

The relationship between eyespot shape and wing shape in the butterfly *Bicyclus anynana*: A genetic and morphometrical approach

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Abstract

The African butterfly, *Bicyclus anynana*, normally possesses circular eyespots on its wings. Artificial selection lines, which express ellipsoidal eyespots on the dorsal surface of the forewing, were used to investigate correlated changes in wing shape. Morphometric analysis of linear wing measurements and wing scale counts provided evidence that eyespot shape was correlated with localised shape changes in the corresponding wing-cell, with overall shape changes in the wing, and with the density/arrangement of scales around the eyespot area.

Introduction

Recent studies describing patterns of morphological diversity between groups of nymphalid butterflies have found that despite the great diversity of wing shapes present, most of this variation was explained by allometric size-scaling effects, i.e., large species were mainly allometrically changed versions of smaller ones (Strauss, 1990, 1992). It appears, thus, that significant evolutionary constraining forces have been governing the sources of wing shape variation. In this paper, we address a

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developmental mechanism of morphological diversification that can account for some of the small size-independent differences in butterfly wing shape and illustrate that, through artificial selection on wing patterns, it is possible to change morphology in localised areas of the butterfly wing.

Quantitative genetic studies of butterfly wing colour patterns (e.g., Brakefield, 1984; Kingsolver and Wiernasz, 1987; Wiernasz, 1989; Holloway and Brakefield, 1995; Monteiro et al., 1994) have not previously been coupled with studies of butterfly wing shape (Ricklefs and O'Rourke, 1975; Johnson and Walter, 1978; Strauss, 1990 and 1992). Both wing pattern and shape (together with wing venation patterns) have played a major role in taxonomic and evolutionary studies of the Lepidoptera (e.g., Strauss, 1990) and it is important to establish whether these are independent characters, or whether they may be coupled through common developmental mechanisms. In this paper we demonstrate a correlation between colour pattern and wing shape in the nymphalid butterfly *Bicyclus anynana*. We also show that there is substantial genetic variation for wing shape present in this species and discuss the potential for natural selection on colour patterns in the evolution of wing shape.

The nymphalid butterfly *B. anynana* has large eyespots near the margin of the adult dorsal and ventral wing surfaces. Each eyespot has a white pupil, a black central disc and an outer gold ring, and is centred midway between adjacent wing veins. The eyespot patterns are determined at the early pupal stage (approximately 24 h after pupation in *B. anynana* reared at 28 °C: French and Brakefield, 1992) by a developmental mechanism consisting of a focal signal that is produced by cells at the centre of the presumptive eyespot and interpreted by the neighbouring cells, leading them to produce different colours with increasing distance from the focus (see Nijhout, 1980a; French and Brakefield, 1992). The nature of the signal is unknown, but a simple, plausible model involves a diffusible "morphogen" that forms a conical concentration gradient around the focus (Nijhout, 1991).

Adult wing colour arises from pigment deposited at very late pupal stage in the cuticle of the overlapping rows of cover scales: large, flattened protrusions from specialised scale-building cells. In nymphalid butterflies, the scale-building cells are first distinguishable in the early pupa as enlarged cells in regular parallel rows running anterior-posterior across the pupal wing epidermis (Nijhout, 1980b, 1991).

The eyespots of *B. anynana* are approximately circular in shape. We applied artificial selection on the shape of the large eyespot of the dorsal forewing (Monteiro et al., 1997). Nine generations of selection led to strong divergence of a FAT and a THIN line with elliptical eyespots elongated in the proximal-distal and anterior-posterior axes, respectively. The divergence demonstrated additive genetic variance for eyespot shape, although estimated values for heritability were only moderate (around 15%). In order to examine whether selection had resulted in asymmetry in signalling from the eyespot focus, we rotated foci on FAT and THIN pupae by 90 degrees and 180 degrees but found no effects on the final shape of the eyespots (Monteiro et al., 1997). These results suggested that selection had acted, not on

the focus, but on the response of the pupal wing epidermis to focal signalling or to its subsequent development to give the adult wing.

Here we test by morphometric analysis whether the shape of the adult wing (or part of the wing) in FAT and THIN lines is distorted in a corresponding manner to the “stretching” observed for the eyespot colour pattern. We also investigated whether changes in the matrix of scales had occurred in the region of the selected eyespot and hence whether the change in eyespot shape was due to scale re-arrangement in the wing epidermis, rather than to the addition of extra pigmented scales along a particular axis.

Material and methods

The butterflies

Samples of eggs from butterflies of the ninth generation of FAT and THIN selected lines (see Monteiro et al., 1997 for details of selection), and from the unselected STOCK population, were reared on young maize plants at 28 °C. About 100 newly-emerged butterflies of each sex and line were frozen and stored for measurement.

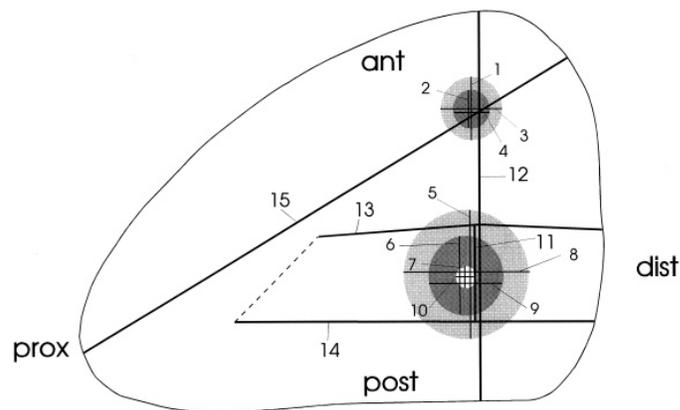


Fig. 1. The dorsal surface of the forewing of *B. anynana*, showing anterior (ant) and posterior (post) eyespots, each consisting of white pupil, black disc and outer gold ring. prox, proximal; dist, distal. Measurements were made of the anterior-posterior (12) and proximal-distal (15) extent of the wing, and along (13, 14) and between (11) the veins that define the “wing-cell” within which the posterior eyespot is centred. Also the anterior eyespot (outer and black disc diameters) and posterior eyespot (outer, black disc and white pupil diameters) were measured in anterior-posterior and proximal-distal axes (measurements 1–4 and 5–10). Measurements 1–11 and 15 cross the centre of the white pupil, but are shown staggered in the figure. Measurement 12 crosses the centre of the two pupils.

The measurements

An image analysis system (see Windig, 1991) was used to perform the linear measurements of the butterfly wings shown in Figure 1. Additionally, this system enabled the measurement of wing area, wing maximum length and the maximum orthogonal length. Also the point of intersection of these latter two lines and the segments of the lines from that point to the distal edge were measured (sub-maximum and sub-orthogonal lengths, respectively). From the maximum length, sub-maximum orthogonal length and sub-maximal length, a measure of wing base length was calculated by squaring (maximum length – sub-maximum length) and adding the square of sub-orthogonal length. Data were stored automatically for later analysis by the MINITAB or SPSS statistical packages.

The measurements (Fig. 1) were chosen in reference to the following questions:

- 1) Has selection for posterior eyespot shape lead to changes in the shape of the corresponding wing-cell (measurements: 11, 13 and 14)?
- 2) Has the whole wing changed shape (12, 15, and wing base length)?
- 3) Has the small anterior eyespot also changed shape (1–4)?
- 4) Have the white pupil, black discs and total diameters of both eyespots in the dorsal surface changed shape in a proportional way (measurements 1–10)?

The analysis

A discriminant function analysis was performed to address the first two questions. The analysis separates the three groups of individuals (FAT, THIN and STOCK), using measurements for these individuals on several variables, by calculating two uncorrelated linear combinations of these variables and summarising all information in two functions. The first function will explain the largest differences between the groups (Simon, 1983; Norusis, 1985; Manly, 1986).

Separate discriminant function analyses were performed here for males and females since males have smaller, more pointed wings than females. A total of six variables were used:

- wing cell height (11) (w-cell-h);
- wing height minus wing cell height (12 – 11) (w-height);
- wing length (15) (w-length);
- average wing cell length $((13 + 14)/2)$ (w-cell-l);
- wing base length (w-base-l);
- wing area.

Scale counts

We examined whether selection for eyespot shape lead to changes in the matrix of cover scales, that is whether the arrangement of scales in the wing-cell of the selected eyespot, had changed in FAT and THIN wings. 70 wings (around 35 per

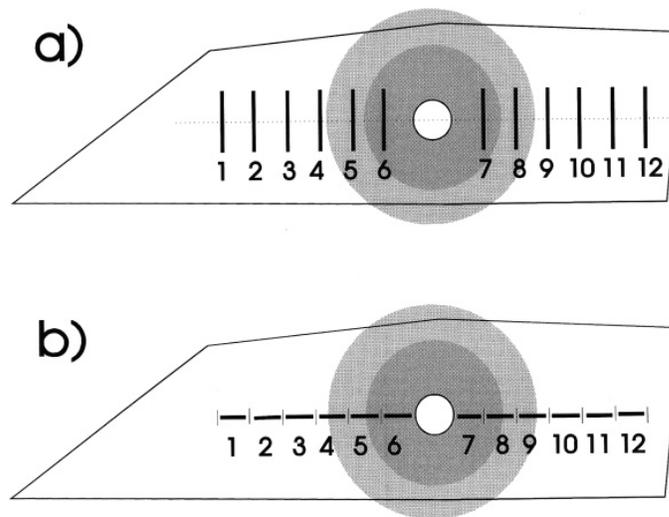


Fig. 2. The wing-cell of the selected posterior eyespot. Scale counts were carried along a) vertical transects of 100 micrometer units (100 m.u. = 1.25 mm), separated by 50 m.u. and b) consecutive horizontal transects of 50 m.u. along the wing-cell midline. The measurements were made to either side of the white pupil, starting from its outer edge.

selected line, 17 for each sex) were attached to microscope slides with Euparal and the number of cover scales were counted in two perpendicular axes, at regular intervals along the wing-cell, starting at the outer edge of the white pupil of the eyespot (Fig. 2). The scale density in the scale rows which run anterior-posterior was measured every 50 micrometer units (100 m.u. = 1.25 mm). This *vertical scale density* was scored as the number of cover scales per 100 m.u. centred around the wing-cell midline (Fig. 2a). The *horizontal scale density* along the wing cell midline was estimated by counting the number of scale rows that fitted in every 50 m.u., using the white pupil as the starting point (Fig. 2b). The measure of vertical scale density includes half the total number of scale building cells present in the row on the pupal wing (the other half form the ground-scales; Nijhout, 1980b).

Results

The change in eyespot shape

Shape was measured as the ratio of the eyespot width to height (diameter along wing-cell midline to diameter across wing-cell midline), for both eyespots on the dorsal surface (Monteiro et al., 1997). The shape of the selected posterior eyespot, in each of its components – the outer gold ring, black disc and white pupil – changed in the selected lines relative to STOCK

butterflies (Fig. 3). Within a line, however, an eyespot had always a “fatter” outer diameter compared to the shape of the more interior black disc and white pupil. The anterior eyespot diverged in shape only in the FAT line where it became “fatter” in both its black and outer ring diameters. For both anterior and posterior eyespots and within lines, males had “fatter” eyespots than females. There was no difference in the size (area) of the selected eyespot between the two selected lines ($F = 0.55$, $p = \text{n.s.}$, $DF = 1, 458$; from a GLM analysis with line and sex as factors).

Discriminant analysis

Differences in wing area occurred between the lines (males: $F = 35.97$, $p < 0.001$, $DF = 2, 292$; females: $F = 50.23$, $p < 0.001$; $DF = 2, 365$): mean wing areas for FAT, THIN and STOCK were 145, 156, and 165 mm² for males and 190, 197 and 220 mm² for females, respectively. For the analysis to discriminate the wings in terms of shape and not size it was necessary to correct each variable for overall wing size. All raw linear measurements and areas were first transformed into natural logarithms. This ensures that the variances of individual measurements are independent of their mean values and linearizes allometric relationships among characters (Bookstein et al., 1985; Strauss, 1987). A pooled within-group regression of the logged linear measurements on logged area (see Fig. 4) was used to produce size-free variables (the residuals). An analysis of covariance was first performed on each of the variables separately to check if there had been any change in the slope (in the allometric relationship between the linear wing measurements and wing area due to selection). All variables, except w-cell-1 in both sexes, were co-linear across the three lines. For smaller wings, w-cell-1 was shorter in FAT butterflies and longer in STOCK wings, but in larger wings the relationship was reversed. THIN wings fell in the middle of the two crossing regression lines. Despite this crossing of the lines (whether real or due to measurement error), the residuals were obtained in the same way as for the rest of the variables (thus some size-dependence will remain in the residuals for w-cell-1). Residuals from 364 females and 293 males were used in two separate analyses. Two discriminant functions were produced in each analysis. Function 1 accounted for 88.1% and 76.7% of the total between-groups variability,

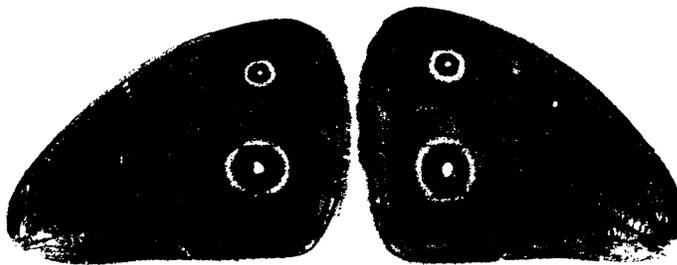


Fig. 3. The dorsal surface of a FAT (left) and a THIN forewing of *Bicyclus anynana*.

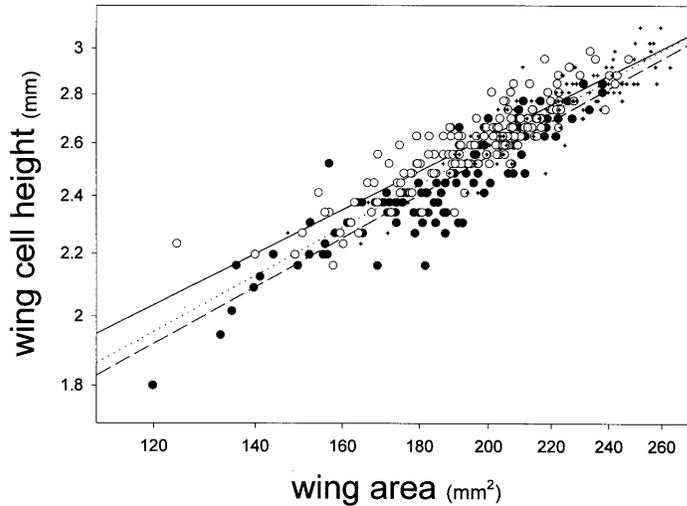


Fig. 4. Relationship between wing cell height and wing area for females of THIN (open circles), FAT (closed circles), and STOCK wings (crosses). Measurements are plotted on a base 10 logarithmic scale. Continuous, dashed and stippled lines are linear regression lines for THIN, FAT and STOCK, respectively.

in males and females, respectively, while function 2 accounted for the remaining 11.9% and 23.3%. Both functions together separated the three groups (test based on Wilks' lambda. Males: $\chi^2 = 109.3$; $df = 10$; $p < 0.0001$, females: $\chi^2 = 127.0$; $df = 10$; $p < 0.0001$) and Function 2 alone contributed to further group separation ($p < 0.01$, for both sexes). Function 1 explains 28% and 23%, in males and females respectively, of the total size-independent variance in wing shape. A further 5.0% (males) and 8.4% (females) of the variation was accounted for by Function 2.

The average scores for Function 1 were negative for FAT, positive for THIN and around zero (males) for STOCK butterflies (Tab. 1). Butterflies with an average negative discriminant score – FAT – had larger w-lengths, w-cell-l and w-base-l (i.e., those variables negatively correlated with the function; Tab. 2) and smaller w-cell-h and w-heights than those wings with an average positive score – THIN – butterflies. The latter had shorter w-lengths, w-cell-l and w-bases and larger w-cell-h and w-heights (see also Fig. 3). STOCK butterflies were intermediate.

Table 1. Average discriminant function scores.

Discriminant functions	Sex	FAT	THIN	STOCK
1	Males	-0.753	0.769	0.077
	Females	-0.757	0.515	0.162
2	Males	-0.139	-0.188	0.317
	Females	-0.111	-0.240	0.487

Table 2. Pooled within-groups correlations between discriminating variables and canonical discriminant functions.

Variables	Males (F1)	Males (F2)	Females (F1)	Females (F2)
w-cell-h	0.367*	0.048	0.767*	-0.250
w-height	0.671*	0.285	0.357	0.742*
w-length	-0.644*	0.382	-0.552*	0.294
w-cell-l	-0.109	-0.420*	-0.267*	0.059
w-base-l	-0.021	0.786*	-0.224*	-0.092

* Denotes largest absolute correlation between each variable and any discriminant function (F1 and F2).

The variables more highly correlated with Function 1 were those that varied most between FAT, THIN and STOCK wings and, therefore, contributed to a greater extent to the separation of the three groups. In males, w-length, w-height, and w-cell-h, in decreasing order of importance, most effectively separated the wings from the three groups. W-base-l and w-cell-l were poorly correlated with this function. In females w-cell-h was the most important discriminator variable, followed by w-length, w-height, w-cell-l and w-base-l.

The average scores for Function 2 were negative and quite similar for FAT and THIN and positive for STOCK. In males, the variables more strongly correlated with this function were w-base-l (positive) and w-cell-l (negative). Thus STOCK wings have a relatively longer w-base-l and shorter w-cell-l than either FAT or THIN wings. In females, w-height (positive) was the only variable more highly correlated with Function 2 than with Function 1. STOCK wings had a larger w-height relative to the selected lines.

In summary, FAT and THIN wings diverged in shape from STOCK wings. This divergence was mostly accomplished by antagonistic changes in linear wing measurements in the selected lines (Function 1). Parallel changes in wing shape occurred in each sex. In females, however, localised changes in the dimensions of the wing-cell (w-cell-h) where the selected eyespot occurred, were the most important, whereas more general wing-shape changes such as w-height and w-length were evident in males. Wing shape differences between both selected lines and STOCK butterflies (Function 2) could have arisen due to some size-dependency of the residuals which was not completely removed from the initial data regression analysis (see Material and methods above).

Scale counts

Scale density was measured in equally spaced intervals along perpendicular axes, within the posterior eyespot wing-cell, of both FAT and THIN wings. However, since THIN wings were larger than FAT wings, two measurements taken at the same absolute distance from the pupil in the two lines, will correspond to different

topological regions of the wing. To compare like with like, the absolute values of the distances from the pupil (50 m.u., 100 m.u., etc.) were divided by wing-cell size (measurement 14 in Fig. 1). The transformed data were then grouped into 13 classes prior to analysis by ANOVA. Class width was calculated by starting from the lowest corrected distance to each side of the pupil and adding increments of 0.14 units. These increments were chosen on the basis of frequency histograms of the corrected distances, so that natural groups of data (closest to the focus) would all fit into the same class. Some of the classes that were more distant from the pupil, and only contained data from females, were removed from the analysis.

A GLM with the data on vertical scale density (i.e. density along a scale row) was done using three factors (sex, line and position along wing-cell; Tab. 3a). Scale density varied with distance from the pupil; there were more scales per 100 m.u. in the distal part of the wing cell than in the proximal part. The relationship was more or less linear (Fig. 5a) until positions 13 and 14, where scale density rose steeply – the area close to the wing margin was composed of very narrow scales. FAT wings had a higher vertical scale density than THIN wings (Fig. 5a) along the whole of the wing-cell. Males had a higher scale density than females and the interaction terms were not significant (Tab. 3a).

A GLM was performed on horizontal scale density (spacing of scale rows) with the same three factors (Tab. 3b). In both lines, scale counts varied with the position along the wing cell, with a higher density of scales occurring towards the margins (Fig. 5b). Scale density was more or less constant in positions 1 through 6, when abruptly, in position 7, the density increased. Positions 7 and 9 always corresponded to measurements made in the region of black scales to either side of the white pupil (position 8 – arrow). The black scales have a characteristically uniform and tight packing, different from either gold or background scales. The density of scales from position 10 onwards increased linearly towards the margin. There was

Table 3. General linear model on scale density, with 3 factors: line (FAT or THIN), sex and position along wing-cell (13 different positions).

Source	a) Vertical scale densities			b) Horizontal scale density			c) Ratio of vertical to horizontal densities		
	DF	<i>F</i>	<i>P</i>	DF	<i>F</i>	<i>P</i>	DF	<i>F</i>	<i>P</i>
Line	1	59.65	***	1	23.98	***	1	10.95	**
Sex	1	36.53	***	1	210.66	***	1	25.80	***
Position	12	252.85	***	12	168.50	***	12	29.73	***
Line*sex	1	0.18	ns	1	1.12	ns	1	2.52	ns
Line*position	12	0.41	ns	12	2.40	**	12	1.47	ns
Sex*position	12	0.79	ns	12	2.64	**	12	1.01	ns
Line*sex*position	12	0.54	ns	12	0.56	ns	12	0.66	ns
Error	683			722			683		
Total	734			773			734		

*** $P < 0.001$, ** $P < 0.01$.

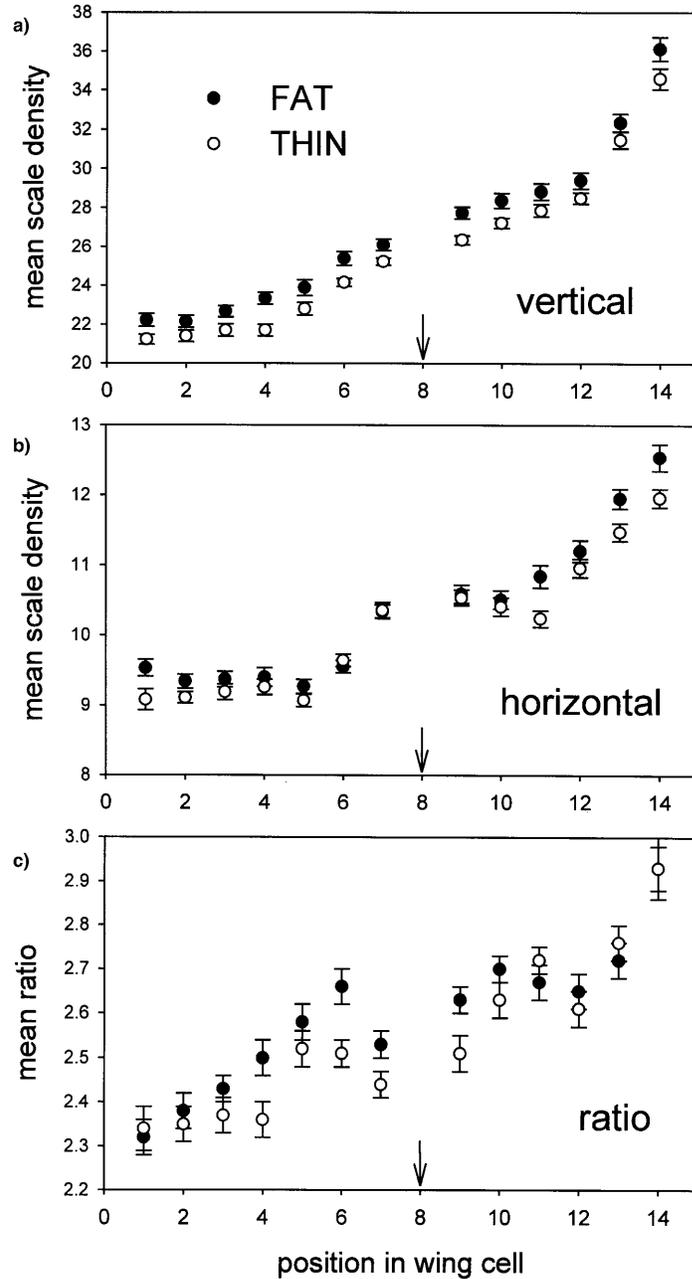


Fig. 5. Scale counts along the wing-cell of the posterior eyespot (mean values \pm one standard error) in the FAT and THIN lines (males and females combined). Arrow represents the white pupil's position. Position 1 is the one closest to the body while position 14 is at the margin of the wing. Top graph - number of scales per 100 m.u. in vertical transects along the wing-cell; Centre - scale number per 50 m.u. in horizontal transects along the wing cell; Bottom - ratio of scale densities (vertical/horizontal counts). Sample size per line in all positions except first and last in each graph is above 23 (mean = 31).

a significant line effect (Tab. 3b): FAT wings have a higher mean scale density than THIN wings. This difference, however, was not so apparent in the area bordering the white pupil but only closer to the body or to the margin (Fig. 5b). The significant interaction between line and position (Tab. 3b) was probably due to this effect. Males had a higher scale density than females and the significant interaction between sex and position was due to a smaller difference between males and females occurring close to the body (positions 1 and 2) and around the pupil (7 and 9) relative to the margin (Fig. 6).

The next and most interesting question is whether the ratio of scale densities, that is the ratio of vertical to horizontal scale counts, varies between the lines. These were calculated by dividing vertical measurements 1, 2, 3, . . . etc. (Fig. 2a) by horizontal measurements 1, 2, 3, . . . (Fig. 2b) respectively, and regrouping the data into the previously calculated distance classes. A GLM with 3 factors (line, sex and distance from focus), performed on the ratio of scale densities data showed (Tab. 3c) a significant effect of position, line and sex. None of the interaction terms were significant. Scale ratio increases towards the margin (Fig. 5c) and FAT wings have a higher average ratio than THIN wings. This difference between the lines, however, is most striking in the middle of the wing-cell, around the pupil. Males have a lower general scale ratio than females.

To understand the extent to which distortion in scale spacing could account for the change in eyespot shape we correlated eyespot shape with scale ratio. We took an average ratio of scale densities closest to the focus (average density of positions 6 and 7 for the vertical scale density and positions 5 + 6 and 7 + 8, for the horizontal scale counts; Fig. 2) and compared it to the ratio of eyespot diameters on the same 70 wings. The correlation between eyespot ratio and scale ratio was 0.49 (significantly different from zero: $t = 4.50$, $p < 0.001$). Thus a significant but

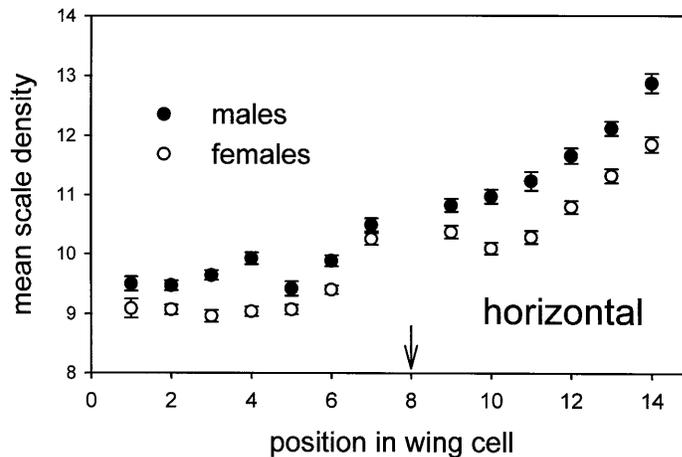


Fig. 6. Horizontal scale counts along the wing cell of the posterior eyespot (mean values with one standard error) for males and females (FAT and THIN combined). Arrow represents position of the white pupil. Sample size per sex from positions 2–14 is above 12 (mean sample size = 30).

small part of the variability in eyespot shape is associated with differences in scale spacing.

Discussion

Shape of the adult wing

The morphometric analysis of the selected lines shows that the wings have changed shape in a parallel manner to the change in the selected eyespot: THIN wings are stretched in the anterior-posterior and compressed along the proximal-distal axis while FAT wings are “flattened”. The responses to FAT and THIN selection have been largely symmetrical judging from the intermediate position of STOCK wings. The response included general changes in wing shape such as those in w-length, w-height and w-base-l, and more localised and intense changes (w-cell-h and w-cell-l) for the wing-cell within which the target eyespot is located. The localised changes, especially in w-cell-h, were more apparent in females, while the males were more effectively discriminated on the basis of more general shape changes such as w-height and w-length.

This result shows that there is additive genetic variance present for the morphology of localised regions of the wing. A similar result was found by Weber (1990, 1992) when selecting antagonistically on wing dimensions in *Drosophila*. He found that well defined allometries were easily broken. Wing regions were more easily contracted or expanded in some dimensions than others but all sub-regions of the wing revealed significant, locally acting, genetic variation. He found that very small regions (less than 100 cells across; Weber, 1992) could respond almost independently, with disproportionately smaller correlated changes happening in the remainder of the wing.

We have shown here, that within a species of butterfly there is potential for evolution of wing shape, independently of allometric size-scaling effects which seem to account for most wing shape variation within and between species (Strauss, 1990, 1991). This potential could also have been used, during evolutionary history, to adjust the wing shape of butterflies in response to selection for changes in aerodynamic performance or for particular flight patterns (Dudley and Srygley, 1994; Kingsolver and Koehl, 1994).

It is not clear why selection for eyespot shape lead to decreases in overall wing size (area) of FAT and THIN wings. The correlation between eyespot shape and wing area was negative for each sex of FAT, THIN and STOCK (but only significant for THIN females: $F = 6.16$, $p < 0.05$), implying an allometric relationship between size and eyespot shape with the “fatter” eyespots occurring in the smaller butterflies. After nine generations of selection a bias of selecting slightly smaller butterflies for the FAT line relative to THIN could account for the significant size changes. The reduced size of the selected (FAT and THIN) versus unselected (STOCK) butterflies, may be due to inadvertent selection for faster developers. A parallel study using molecular markers (M. van Eeken, unpubl. data) showed only a small loss of heterozygosity, and high egg fertility in the selected

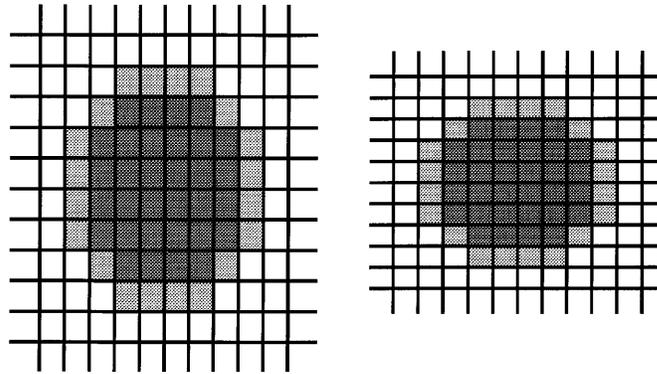


Fig. 7. The “shape” of the matrix of scale-cells around the eyespot area. Left – THIN, right – FAT. See text for explanation.

lines relative to STOCK, implying that our method of selection was effective in minimising the effects of drift and inbreeding.

Through scale counts we found that vertical scale density varied within a butterfly wing. Inside the studied wing cell, scale spacing along a row decreased from proximal towards the margin. Rows of scales also occurred closer to each other near the margin than proximally in the wing-cell. On average, throughout the wing-cell, FAT wings had a higher scale density along both horizontal and vertical axes. This could be due to a difference in the size of the epidermal cells and hence, also explain the observed differences in wing area. We also found that there were localised differences between FAT and THIN wings in the ratio of scale densities around the eyespot area. This effect can be visualised with reference to a matrix of scales, with columns and rows of rectangular cells (Fig. 7). Each rectangle corresponds to the average space taken by a single cover scale. The scale can be larger or smaller than the rectangle if it overlaps with the neighbouring scales or if it has some space around it. FAT and THIN wings differ from each other in the size of each rectangle within this matrix. The size scaling of the matrix in FAT and THIN wings occurred in a proportionate way throughout most of the wing cell but in the region around the focus, the shape of the rectangles (ratio of scale densities) changed. In this area, FAT wings have more scales within a row (vertical density) but a similar spacing of rows from that of THIN wings, i.e., the rectangles became “flatter”.

Development of the wing

The FAT and THIN lines of *B. anynana* were generated by artificial selection on eyespot shape. The elliptical eyespots reflect changes in the developmental properties of the wing epidermis, rather than asymmetry in the central focus that induces the pattern of scale cell pigmentation during the early pupal stage (Monteiro et al., 1997). The timing of early pupal development is best known in another nymphalid butterfly, *Precis coenia*, where apolysis of the pupal wing epidermis begins at 12 h

after pupation at 26 °C and coincides with the start of a period of cell divisions (Nijhout, 1980b). The appearance of rows of enlarged scale-building cells begins at 20 h and is complete 16 h later. Sporadic mitosis still occur after this period in the undifferentiated epidermal cells but no significant changes in cell number or cell arrangements take place in the period between 36 and 48 h (Nijhout, 1980b). Signalling from the focus to specify the colour pattern in *Precis* begins prior to pupation and is completed about 48 h later (Nijhout, 1980a), and thus fully overlaps these cellular events.

Our results show that change in eyespot shape is associated with corresponding changes in wing shape (particularly in the region of the eyespot) and in the matrix of wing scales comprising the eyespot. The differences in scale densities observed between the selected lines, however, cannot account for the whole change in eyespot shape. During wing development there are separate, as well as common periods, where deformation of the wing epidermis and deformation of the wing pattern can occur. The wing starts to increase in size during the larval stage (as invaginated imaginal pouches) before pattern formation takes place on the evaginated pupal wing (Nijhout, 1991). Eyespot determination overlaps the later period of wing growth, during the pupal stage. At present, it is not clear to what extent the differences in adult wing morphology result from differences in the early pupal wing (in shape and in the establishment of the rows of scale-building cells), or in the morphogenesis that occurs during and after eyespot specification, as the pupal epidermis grows and expands to form the adult wing. Similarly, a part of the change in eyespot shape may reflect asymmetry in the propagation of the focal signal through the pupal epidermis, or a subsequent deformation of the epidermis (after pattern specification).

Evolution of eyespot shape

In our FAT and THIN lines, quantitative genetic variation underlies the production of ellipsoidal eyespots, but this is not the only potential source of variation for changing eyespot shape in butterflies. The ringlet butterfly (*Aphantopus hyperantus*) an European satyrine, is polymorphic for a locus with a recessive allele that changes the shape of all eyespots in the wing margin from circular to ellipsoidal – “fat” (Ford, 1945; Collier, 1956; Revels, 1975). It is unknown whether this gene is causing similar wing-shape scale matrix changes as in *B. anynana*, in a single step, or whether it is affecting the focal determination mechanism. Genetics of species of *Heliconius*, *Hypolimnas*, *Papilio* and other genera also show that single genes can produce extensive change of colour pattern elements without influencing wing shape (see Sheppard et al., 1985; Nijhout et al., 1990 and Nijhout, 1991).

In the FAT line we found a positive genetic correlation for the shape of anterior and posterior eyespots, indicating that genes regulating scale spacing, wing growth or colour pattern formation may be shared, to a certain extent, in both areas of the wing. Genetic correlations between characters may reflect the action of natural selection in attempting to couple and restrict certain degrees of morphological

variation (especially in patterns of functional significance such as eyespots). Alternatively, genetic correlations may reflect an ancestral state of common developmental organisation of homologous patterns or wing regions (Monteiro et al., 1994). Future experiments with *B. anynana* will examine how readily anterior and posterior eyespots can be uncoupled under antagonistic selection and, thus, help in quantifying the amount of independent genetic variation affecting those eyespots.

Examination of photographs of about 60 species of *Bicyclus* showed that their eyespots are nearly always circular in shape. Only two species (*B. ena* and *B. procures*) appear to have slightly "fat" anterior eyespots. Additive genetic variation for eyespot shape (as in our stock of *B. anynana*) does not seem to have been an important factor in diversification in colour pattern or wing shape within this genus. However, this may not always be the case. Wherever changes in either colour pattern or wing shape are favoured through selection, correlated responses of the type we have demonstrated in *B. anynana* may lead to biases in the pattern of evolution of certain combinations of pattern and wing shape. The outcome will depend on the developmental and genetic basis of the correlations and on the strengths of directional and stabilising selection. Further work will examine, in more detail, eyespot and wing morphology across the genus *Bicyclus* which contains around 80 species (Condamin, 1973).

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References

- Bookstein F., B. Chernoff, R. Elder, J. Humphries, G. Smith and R. Strauss. 1985. Morphometrics in Evolutionary Biology. Academy of Natural Sciences of Philadelphia.
- Brakefield, P. M. 1984. The ecological genetics of quantitative characters in *Maniola jurtina* and other butterflies, pp 167–190. In R. I. Vane-Wright and P. R. Ackeryn (Eds.), The Biology of Butterflies. Academic Press, London.
- Collier, A. E. 1956. A new aberration of *Aphantopus hyperantus* Linn. ab. *lanceolata* Shipp. Entomologist's Rec. J. Var. 68: 1–2.
- Condamin, M. 1973. Monographie du genre *Bicyclus* (Lepidoptera Satyridae). Mem. Inst. Fond. Afr. Noire 88: 1–324.
- Dudley R. and R. B. Srygley. 1994. Flight physiology of neotropical butterflies: allometry of airspeeds during natural free flight. J. exp. biol. 191: 125–139.
- Ford, E. B. 1945. Butterflies. Collins, London.
- French, V. and P. M. Brakefield. 1992. The development of eyespot patterns on butterfly wings: morphogen sources or sinks? Development 116: 103–109.
- Holloway, G. J. and P. M. Brakefield. 1995. Artificial selection of reaction norms of wing pattern elements in *Bicyclus anynana*. Heredity 74: 91–99.

- Johnson, J. W. and E. Walter. 1978. Similarities and differences in forewing shape of six California *Catocala* species (Noctuidae). *J. Res. Lepid.* 17: 231–239.
- Kingsolver, J. G. and M. A. R. Koehl. 1994. Selective factors in the evolution of insect wings. *Annu. Rev. Entomol.* 39: 425–451.
- Kingsolver, J. G. and D. C. Wiernasz. 1987. Dissecting correlated characters: adaptive aspects of phenotypic covariation in melanization pattern of *Pieris* butterflies. *Evolution* 41: 491–503.
- Manly, B. F. J. 1994. *Multivariate Statistical Methods: A Primer*. 2nd ed. Chapman and Hall, London.
- Monteiro, A. F., P. M. Brakefield and V. French. 1994. The evolutionary genetics and developmental basis of wing pattern variation in the butterfly *Bicyclus anynana*. *Evolution* 48: 1147–1157.
- Monteiro, A. F., P. M. Brakefield and V. French. 1997. The genetics and development of an eyespot pattern in the butterfly *Bicyclus anynana*: response to selection for eyespot shape. *Genetics* 146: 287–294.
- Nijhout, H. F. 1980a. Pattern formation on lepidopteran wings: Determination of an eyespot. *Dev. Biol.* 80: 267–274.
- Nijhout, H. F. 1980b. Ontogeny of the colour pattern on the wings of *Precis coenia* (Lepidoptera: Nymphalidae). *Dev. Biol.* 80: 275–288.
- Nijhout, H. F. 1991. *The Development and Evolution of Butterfly Wing Patterns*. Smithsonian Institution Press, Washington.
- Nijhout, H. F., G. Wray and L. E. Gilbert. 1990. An analysis of the phenotypic effects of certain colour pattern genes in *Heliconius* (Lepidoptera: Nymphalidae). *Biol. J. Linn. Soc.* 40: 357–372.
- Norusis, M. J. 1985. *SPSS Advanced Statistics Guide*. McGraw-Hill, New York.
- Revels, R. 1975. Notes on breeding the ringlet: *Aphantopus hyperantus* ab. *pallens* and ab. *lanceolata*. *Ent. Rec.* 87: 283–285.
- Ricklefs, R. E. and K. O'Rourke. 1975. Aspect diversity in moths: a temperate-tropical comparison. *Evolution* 29: 313–324.
- Sheppard, P. M., J. R. G. Turner, K. S. Brown, W. R. Benson and M. C. Singer. 1985. Genetics and the evolution of Mullerian mimicry in *Heliconius* butterflies. *Philos. T. Roy. Soc. B* 308: 33–613.
- Simon, C. 1983. Morphological differentiation in wing venation among broods of 13- and 17-year periodical cicadas. *Evolution* 37: 104–115.
- Strauss, R. E. 1987. Allometry and relative growth in evolutionary studies. *Syst. Zool.* 36: 72–75.
- Strauss, R. E. 1990. Patterns of quantitative variation in Lepidopteran wing morphology: the convergent groups Heliconinae and Ithomiinae (Papilionoidea: Nymphalidae). *Evolution* 44: 86–103.
- Strauss, R. E. 1992. Lepidopteran wing morphology: The multivariate analysis of size, shape, and allometric scaling, pp 157–178. *In* J. T. Sorensen and R. Footitt (Eds.), *Ordination in the Study of Morphology, Evolution and Systematics of Insects*. Elsevier, Amsterdam.
- Weber, K. E. 1990. Selection on wing allometry in *Drosophila melanogaster*. *Genetics* 126: 975–989.
- Weber, K. E. 1992. How small are the smallest selectable domains of form? *Genetics* 130: 345–353.
- Wiernasz, D. C. 1989. Female choice and sexual selection of male wing melanin pattern in *Pieris occidentalis* (Lepidoptera). *Evolution* 43: 1672–1682.
- Windig, J. J. 1991. Quantification of Lepidoptera wing patterns using an image analyzer. *J. Res. Lepid.* 30: 82–94.

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