

# Female *Bicyclus anynana* butterflies choose males on the basis of their dorsal UV-reflective eyespot pupils

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Sexual and natural selection pressures are thought to shape the characteristic wing patterns of butterfly species. Here we test whether sexual selection by female choice plays a role in the maintenance of the male wing pattern in the butterfly *Bicyclus anynana*. We perform one of the most extensive series of wing pattern manipulations in butterflies, dissecting every component of the ‘bulls-eye’ eyespot patterns in both ventral and dorsal wing surfaces of males to test the trait’s appeal to females. We conclude that females select males on the basis of the size and brightness of the dorsal eyespot’s ultraviolet reflecting pupils. Pupil absence is strongly selected against, as are artificially enlarged pupils. Small to intermediate (normal sized) pupils seem to function equally well. This work contradicts earlier experiments that suggest that the size of dorsal eyespots plays a role in female choice and explains why male dorsal eyespots are very variable in size and often have indistinct rings of coloration, as the only feature under selection by females seems to be the central white pupil. We propose that sexual selection by female choice, rather than predator avoidance, may have been an important selective factor in the early stages of eyespot evolution in ancestral Lepidopteran lineages.

**Keywords:** sexual selection; butterfly; *Bicyclus anynana*; wing patterns; ultraviolet; eyespots

## 1. INTRODUCTION

There is spectacular diversity in wing patterns across the Lepidoptera. Despite this diversity, wing patterns are fairly invariant within a species. Museum collections of specimens from a 100 years ago still look identical to specimens collected today. Yet, when species are brought into the laboratory and are subject to artificial selection, they display large amounts of additive genetic variance for wing patterns and can rapidly evolve into phenotypes more characteristic of different species (reviewed in Brakefield 1998; Beldade & Brakefield 2002; McMillan *et al.* 2002). What selective factors are maintaining a species characteristic pattern in natural populations?

Wing colour and colour patterns in the Lepidoptera play a role in both inter- and intra-specific communication as well as in sexual selection (Silberglied & Taylor 1973, 1978). In addition to ‘visible’ colour, the wings of many butterflies bear a wide variety of ultraviolet (UV) reflective patterns that are usually confined to dorsal wing surfaces (Silberglied 1979). Butterflies have excellent colour discrimination across a broad visible and UV spectrum (Kinoshita *et al.* 1999; Briscoe & Chittka 2001) and sexually dimorphic patterns are often better expressed in UV light, rather than in visible light (Silberglied 1979). These patterns are also more pronounced in males than in females of the same species, as seen in many nymphalids and pierids (Eguchi & Meyer-Rochow 1983), suggesting their maintenance via female choice.

The wing pattern of the African butterfly, *Bicyclus anynana* (Nymphalidae, Satyrinae), consists of a series of marginal eyespots on both the ventral and dorsal wing

surfaces and displays relatively little sexual dimorphism. The nine marginal eyespots present on the ventral wing surfaces are exposed in a resting butterfly and are thought to play a role in predator deflection (Brakefield & Larsen 1984), whereas the dorsal eyespots are not visible while a butterfly is at rest. It has been suggested that the dorsal forewing eyespots play a role in sexual selection as experiments indicated that females appear to choose males with larger dorsal eyespots (Breuker & Brakefield 2002). These experiments, however, were primarily targeting the effect of fluctuating asymmetry on female choice and were not specifically devised to test the effect of symmetrical male wing pattern variation on female choice. Here we re-examine eyespot size, as well as male size, and 10 other independent permutations of a male’s wing pattern, paying special attention to UV patterns on both dorsal and ventral surfaces, and ask which, if any, of these colour patterns play a role in female choice.

## 2. MATERIAL AND METHODS

### (a) *Experimental animals*

A *Bicyclus anynana* laboratory colony was established in Buffalo, New York, in 2002, from hundreds of eggs collected from a laboratory colony in Leiden, The Netherlands, which were originally established from 80 gravid females collected in Malawi in 1988. A mutant colony, *Missing*, was also established in Buffalo from eggs from the colony in Leiden from a spontaneous laboratory mutation that occurred in 1997. Larvae were raised on young maize plants in a climate room at 27 °C with a 12 : 12 light to dark cycle and 80% relative humidity. Males and females were separated on the day of eclosion from pupa (day 0). Females generally do not accept a mate until they are 2 days old; therefore, we gave

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single virgin females that were 2–5 days old the choice between two 2 to 5 day old virgin males in a cylindrical hanging net cage (30 cm diameter × 40 cm height). Males used in each trial were of the same age. Alternative treatments (described below) were applied to the two males. The female was added to the cage immediately prior to mate choice experiments. After a mating occurred, the pair was identified and all three butterflies were sacrificed and kept in a freezer for later analysis. Trials in which no mating occurred within three hours were excluded from the analysis. All trials were started between 11.00 and 12.00 each day and ended between 14.00 and 15.00. The trials were run in a greenhouse at approximately 27 °C. A total of 12 experiments were carried out, each consisting of approximately 50 trials. Each experiment was conducted over a consecutive 10-day period.

### (b) *Treatments*

We gave female butterflies the choice between two males that varied independently in body size, eyespot size, eyespot colour composition and eyespot UV reflectivity. We also tested males that varied in more than one trait simultaneously (figure 1). For some experiments, we chose males from the laboratory population with extreme natural trait variation; in other experiments we manipulated the pattern of males with paints and other chemicals to test trait significance. Pattern manipulations were performed on the day after eclosion and were applied using an ultra-fine paintbrush.

In order to test whether females have a preference for male body size, ventral eyespot size and ventral eyespot number, females were given the choice between large and small wild-type males (figure 1*a*), between males with large or small ventral eyespots (figure 1*b*), and between wild-type males and mutant males missing eyespots 3 and 4 on their ventral hind wings (figure 1*c*), respectively. Body and eyespot size variants were picked from the large pool of phenotypes that segregate naturally in our laboratory population. To examine whether the ventral eyespots' central pupil plays a role in female choice, we blocked the pupils by applying Testers® model enamel paint (flat black No. 1149) on all nine ventral eyespots (figure 1*d*). To serve as a control for smell and colour, we applied the paint on to the black scales surrounding the pupil of all nine eyespots in control males (figure 1*d*).

Our investigations on the dorsal wing pattern tested whether females select males based on eyespot size (figure 1*e*), presence and absence of a gold ring of scales (figure 1*f*), and presence or absence of a white pupil (figure 1*g*). The first two experiments relied on pairing males with natural variation in trait size, whereas the third relied on a similar pupil manipulation to the one applied to ventral eyespot pupils.

In order to examine whether dorsal pupil size influenced female choice, we first reduced and then enlarged the eyespot's natural pupil in two separate experiments. We applied black paint around the edges of the pupil in order to reduce the pupil size of both anterior and posterior dorsal eyespots (figure 1*h*). In control males we applied the paint around the black inner disc of both eyespots (figure 1*h*). Enlargement of the eyespot's central pupil was performed by applying white Paper Mate® correction fluid on both anterior and posterior pupils on the dorsal forewings (figure 1*i*). In control males the fluid was applied to the pupils of the wild-type male (figure 1*i*) but these were not enlarged.

To test the significance of UV-reflecting pupils at an eyespot's centre on the dorsal forewing, we reduced the UV reflectivity using a 2 mg ml<sup>-1</sup> solution of rutin (quercetin

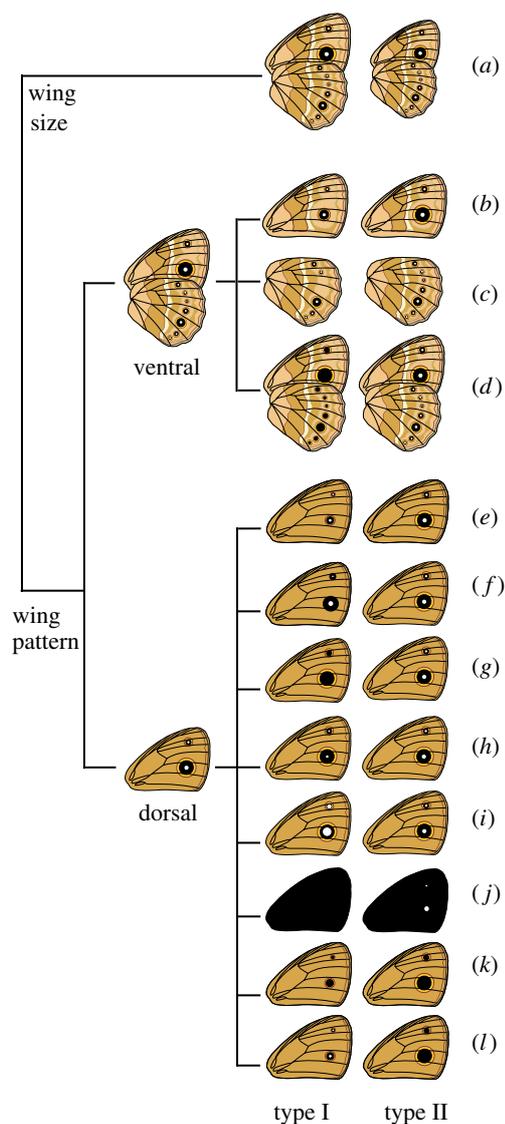


Figure 1. Females were given a choice between type I and type II males in each of twelve experiments, involving wing size differences (*a*), ventral pattern difference (*b–d*) and dorsal pattern differences (*e–l*). *a* wing size differences; *b* eyespot size; *c* missing eyespots on hindwing; *d* absence of pupil; *e* eyespot size; *f* absence of gold ring; *g* absence of pupil; *h* reduction of pupil; *i* over-expression of pupil; *j* UV-reflectivity of pupil; *k* eyespot size with no pupils; *l* eyespot size where small eyespots have pupil and large eyespots have no pupil.

dihydrate) dissolved in ethanol. Rutin is a bioflavonoid found in many plants and is a highly UV-absorbing pigment that is known to be sequestered in the wings of many butterflies during larval development (Schittko *et al.* 1999; Knüttel & Fiedler 2001). This solution was applied to both the anterior and posterior pupils on the dorsal forewing (figure 1*j*). Control males were lightly dabbed with the solution on the gold ring of each eyespot (figure 1*j*). To test if females have a preference for eyespot size in the absence of the UV-reflective white scales on both dorsal eyespots, pupils were blocked with the rutin solution in two types of male showing significant natural variation in eyespot size (figure 1*k*). In order to test the relative significance of UV pupil reflectivity and eyespot size, we blocked pupils with rutin solution in males with large eyespots and, as a control, blocked an area of similar size in the gold scales region of butterflies with small eyespots (figure 1*l*).

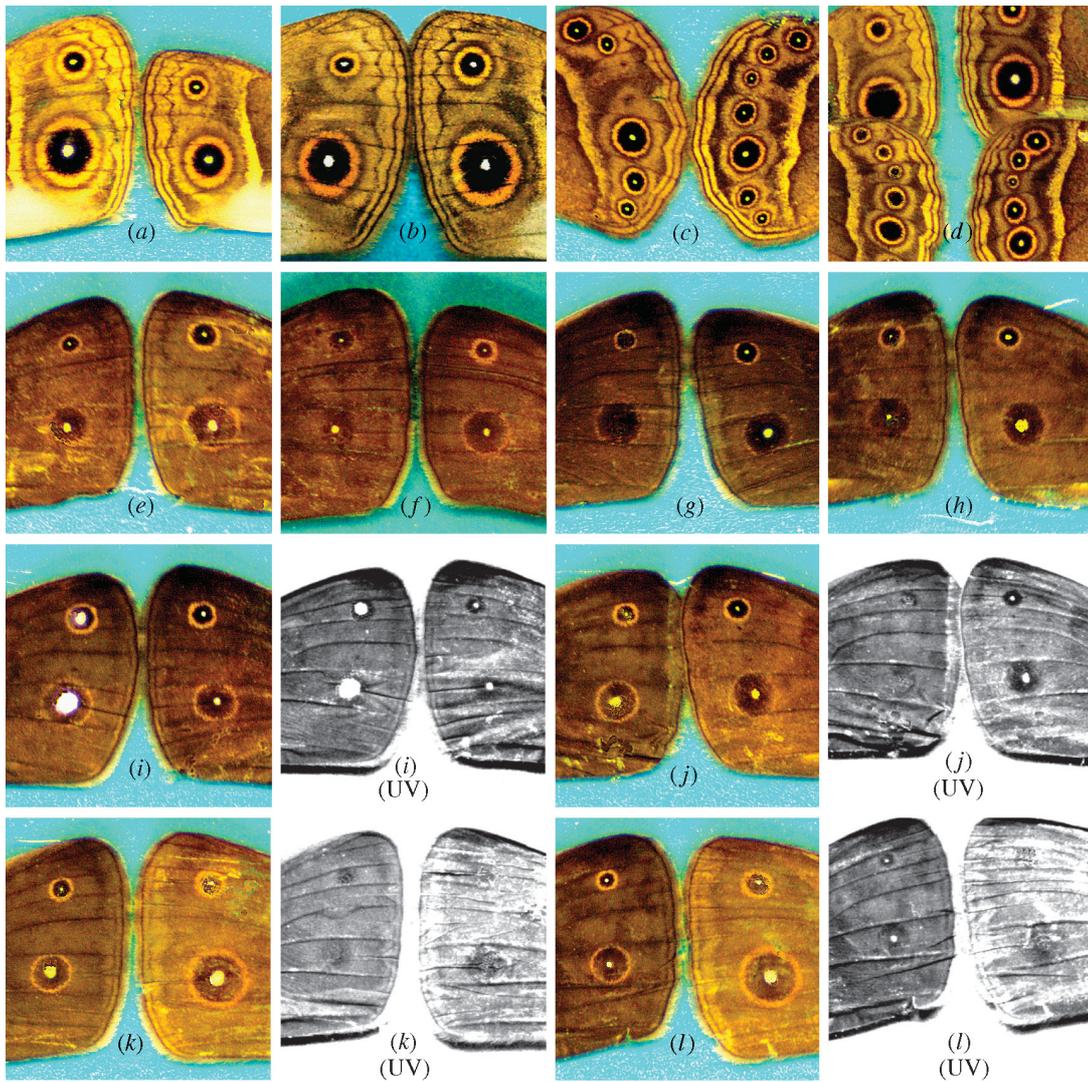


Figure 2. Representatives of the test males in each experiment outlined in figure 1. Males on the left of each panel represent type I males; males on the right are type II males. Black and white photographs show patterns reflecting in the UV.

### (c) Measurements

In order to show that the trait parameters under investigation were significantly different in the paired males in each experiment, and that all other variables were not significantly covarying with our test variable, we carried out a series of measurements in the wing pattern of males. The dorsal and ventral surfaces of all male forewings were digitally acquired with a Panasonic CCTV colour camera model WV-CL320 using BTV Pro version 5.4.1 software. Measurements were carried out without knowledge of mating order, day and trial. Diameter measurements (in mm) of the gold outer ring, the black inner disc and the central white pupil of both the anterior and posterior eyespots on both wing surfaces were carried out using Object-image version 2.08 (Vischer *et al.* 1994). The distance between anterior and posterior pupils on ventral forewings (interpupil distance) was used as an index of wing size as well as overall body size.

To detect UV pattern reflectivity, males were photographed using a Fuji Fine Pro S2 digital camera mounted with a 78 mm UV lens. All wings were first photographed in natural light and, subsequently, under UV light with a  $2' \times 2'$  UV bandpass filter that selectively transmits light with a central wavelength (CWL) of 355 nm and a full width half maximum (FWHM) of 53 nm (Edmond Optics

No. NT46-048; figure 2). UV light was provided by a Mineralight<sup>®</sup> Lamp model UVGL-58. Only long-wave UV light was used as a light source, as the short wave was not transmitted through the filter. Camera exposure was first calibrated using a dandelion and adjusted so that the photo showed good contrast between the central and peripheral flowers in the composite. The UV reflectivity of dorsal forewing pupils was analysed using the grayscale mode in Adobe Photoshop 7.0.1, after removing the red and green channels. The difference in relative brightness of pupil reflectivity was assessed using Photoshop's mosaic feature with a cell size of four pixels per square. These values were then averaged across both dorsal forewing pupils to yield an overall measure of UV pupil brightness.

### (d) Statistical analyses

To examine whether there was a female preference for one of the two paired male phenotypes, we used a sign test. Preferred males of one type were given a '+' value, preferred males of the other type were given a '-' value and the sum of the '+s' and '-s' across trials was calculated. The probability of this value being significantly different from zero, that is, of there being non-random mate choice, was obtained from the IFA (Institute of Phonetic Sciences) Web services site (<http://www.fon.hum.uva.nl/Welcome.html>).

To test for differences in wing size, eyespot colour ring size and UV reflectivity between successful (mated) and unsuccessful males, we performed a Paired-*t* test using Minitab Statistical Software, version 13.32.

### 3. RESULTS

In most trials, both males actively courted the female before she copulated with one of them. We also observed very little, if any, male–male competition over the female. Once the female became receptive to one male and the pair initiated copulation, the losing male sometimes tried to disrupt copulation but was never successful.

There was no indication that females discriminated between males with different wing sizes, different ventral eyespot sizes, seven versus five ventral hindwing eyespots or with or without pupils on their ventral eyespots (table 1*a–d*), suggesting that male ventral wing patterns and overall body size do not play a role in female choice.

When we tested pattern variants on the dorsal side we found no evidence of female preference for males with overall larger eyespots (table 1*e*), contrary to previous experiments (Breuker & Brakefield 2002). Females also did not appear to discriminate between males with or without the presence of the eyespot's outer gold ring (table 1*f*). When females were given the choice between males with pupils blocked with black paint and males with the pupils visible, they significantly preferred the latter (table 1*g*). When females were presented a choice between a male with a significantly reduced pupil and a wild-type male (table 2*h*), females showed no preference for either phenotype (table 1*h*). However, when females were given the choice between males with enlarged pupils and males with average pupil sizes (table 2*i*), females preferred the males with the average pupils (table 1*i*). In summary, females disliked males with either very large or no pupils but did not discriminate between males with small to intermediate sized pupils. These results suggest that female *B. anynana* butterflies exert moderate stabilizing sexual selection on the size of the male dorsal eyespot's central white pupil.

To the human eye, untreated pupils and those treated with rutin were indistinguishable. However, when the wings were analysed for UV reflectivity, rutin-free individuals reflected three times more UV light than rutin-treated males (table 2*j*) and females preferred to mate with the former (table 1*j*). In experiments where females were given the choice between males with different dorsal eyespot sizes with their pupils absent, there was no preference for either phenotype (table 1*k*). When given the chance to mate with males with significantly larger eyespots (table 2*l*) with the pupils blocked with rutin or with males with overall smaller eyespots but with pupils present, females significantly preferred the latter (table 1*l*). These results suggest that females can detect differences between males with varying UV pupil reflectance patterns and that males with brighter pupils are more attractive.

By having carefully chosen males with very similar phenotypes for all paired experiments, we confirmed that the only traits that were significantly different between paired males were the ones under study (table 2).

Table 1. Male types preferred by females when given a choice between two males (treatment types *a* to *l* are described in figure 1; rows in bold italics indicate experiments in which females showed a significant preference ( $p < 0.05$ ) for one of the two male types—I or II as described in figure 1).

treatment type	no. trials	no. mated type I	no. mated type II	<i>p</i> -value	choice type
<i>wing size</i>					
<i>a</i>	50	27	23	0.67	—
<i>ventral pattern</i>					
<i>b</i>	50	26	24	0.89	—
<i>c</i>	45	23	22	1.00	—
<i>d</i>	53	24	29	0.58	—
<i>dorsal pattern</i>					
<i>e</i>	50	22	28	0.48	—
<i>f</i>	50	26	24	0.89	—
<i>g</i>	<b>50</b>	<b>16</b>	<b>34</b>	<b>0.02</b>	<b>II</b>
<i>h</i>	51	24	27	0.78	—
<i>i</i>	<b>50</b>	<b>17</b>	<b>33</b>	<b>0.03</b>	<b>I</b>
<i>j</i>	<b>51</b>	<b>17</b>	<b>34</b>	<b>0.02</b>	<b>I</b>
<i>k</i>	50	25	25	1.11	—
<i>l</i>	<b>50</b>	<b>33</b>	<b>17</b>	<b>0.03</b>	<b>I</b>

### 4. DISCUSSION

Female *B. anynana* choose males on the basis of the size and UV reflectivity of the dorsal eyespot's central white pupil. The lack of a preference for other pattern elements, however, cannot be ruled out entirely. It is possible that under other experimental conditions, that is, larger number of trials and larger flight cages, different mating preferences could have been observed. Ventral eyespot patterns do not seem to play a role in sexual selection and their presence is probably best explained by their role in predator deflection as suggested in previous work (Lyytinen *et al.* 2003). Most studies to date involving UV-influenced mate choice have focused on species that possess extremely conspicuous UV wing patterns. For example, the majority of research has focused on the lycaenid and pierid families whose members have extensive patches of UV wing coloration (Meyer-Rochow & Jarvilehto 1997; Knüttel & Fiedler 2001). Although a recent study has shown that small patches of coloured scales play a role in male mate choice (Fordyce *et al.* 2002), our study is the first to show that small UV-reflective patches of scales significantly influence sexual selection by female choice.

In butterflies, receptivity usually involves a combination of visual and olfactory stimuli; the use of pheromones being more important close up and visual signals being more important at a distance (Silberglied 1984). During courtship, a male *B. anynana* approaches a female at an angle to her side and opens and closes his wings rapidly, apparently in an attempt to spread a plume of pheromones. Next, he flutters around her, his wings flickering in rapid succession. It is usually following this stage that the female actively rejects a courting male, suggesting that she may be actively choosing a male based on visual cues. Many insects are sensitive to interrupted or 'flicker' photic stimulation (Meyer-Rochow 1991) and it appears that female *B. anynana* can observe such stimuli and distinguish the level of a male's central pupil's UV reflectivity.

Table 2. Wing size and eyespot colour ring size (gold, black, white) differences between type I and type II males in each treatment (*a* to *l* as shown in figure 1; rows in bold italics indicate significant differences ( $p < 0.05$ ) in trait size between type I and type II males.)

treatment type	average anterior eyespot measurements (mm)			average posterior eyespot measurements (mm)			wing size (mm)	
	gold	black	white	gold	black	white	interpupillary distance	pupil reflectivity (%)
<i>wing size</i>								
a	I=1.42, II=1.38	I=1.03, II=0.99	I=0.21, II=0.22	I=2.29, II=2.25	I=2.00, II=2.01	I=0.42, II=0.42	<b>I=5.08,</b> <b>II=4.28</b>	—
<i>ventral pattern</i>								
b	<b>I=1.04,</b> <b>II=1.43</b>	<b>I=0.80,</b> <b>II=1.05</b>	<b>I=0.15,</b> <b>II=0.27</b>	<b>I=1.44,</b> <b>II=2.42</b>	<b>I=1.70,</b> <b>II=2.01</b>	<b>I=0.22,</b> <b>II=0.42</b>	I=4.34, II=4.54	—
c	I=1.39, II=1.39	I=1.01, II=1.03	I=0.27, II=0.25	I=2.36, II=2.37	I=2.11, II=2.08	I=0.45, II=0.45	I=4.59, II=4.67	—
d	I=1.67, II=1.75	I=1.15, II=1.17	<b>I=0.00,</b> <b>II=0.37</b>	I=3.30, II=3.30	I=2.46, II=2.47	<b>I=0.00,</b> <b>II=0.61</b>	I=4.79, II=4.80	—
<i>dorsal pattern</i>								
e	<b>I=1.05,</b> <b>II=1.52</b>	<b>I=0.80,</b> <b>II=1.05</b>	<b>I=0.22,</b> <b>II=0.42</b>	<b>I=1.70,</b> <b>II=2.42</b>	<b>I=1.44,</b> <b>II=2.01</b>	<b>I=0.22,</b> <b>II=0.42</b>	I=4.46, II=4.54	—
f	<b>I=0.00,</b> <b>II=1.45</b>	I=1.00, II=0.99	I=0.24, II=0.23	<b>I=0.00,</b> <b>II=2.33</b>	I=2.00, II=2.06	I=0.42, II=0.40	I=4.78, II=4.77	—
g	I=1.08, II=1.06	I=0.84, II=0.85	<b>I=0.00,</b> <b>II=0.35</b>	I=1.69, II=1.77	I=1.50, II=1.37	<b>I=0.00,</b> <b>II=0.55</b>	I=4.51, II=4.36	—
h	I=1.53, II=1.53	I=1.16, II=1.14	<b>I=0.16,</b> <b>II=0.32</b>	I=2.49, II=2.48	I=2.13, II=2.08	<b>I=0.26,</b> <b>II=0.44</b>	I=4.79, II=4.78	—
i	I=1.42, II=1.36	I=1.06, II=1.03	<b>I=0.53,</b> <b>II=0.25</b>	I=2.31, II=2.29	I=2.03, II=2.00	<b>I=1.02,</b> <b>II=0.48</b>	I=4.79, II=4.69	—
j	I=1.70, II=1.73	I=1.14, II=1.15	I=0.35, II=0.36	I=3.33, II=3.29	I=2.47, II=2.47	I=0.63, II=0.62	I=4.76, II=4.79	<b>I=33,</b> <b>II=100</b>
k	<b>I=1.01,</b> <b>II=1.54</b>	<b>I=0.79,</b> <b>II=1.05</b>	I=0.00, II=0.00	<b>I=1.41,</b> <b>II=2.51</b>	<b>I=1.41,</b> <b>II=2.10</b>	I=0.00, II=0.00	I=4.59, II=4.65	—
l	<b>I=1.14,</b> <b>II=1.64</b>	<b>I=0.80,</b> <b>II=1.15</b>	<b>I=0.21,</b> <b>II=0.00</b>	<b>I=1.65,</b> <b>II=2.50</b>	<b>I=1.36,</b> <b>II=2.02</b>	<b>I=0.22,</b> <b>II=0.00</b>	I=4.54, II=4.60	—

Our results differed significantly from previous work by Breuker & Brakefield (2002). They found that wild-type females preferred to mate with males possessing wild-type eyespots rather than males with experimentally reduced eyespots. In an effort to examine whether female *B. anynana* chose mates based on left–right symmetry of their eyespots, the researchers manipulated eyespot size variation by damaging the focal (central signalling) area of both dorsal forewing eyespots three hours after pupation (Breuker & Brakefield 2002), leading to complete or partial reduction of the adult eyespots. Many males within the small eyespot category, however, may have possessed eyespots with no UV-reflecting pupil as early focal damage can reduce the eyespot to just its peripheral gold scales or completely eliminate it (French & Brakefield 1992). Therefore, males possessing the largest eyespots would have contained UV-reflecting pupils while the focal damage eliminated the pupil in the smallest eyespots. This may explain why females preferred males with the largest eyespots.

There is extensive variation in male dorsal eyespot characteristics within our laboratory stock population of *B. anynana*. For example, some eyespots are small, some large, and many lack the outer gold ring of scales. In some cases, the male's dorsal white pupils are also absent (as well as the other colour rings). This suggests that selection, rather than constraints on the generation of males without dorsal pupils, is the most important factor in maintaining the white pupils in our stock population.

Our study suggests that female preference for pupil size is stabilizing but only when pupil size falls beyond extreme values, that is, females show no preference for males with either very small or intermediate pupil sizes but strongly discriminate against those with very large or no pupils on their dorsal forewings.

In contrast to male dorsal eyespots, females within our population possess dorsal eyespots with clearly contrasting rings of coloration. It is unknown why females retain these dorsal eyespots and why they remain so conspicuous. Explaining the maintenance of dorsal eyespots in females awaits further experimentation.

We found no evidence that females choose their mates based on ventral wing patterns. Instead, the marginal eyespots on the ventral wing surfaces of many Lepidoptera are assumed to function as a means of avoiding predation by misdirecting the attacks of predators away from the body and towards the wing margin (Blest 1957; Wourms & Wasserman 1985; Lyytinen *et al.* 2003).

In considering the early stages of eyespot evolution, it has been proposed that the 'bull's eye' concentric ring structure evolved from simpler spot patterns (Nijhout 1994; Brunetti *et al.* 2001). Spots of typically white scales are still present today in the centre of the wing, straddling a cross-vein, in many moths and also in some pierid butterflies (i.e. the elephant hawk-moth, *Deilephila elpenor*, and the Clouded Yellows and Clouded Sulphurs, *Colias* sp.). Since it is unlikely that such patterns are effective at deflecting attacks towards the wing margin, due to their

central placement on the wing and their inaccurate mimicry of vertebrate eyes, they require a different selective explanation for their occurrence and persistence in these lineages. Sexual selection by female choice, as described here for *B. anynana*, could be such a selective agent. It has recently been found that hawk-moths are actually able to discriminate colour, as well as UV-reflective patterns, with high acuity in dim start-light conditions (Warrant *et al.* 1999; Kelber *et al.* 2002). Sexual selection could have maintained the early stages of eyespot evolution in such basal, night-flying, moth lineages before the 'bulls-eye' phenotype and associated predator-deflective function evolved.

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