see Condamin 1973). All species, however, appear to fall well within the range of the selected eyespot phenotypes of *B. anynana*. Some species have completely black dorsal eyespots and ventral ones with only a narrow gold ring (e.g., *B. angulosus* and *B. cottrelli*). Others have a broad gold ring on both wing surfaces (e.g., *B. ena* and *B. trilopus jacksoni*). In these latter species, the background color surrounding the eyespots also tends to be light in color, reducing the conspicuousness of the gold ring.

This study shows that artificial selection can produce rapid changes in epidermal response to focal signaling, yielding lines with mostly "black" eyespots or with much more prominent outer gold rings: phenotypes not present in the stock or in field collected material of *B. anynana* (P. M. Brakefield, pers. obs.). Visual selection in the field may tend to favor an intermediate eyespot phenotype in most *Bicyclus* species, perhaps because of its effect on conspicuousness. However, some form of disruptive selection (e.g., sexual selection vs. visual selection by predators) may account for the occurrence of high additive genetic variance for eyespot color composition. Continued investigation into the interaction between such selective forces and constraints dictated by the properties of the developmental mechanism will help elucidate further intra- and interspecific patterns of variation of color patterns on butterfly wings.

ACKNOWLEDGMENTS

We would like to thank H. van Rijnberk for writing the measurement program in the image analysis system; H. van Rijnberk and P. de Jong for helpful discussions on some of the analysis; F. Nijhout, L. Meffert, and an anonymous reviewer for their comments on the manuscript; E. Schlatmann and B. de Winter for growing maize for caterpillars; and A. 't Hooft and P. Mulken for the photographs. AM was supported by a Ciência grant from Junta Nacional de Investigação Científica e Tecnológica (JNICT).

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Corresponding Editor: L. Meffert