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Correlations between scale structure and pigmentation in butterfly wings

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SUMMARY We examined the correlation between color and structure of wing scales in the nymphalid butterflies *Bicyclus anynana* and *Heliconius melpomene*. All scales in *B. anynana* are rather similar in comparison to the clear structural differences of differently pigmented scales in *H. melpomene*. Where scale structural differences in *H. melpomene* are qualitative, they seem to be quantitative in *B. anynana*. There is a "gradient" in the density of some structural elements, the cross ribs, in the scales of *B. anynana*: black, gold, and brown scales show progressively lower cross rib density within an individual. There is, however, high individual variation in the absolute cross rib densities (i.e., scales with a particular color and cross rib density in one individual may have a different color but similar density in another individual). By ectopically inducing color pattern during early pupal development, we examined whether a scale's color and its microstructure could be uncoupled. The effect of these manipulations appears to be different in *B. anynana* and *H. melpomene*. In *Bicyclus*, "black" scales induced by wing damage at an ectopic location normally containing brown scales acquire both an intermediate structure and color between that of brown and normal black scales. In *Heliconius*, however, intermediate colors or scale structure were never observed, and scales with an altered color (due to damage) always have the same structure as normal scales with that color. The results are discussed on the basis of gene expression patterns, variability in rates of scale development and pigment, and scale sclerotization pathways.

INTRODUCTION

Mathematical modeling approaches to the evolution of butterfly wing patterns have focused on how the position, strength, and distribution of organizing sources, sinks, and gradients is established and modulated, and how it may determine eventual phenotype (Nijhout 1991; Nijhout and Paulsen 1997). Molecular developmental approaches have focused on variation of very early expression of transcription factors that later correlate with scale coloration, across species, selection lines, and seasonal forms (Carroll et al. 1994; Brakefield 1996, C. Brunneti et al. 2001). The regulation of pigment synthesis in different regions of the wing, and sometimes in different genotypes of color pattern polymorphisms, has also received some attention (Koch et al. 1998). However, the potential for variation and evolution occurring via change in scale structure and distribution, and through interactions between scale type and pigmentation, has not been considered in any detail. In this paper we begin to explore this potential through descriptive and experimental studies in two different species of butterflies. In particular, we examine whether the developmental processes that result in pigment production and in scale morphology and cuticular patterning are developmentally linked.

Butterfly wing patterns, in the visible or the ultraviolet spectrum (Eguchi and Meyer-Rochow 1983), are known to play a role in sexual selection (e.g., Bernard and Remington 1991; Meyer-Rochow 1991; Burghardt et al. 2000), in crypsis (Brakefield 1990; Brakefield et al. 2000), in thermoregulation (Kingsolver and Watt 1983; Kingsolver 1995), and in aposematism and mimicry rings (Mallet et al. 1998; Joron and Mallet 1998; Kapan 2001). The wing scales that convey those patterns are intricately designed protein-chitin extensions of epidermal wing cells, arranged in rows that run along the anterior-posterior axis of the wing (Nardi and Magee-Adams 1986). They display a single color and their particular arrangement on the wing determines the pattern characteristic of each butterfly species. Moreover, the color of scales can either originate from a pigment or from the light reflectance on the microstructure of the scale surface (Nijhout 1991). Most scale colors are caused by pigments, such as melanins (black, brown), ommatins (red, brown), and pteridines (white, yellow, red) (Nijhout 1980; Koch 1992; Koch and Kaufmann 1995). Structural colors, how-

ever, including UV reflectant and iridescent scales, are also found in many different butterfly families (Ghiradella 1984, 1985; Vukusic et al. 1999). In this case, the particular architecture of the surface of the scale determines scale color rather than pigments incorporated into the scale.

Early research on scale morphology mainly concentrated on scale shape (Köhler and Feldotto 1937; Köhler 1943), whereas later work began to examine scale structure by electron microscopy (Burgeff and Schneider 1979; Gilbert et al. 1988). For *Heliconius* butterflies, Gilbert et al. (1988) defined four distinguishable scale types, based on differences in scale morphology and pigmentation. Here we characterize "scale types" in wing scales of the tropical butterfly *Bicyclus anynana* and confirm the scale types for *H. melpomene*.

The dorsal wing pattern of *B. anynana*, an African satyrid (Lepidoptera; Nymphalidae; Satyrinae), is characterized by a series of conspicuous eyespots made up of concentric rings of white (central pupil), black, and an outer gold ring in a predominantly brown background coloration. *H. melpomene* (Lepidoptera; Nymphalidae; Heliconiinae) has mainly black wings with patches of red, yellow, and white scales. In addition to describing the normal structure and pigment of particular wing scales, we examined the structure of scales in manipulated wing pattern for both *B. anynana* and *H. melpomene*. In these species it is possible to induce a change in scale color at a broad ectopic location of the wing by piercing the wing epidermis with a fine tungsten needle during the early pupal stage (French and Brakefield 1992; Niek van Til, unpublished). Using this technique we tested whether it is possible to uncouple a scale's pigmentation from its structure (i.e., whether a shift in the coloration of a scale can occur without a corresponding change in shape or ultrastructure of the scale). Are the developmental processes that result in pigment production and in scale shape and cuticular patterning developmentally linked?

MATERIALS AND METHODS

Eggs from *B. anynana* were collected on a small maize plant from a large population of our laboratory stock (>75 females). The larvae were reared on young maize plants at 27°C ± 1°C, >80% relative humidity, and a 12:12 h photoperiod. Under these conditions the larvae develop into wet season form butterflies with conspicuous marginal eyespots (Brakefield and Reitsma 1991). Prepupae were collected daily and checked every 10–15 minutes to score individual pupation times. Subsequently the pupae were manipulated by means of micro-cautery (i.e., piercing of the pupal forewing disk with an unheated, fine tungsten needle). This was done at 12–18 h after pupation, when the effect is largest; pupae that are younger or older react less strongly to the manipulation (Nijhout 1985; French and Brakefield 1992; Brakefield and French 1995). Cauteries were done only in positions on the distal portion of the wing as no ectopic eyespots are formed in the proximal portion (Brakefield and French 1995; Fig. 1). Adult butterflies were frozen shortly after eclosion. All manipulated butterflies were screened for ectopic eyespots on the dorsal forewing surface (i.e., eyespots formed centered around the damaged epidermis, with a black center and gold ring, but no white central pupil).

Piercing pupal wings of *H. melpomene* with a tungsten needle (in this case with a heated tip) also leads to changes in the coloration of the scale cells around the site of damage (Niek van Til, unpublished). We used manipulated *H. melpomene* wings operated earlier by van Til to score scales with altered coloration, as well as fresh individuals reared in the laboratory to score normal scale structure and pigmentation. Eggs from these butterflies were collected on passion fruit plants (*Passiflora caerulea* L.), from a large population of animals reared from a stock obtained from the Amsterdam Zoo. The stock is derived from a mixture of different color pattern races of this species. The larvae were reared at 27°C ± 1°C, >70% relative humidity and a 12:12 h photoperiod. Pupae were operated at 1–3 h after pupation with a heated tungsten needle in various positions of the wing. Adults were frozen upon emergence.

A number of unmanipulated butterflies, together with some of the successfully cauterized ones, were selected for scanning electron microscopy (SEM). Their wings were cut to the appropriate size and mounted onto SEM "stubs" (specimen holders; diameter 12.5 mm, height 10 mm) with a fine pair of scissors. The cut wings were carefully attached to the stubs with silver paint (Silver Print; BAL-TEC GmbH; Wielsiepen, Germany) to ensure a good conductivity from the specimen to the stub under the electron microscope. The silver paint was left to air-dry for at least 24 h, after which color images were made of the stubs using a Leica stereo microscope (Leica Microscopy Systems Ltd.; Heerbrugg, Switzerland) (10–40×

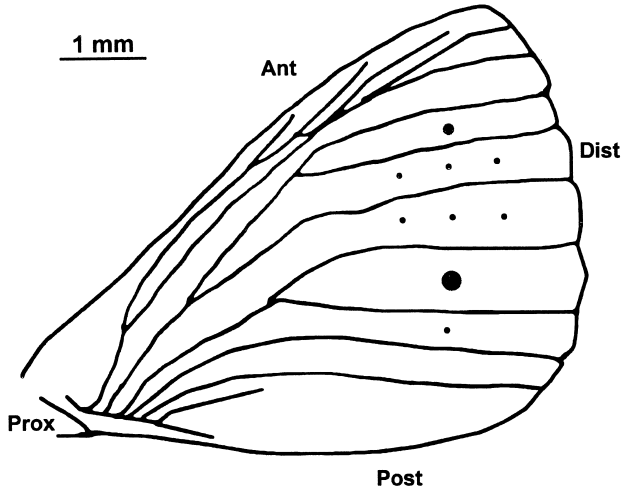

Fig. 1. Dissected pupal wing disk of *B. anynana*. The positions in which the anterior and posterior eyespot will develop are indicated via the two largest dots. Cautery positions are indicated with the smaller dots. Anterior (Ant), posterior (Post), proximal (Prox), and distal (Dist) side of the wing. The veins, visible through the pupal cuticle, and the eyespot centers, which show as small, elevated bumps on the surface of the cuticle, were used as landmarks to perform the operations. (Modified after Brakefield and French 1995.)

magnification) fitted with a DC200 (Leica Microscopy Systems Ltd.; Heerbrugg, Switzerland) digital camera. Markings were made on the wings by taking some scales off the wing surface with a fine tungsten needle. The position of these markings was indicated on the color images. Hence, the markings, together with the color images, were used to orient the wing under the electron microscope. After the silver paint had completely dried (48–72 h) the stubs were coated with gold in an E5100 sputter coater (Polaron Equipment, Thermo VG Scientific; East Grinstead, UK) for 3 min. This deposited a gold layer of approximately 10 nm over the wings (Gerda Lamers, personal communication). The specimens were viewed in a JEOL JSM 6400 scanning electron microscope (JEOL Ltd.; Akishima, Japan).

Bicyclus anynana

Scale shape was examined both with a light microscope and with the SEM, scale structure only with the SEM. A first exploration of scales of different colors in different positions on the wings did not show any large differences in shape or structure. Differences in the density of longitudinal ridges between scales of different colors were too small to distinguish scales. The scale structure of the normal wing pattern of *B. anynana*, therefore, was studied by means of differences in the more detailed elements: the cross ribs (see Fig. 2). SEM images (magnification 4300×) of differently colored scales were used to count the number of cross ribs along a scale transect 14 μm in length (i.e., the equivalent of 60 mm in an image at 4300× magnification). This was done for five standardized positions (but chosen by eye) in each scale (Fig. 2B). An average of two to three scales of each color (black, gold, and brown) was analyzed per individual. Analyzed black scales were from the center of the posterior eyespot, gold from the eyespot's outer ring, and brown scales from a proximal position adjacent to the eyespot. The dorsal forewings of seven different individuals (all female) were used for this. For each individual the same number of scales was measured for each color. We used nested analysis of variance, with scale transect position nested within scale, and scale number nested within individual to test for differences in cross rib densities between the differently colored scales.

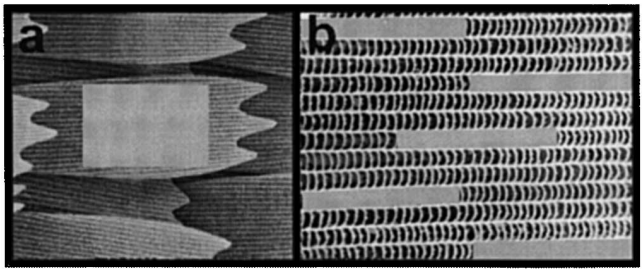

Fig. 2. Scanning electron microscopic (SEM) images at two different magnifications of cover and ground scales (hidden under the cover scales) of *B. anynana*. (A) Marked in gray is the area used for examination of the scale's ultrastructure. (B) A representative image as used for examination of these structures. The five uniformly shaded bars (each with a length of 14 μm) indicate the approximate positions used to count the cross rib densities in each scale. The horizontal structures are the longitudinal ribs, the smaller vertical structures are the cross ribs.

The cautery-induced wing pattern of *B. anynana* was examined in the same way as the normal pattern. Gold scales were not analyzed because the gold ring was very thin in some of the examined animals. The density of cross ribs in "black" scales, induced on the experimental wing, was compared with that of normal black scales at several positions and with that of brown scales at corresponding positions on the control wing. Altogether this resulted in five colors: The induced black (iB), the black of the anterior (aB) and posterior (pB) eyespot on the experimental wing, and the black (Bl) and brown (Br) of the control wing (Fig. 3). The black on the control wing was taken from the eyespot closest to the position of cautery (in most cases the large, posterior, eyespot; in only two cases, the anterior one). For all five colors three scales were analyzed per individual. The cross rib counts were again done at five positions in each scale (Fig. 2B). All but one of the nine examined animals were cauterized in one or two positions between the two eyespots (Fig.

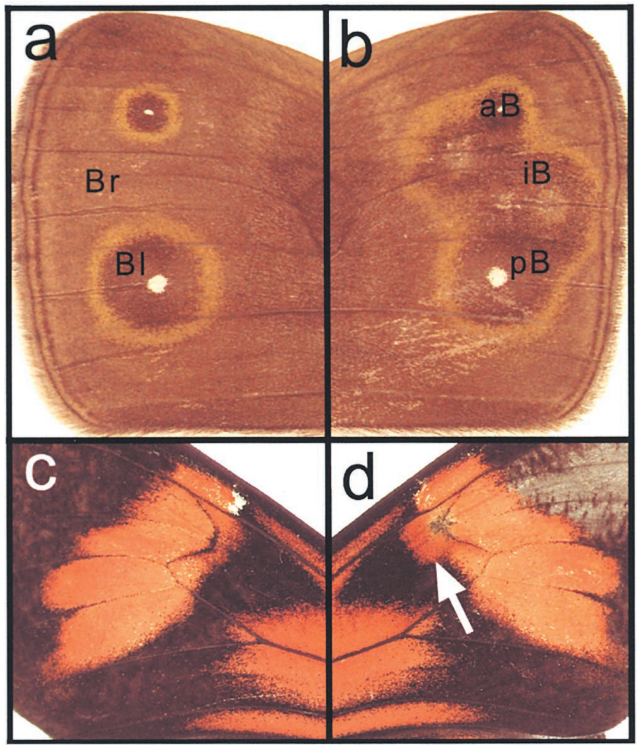

Fig. 3. Dorsal forewings of a cauterized *B. anynana* and *H. melpomene*. Control (A) and experimental (B) wing of *B. anynana*. The experimental wing was cauterized in two different positions. In these positions ectopic eyespots were formed, which fused with the normal eyespots. Control wing: Br = brown scales; Bl = black scales; experimental wing: aB = black scales from the anterior eyespot; iB = cautery induced black scales; pB = black scales from the posterior eyespot. The scales examined in the SEM are situated approximately in the areas covered by the abbreviations. (These are also the areas for which the color intensities were determined.) *H. melpomene* control (C) and experimental (D) wing. Due to cautery several red scales differentiated in a normally black area (arrow). The site of damage is visible next to the cross vein. Distal to the position of cautery is an area with partial scale loss.

1). One individual was cauterized posterior to the large (posterior) eyespot.

Color intensities of the scales around manipulated areas were compared to those of homologous positions on the control wing and to unmanipulated areas of different colors (Figs. 3A, 3B) in the same five areas where scale structures were examined. Each of the areas examined was approximately the size of the labels applied in Figs. 3A and 3B. The digital photos of the mounted wings on the SEM stubs (before gold coating) were analyzed with SigmaScan Pro 5.0 (SPSS Science; Chicago, Illinois, USA) by means of the "average intensity" measure. This measure (the average RGB value) assigns a value between zero (totally black) and 256 (pure white) to a selected area. Several other measures were also tested (e.g., average red, green, or blue), but these were all highly correlated to the average RGB value.

Heliconius melpomene

We examined the normal structure of the differently colored scales (black, red, yellow, and white) in several freshly frozen animals from our laboratory stock. The manipulated pattern was examined in some dried specimens cauterized earlier by Niek van Til. The manipulations were performed on only one wing so that the other wing could serve as a control (Figs. 3C–3D). Wings were removed from both the fresh, unmanipulated, and the older, cauterized, animals. The wings were then cut to shape and mounted, dorsal surface up, onto SEM stubs (as described for *B. anynana*). Color images were made of these stubs for later orientation. The stubs were then coated with gold and analyzed in the electron microscope.

RESULTS

Bicyclus anynana

We examined cover scales only since ground scales are covered almost completely by neighboring cover scales. In general there are no striking differences in the shape of the differently colored (cover) scales with the exception of the white scales. White scales are more densely packed on the wing surface then the other scales (Figs. 4B–4E), making the white eyespot focus easily distinguishable, even at low magnifications (Fig. 4A). There are small shape differences between scales in different positions on the wing, but these do not relate to color. After gold coating, however, black scales were often curled up. This was probably an artifact of the coating itself, as it was not observed in the digital photos made before the coating. Black, gold, and brown scales of *B. anynana* are also similar in structure. White scales, however, have large numbers of trabeculae (pillarlike structures that connect the upper and lower surfaces of a scale; Fig. 5A), which are almost completely absent in the colored scales. The only structural difference detected between black, gold, and brown scales was in the density of their cross ribs (Table 1; Figs. 5B–5D). Black scales have the largest number of cross ribs, brown the least, and gold scales are intermediate. This relationship was found in all individuals. However, among individuals, there is wide variation in the precise density of cross ribs for each color (Fig. 6A). Thus, an average number of cross ribs for a particular color in one individual can be equal to that of another color in another individual.

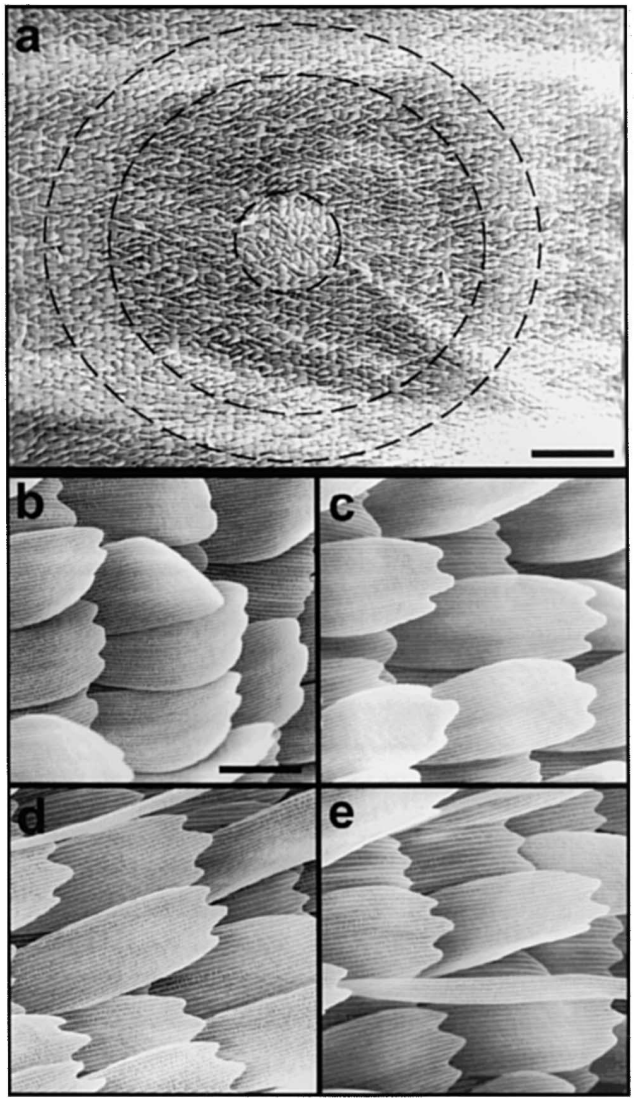

Fig. 4. Scales from the posterior eyespot of *B. anynana*. (A) An overview SEM image showing the posterior eyespot on the right dorsal forewing of *B. anynana*. In the middle the white pupil is clearly visible because of its high scale density. The black ring is also distinguishable, but this is mainly due to an artifact; most black scales are curling to one side. Scale bar, 500 μm. We have highlighted the approximate color scale boundaries with a dashed line. (B–E) Scale shapes of white, black, gold, and brown scales, respectively. At this magnification the longitudinal ridges running along the scale surface are already visible. The scale bar for these images, in (B), is 30 μm long.

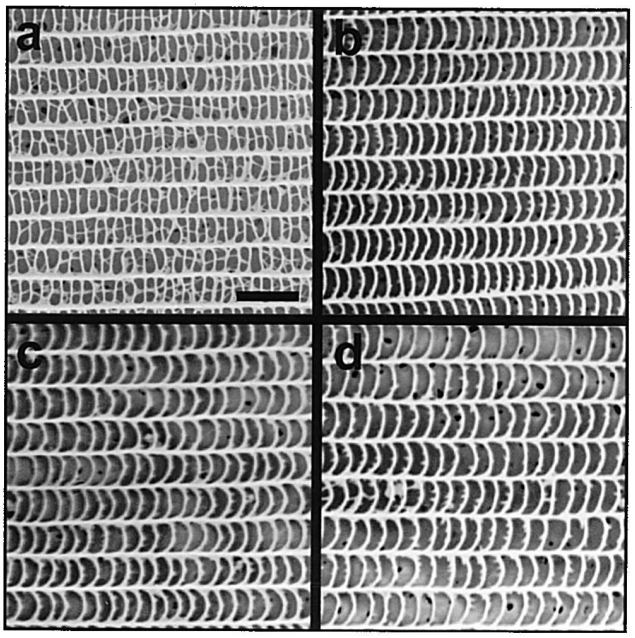

Fig. 5. Scale structure of white (A), black (B), gold (C), and brown (D) scales of *B. anynana*. In the white scales there are many trabeculae (connections of the upper and lower scale surfaces) visible in the windows formed by the longitudinal and cross ribs. The black, gold, and brown scales only differ in the density of their cross ribs. Scale bar, 3 μm.

Cautery-induced black scales have a cross rib density intermediate between that of brown and normal black scales (Fig. 6B). This relationship holds true, however, only among scales of each individual. Between individuals we find that the cross rib density of the normal black scales of one individual may be similar to that of the induced black or brown scales in a different individual. Over all wings the induced black scales have an intermediate cross rib density between the normal black and brown scales. In cauterized animals, the mean cross rib density of scales of different colors shows a negative relationship to their color intensity (Fig. 6C; $r = -0.554$, $P = 0.001$). Scales with high cross rib densities are darker (low color intensity) than scales with low cross rib densities. This relationship holds also at the individual level (not shown).

Heliconius melpomene

Scale shape in *H. melpomene* is similar across all differently colored scales. There are slight differences, however, between scales of the same color at the proximal compared to the distal end of the wing. These differences occurred in scales of all colors. The structure of white/yellow, black, and red scales on the dorsal wing surfaces of unmanipulated butterflies is comparable to that described in Gilbert et al. (1988) in four different species of *Heliconius*, including *H. melpomene*. The major scale types defined by these authors and correspondent associations with particular pigmentation was confirmed in this study (Table 2). Examination of the cauterized animals showed that scales in areas with an altered color also have an altered structure, namely, the appropriate structure for the new color. Scales in an area that should normally have been black but that is now red due to cautery have the same structure as normal red scales (Fig. 7). The same effect was found for scales that changed from red to black.

DISCUSSION

As already pointed out by Gilbert et al. (1988), it is still not clear how genes determine both color and ultrastructure in a butterfly scale. At one extreme, scale morphology and pigmentation could be determined separately, by different developmental pathways, which could make any combination of structure and color possible. On the other hand, genes that determine pigment synthesis in a developing scale may pleiotropically also determine their structure. In support of the first hypothesis, there is evidence that at least two types of pigment can be deposited in scales of *Heliconius* belonging to the same scale type (Gilbert et al. 1988). This is the case for type III scales, which can be either red or brown; type II scales, which can have two shades of black; and type I scales, which can be either white or yellow. On the other hand, support for the second hypothesis comes from the fact that in *Heliconius* there does not seem to be a completely free association of pigments with scale types, but certain pigments (belonging to the same pathway) are always restricted to a certain scale type. Additional support for this hypothesis is also given by the results of the present study. The main findings here are as follows.

Firstly, black scales of *Bicyclus* are of a similar type (type II) as black scales in *Heliconius*. Although we did not attempt to characterize the pigments that make up the gold, brown, or black scales of *Bicyclus*, we observe that all these scales belong to scale type II, which in *Heliconius* corresponds to scales containing melanin incorporated in the walls of the ridges and cross ribs (Gilbert et al. 1988). It is possible that also in *Bicyclus* only the melanin pathway is involved with slight modifications in each of the colored scales.

Secondly, disrupting the normal pattern differentiation process by ectopically inducing scales of a different color carries along a corresponding modification of the ultrastructure of the scales. This effect, however, seems to differ in *Heliconius* and *Bicyclus* wings. Whereas in *Heliconius* the change from red to black and black to red scales carries along a change from scales of type III to type II, and type II to type III, respectively, in *Bicyclus* the changes are more subtle as they involve quantitative modifications within a similar

Table 1. Analyses of variance to detect differences in cross rib density between scales of different colors in *Bicyclus anynana*.

Colors	Source of variation	DF	Adj. MS	F value	p
Black, gold, and brown	color	2	165.491	7.78	0.003**
	individual (color)	18	23.597	2.39	0.013*
	scale (color individual)	36	9.865	8.16	0.000***
	error	228	1.209		
Black and brown	color	1	328.534	16.20	0.001***
	individual (color)	12	22.441	2.33	0.038*
	scale (color individual)	24	9.640	7.57	0.000***
	error	152	1.274		
Black and gold	color	1	108.536	4.66	0.049*
	individual (color)	12	26.036	2.67	0.019*
	scale (color individual)	24	9.739	8.08	0.000***
	error	152	1.205		
Gold and brown	color	1	59.405	2.93	0.109 ns
	individual (color)	12	22.313	2.18	0.050*
	scale (color individual)	24	10.216	8.90	0.000***
	error	152	1.147		

The counts per scale are nested within individual, which are nested within color. This nested analysis accounts for the fact that not all values can be assumed to have similar levels of variation: the five counts within single scales may show lower levels of variability than counts between scales, or those between individuals. Only the difference between gold and brown scales is not significant. In all analysis there are also significant individual differences in cross rib density for scales of the same color and different cross rib densities between scales of the same color within the same individual. *P* values indicated with NS are nonsignificant, with * are significant at the 5% level,** at the 1% level, and*** at the 0.1% level.

scale type (type II). The differences between the differently colored scales at the ultrastructure level are small and relate to differences in the density of cross ribs. Black scales having the highest density of cross ribs, gold intermediate, and brown the lowest density.

Developmental mechanisms to explain the relative coupling of a scale's pigment to its ultrastructure in *Heliconius* have been put forward by Gilbert et al. (1988) and involve a genetic switch mechanism coordinated by 2 "selector genes," *M* and *X*. These genes would regulate morphological decisions and pigment pathways simultaneously. For type I scales (structural white) these authors proposed a null state that develops if neither selector gene is switched on. If selector gene *M* is turned on then type II scales develop. If selector genes *M* and *X* are turned on simultaneously then type III scales develop. In this model, selector gene *X* can only be expressed in *M*-expressing cells. In addition to these two genes, however, a couple of extra regulatory genes have to come into play for the different pigments to be synthesized within each of the scale types, as mentioned above.

Scale color differences in *Bicyclus anynana*, apart from the white pupil scales, seem to be generated within a single scale type as defined by Gilbert et al. (1988). Here, we are mostly concerned, then, with the developmental decisions that follow those of the putative *Bicyclus M* homolog (leading to type II scales throughout the wing with the exception of the white pupil scales). Recent research, however, has revealed that the domain of expression of two transcription factors (proteins that regulate the activity of other genes by binding to nuclear DNA) maps very closely with the putative black and gold scale domains of an eyespot in *B. anynana*. Antibodies against *Spalt* (*Sal*) and *Engrailed* (*En*) proteins are found at high concentration in the black and gold ring eyespot domains, respectively (C. Brunetti et al. 2001). Both gene products were also found in the eyespot focus, where white scales will differentiate. To extrapolate from this information and develop a working model of scale development we propose the following two alternative mechanisms:

1. The different colors (black, gold, and brown) are each caused by a *different* pigment but possibly are all involved in the same melanin pathway. Modifications of this pigment pathway may be controlled by the *Sal* and *En* selector genes. As a consequence of the pleiotropic and direct action of these genes, a separate developmental cascade could lead to the particular pattern of cuticular sclerotization and to the cross rib densities observed.
2. The black and brown scales are caused by different amounts of the *same* pigment. The same may also apply to the gold scales, but here a slight modification of the pigment may be necessary. Since it is likely that in all these scales the pigment is found inside the cuticular mass of the scale (Downey and Allyn 1975; Gilbert et al. 1988; Nijhout 1991), more cuticle mass may contain more pigment. Because the black scales have a denser cross rib structure, they can incorporate more pigment molecules than the brown or gold scales, which have a less dense structure. Absolute cross rib densities in

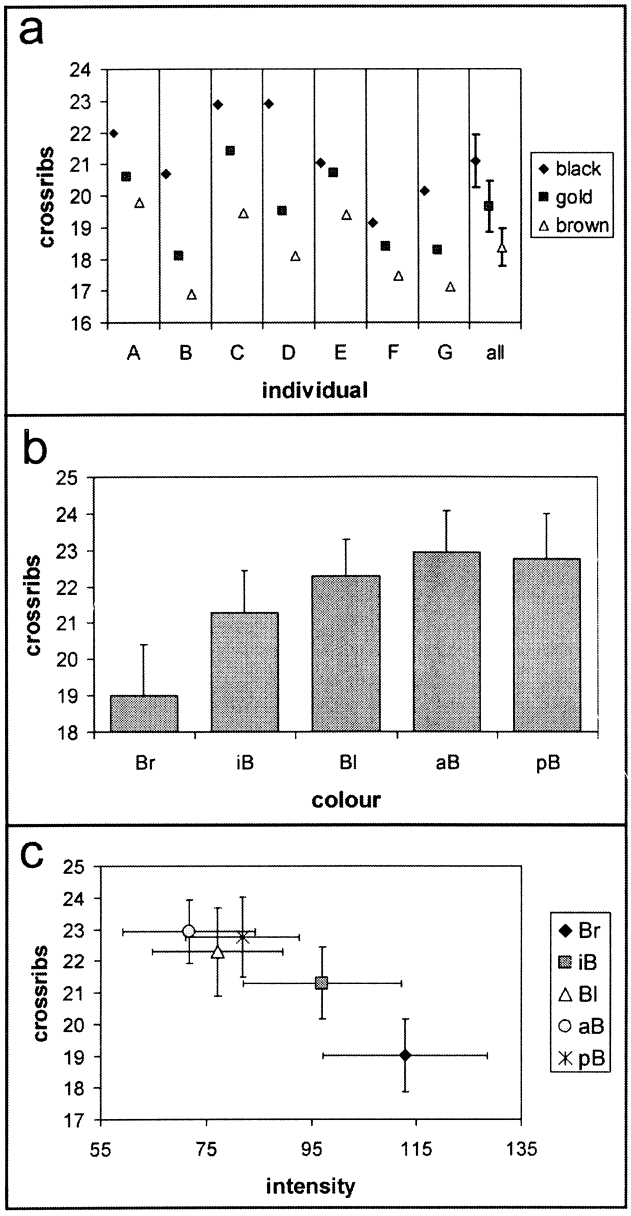

Fig. 6. Relationship between scale cross rib density in *B. anynana* and scale color. (A) The mean number of cross ribs for each scale color measured in seven wild-type individuals (A–G) and for all of them combined. Only one scale of each color was analyzed from individual A. Five scales of each color were analyzed from individual E. From all other individuals we examined two or three scales per color. Bars (only for the combined data of all individuals) represent 95% confidence intervals, which are corrected for the fact that the data are nested. (B) Mean cross rib density for scales of different colors in cauterized wings. Data are shown as overall means of nine manipulated individuals, with three scales per color analyzed from each individual (five measurements per scale). Bars represent 95% confidence intervals, based on mean cross rib densities per individual. See Fig. 3 for color notations. (C) The relationship between cross rib density and color intensity. Data points represent the five different black and brown areas in cauterized *B. anynana* butterflies (see Fig. 3 for explanation of legend.) The data are given with 95% confidence intervals, based upon an average cross rib value for each of the nine examined individuals. Cross rib density was calculated based on three scales per color per individual. Confidence intervals for color intensity are based on the nine average color values from each individual. Color intensity and cross rib density were measured in the same area.

Table 2. Scale types and pigmentation in *Heliconius* butterflies (from Gilbert et al. 1988).

	Pigmentation				
Scaletype	None	Yellow	Red	Brown	Melanic
I	X/0	X/0			
II					X/0
II′					X/–
III			X/0	X/–	

X: possible pigmentation of a scale and the matching scale type(s) found by Gilbert et al. (1988) 0: likewise, but as found in this project just for *H. melpomene*. The minus sign (−) indicates scales that were not examined in the present study. Yellow pigmentation is caused by 3-OH kinurenin; red and brown by ommatins; black by a melanin.

scales, however, are insufficient to determine their coloration when comparisons are made across individuals. To accommodate this observation, we propose the following mechanism: selector genes, *Sal* and *En*, are involved in controlling pleiotropically two separate developmental cascades, one leading to the production of a single pigment, the other leading to the cuticular sclerotization pattern. The genetic switch, however, has to have the following properties: the selector gene that determines a denser cross rib structure (e.g., *Sal* in the black scales) also directs the production of large amounts of pigment. The alternate selector gene that determines a wider spacing between the cross ribs (e.g., *En* in the gold scales) also directs the production of small amounts of pigment. Now we have to envisage that the evolutionary process is continually generating new variants within a population. If neutral, these variants may be maintained as part of the standing genetic variance in the population. In this case, variability within each of the developmental pathways is likely to exist in effector genes downstream of the initial selector gene switch (Carroll 1994; Stern 2000). These genes help modulate or modify the absolute value of the phenotype at the end of each of these cascades. This makes it possible to envisage scales with similar cross rib densities but displaying different colors, or vice versa, even when only the single pigment is involved. Evolution of downstream effector genes would also have to play a role in the first hypothesis to explain variability across individuals.

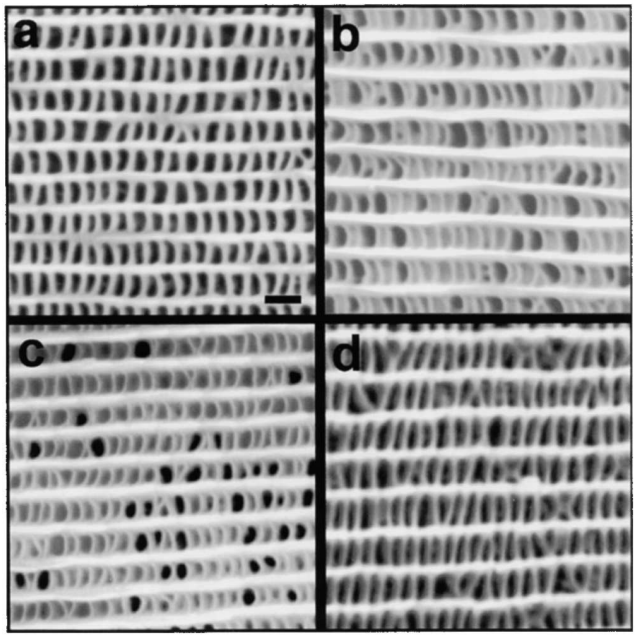

Fig. 7. *H. melpomene* scale structure of normal black (A), induced red (B), normal red (C), and induced black (D) scales. The induced red scales are in a position on the wing where the scales normally (i.e., without manipulation) would have become black and vice versa. There are no visible structural differences between normal and induced scales of the same color. Scale bar, 1 μm.

But how do we relate these hypotheses with what is known about ectopic eyespots produced through wing damage? Although the effect of wing damage at the cellular level in *B. anynana* has never been investigated, Takayama and Yoshida (1997) and Takayama et al. (1997) have looked at the effects of wing cauteries on the small white butterfly *Pieris rapae*. This butterfly is mostly white with black wing tips. By cauterizing the pupal wing area where black pigment would normally develop, at 1–3 days after pupation they obtained a roughly circular patch of white scales, of varying diameters, within the black wing tip area. By analyzing through histology and microscopy the pupal wing epidermis after cautery was applied, they also found a roughly circular patch of epidermal cells with delayed development. The diameter of this patch of cells steadily reduced as the wings were observed at increasingly later times after the damage was applied. The authors argued that rates of scale development were instrumental in determining the final color pigment of the scales. They also observed that white scales were either developing faster (the normal, noncauterized scales) or slower (the induced white scales within the black wing regions) than black colored scales. The decision of whether a cell produces one or the other pigment would depend on that cell's developmental state at a particular threshold developmental period. From experiments on the nymphalid butterfly *Precis coenia*, it is known that scale color development is set against a declining ecdysteroid hormone titer in the late pupa (Koch 1995). Hormone level could provide such a threshold in the process of making a scale coloration decision.

In *B. anynana* differently colored scales also mature at different rates (Koch et al. 2000). Maturation refers to the process of synthesizing the actual pigment and becoming rigid enough to stand upright when a pupal wing is air-dried one day prior to adult emergence. In *B. anynana* the white pupil scales mature first, followed by the gold ring scales, followed by the black disk scales, and finally by the brown background scales. If early in the pupal period, when damage is applied, the scale building cells delay their development, this may have consequences later in development and in the final color pigment synthesized by these cells.

Future research on localization of the *En* and *Sal* genes in areas following wing damage in *Bicyclus anynana* would help elucidate whether their expression is necessary for color and ultrastructure differentiation or whether there are alternative mechanisms that lead to the same phenotype, bypassing the expression of these transcription factors. These mechanisms would be based on an overall delay in scale development and would imply that the function of the transcription factors could be nothing more than a control mechanism that either accelerates or delays rates of scale development. We are currently pursuing this hypothesis by cooling a small area of the wing during the pupal stage to delay local epidermal cell development. In addition, experiments with known translation inhibitors such as RNAi and morpholinos, targeting *En* and *Sal*, will help determine their causal involvement with the differentiation of the differently colored scales. On another front, characterization of the different colors in terms of their pigment composition is needed for *Bicyclus anynana* to enable us to differentiate between the two above hypothesis. Work focusing on the later stages of pigment deposition, pigment synthesis, enzyme regulation, and the role of insect hormones in specific pigment synthesis pathways has been developed in other butterflies (Koch and Nijhout 1990; Ishizaki and Umebachi 1990; Koch 1991; Hiruma and Riddiford 1993; Koch and Kaufmann 1995; Koch et al. 1998). Future work should attempt to link the information of scale developmental rates in normal and cauterized wings with that of early selector genes, operating in the first hours after pupation, with scale sclerotization and pigment synthesis pathways, operating during the whole pupal period and during the last days of pupation, respectively, for a full understanding of the whole scale differentiation process. Divergence and differentiation in the genetics and development of the color scales, including their fine structure, probably makes an important contribution to the evolution of the dramatic morphological diversity of butterfly wing patterns.

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