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Female choice depends on size but not symmetry of dorsal eyespots in the butterfly *Bicyclus anynana*

Casper J. Breuker[*] **and Paul M. Brakefield**

Evolutionary Biology, Institute of Evolutionary and Ecological Sciences, Leiden University, PO Box 9516,
2300 RA Leiden, The Netherlands

The eyespots on the ventral wings of *Bicyclus anynana* butterflies are exposed when at rest and interact with predators. Those on the dorsal surface are not exposed in this way, and may be involved in courtship and mate choice. In this study, we examined whether the size and fluctuating asymmetry (FA) of dorsal eyespots are reliable signals of male quality. High developmental stability is considered to result in low FA, and to be associated with high quality. Individuals of high quality are predicted to produce sexually selected traits that are large and symmetrical, at a relatively low cost. In this study, we manipulated eyespot development to uncouple eyespot size and FA in order to examine their independent roles in signalling to the female. Individual females in cages were given the choice between two or three males differing in eyespot traits. The results indicate that although size *per se* of the eyespots is used as a signal, FA and wing size are not. We discuss the use of FA in studies of sexual selection and aspects of sexual selection on dorsal eyespot size.

Keywords: *Bicyclus anynana*; eyespots; fluctuating asymmetry; sexual selection; signal; size

1. INTRODUCTION

Selection on characters that give certain individuals a mating advantage over others of the same sex is called sexual selection. Characters under sexual selection range from traits that improve fighting ability within one sex to those that signal the quality of the bearer to the opposite sex. Traits solely involved in signalling are usually called ornaments. Examples include tail length in various bird species (Fitzpatrick 1998) and the eye stalk in stalk-eyed flies (David *et al.* 1998). Sexual selection has generally been regarded as a process that mainly affects males, because variance in mating success is typically greater in males than females (Halliday 1994). According to the handicap principle, high-quality individuals are able to produce ornaments of a particular (large) size at a relatively lower cost than lower-quality individuals (Zahavi 1975).

High-quality males are also predicted to be developmentally more stable, and thus show higher bilateral symmetry (Møller 1990; Markusson & Folstad 1997; Hunt *et al.* 1998; Pryke *et al.* 2001; but see Hunt & Simmons (1998) and Bjorksten *et al.* (2000)). Fluctuating asymmetry (FA) is a population phenomenon defined as random deviation from perfect bilateral symmetry in a morphological trait. The differences between the left and right side have a mean of zero and are normally distributed (Palmer & Strobeck 1986, 1992; Palmer 1994). Higher levels of developmental noise (DN) due to environmental or genetic stress may result in larger differences between the left and right side, hence in higher FA (Van Dongen 2001; Klingenberg 2002; Nijhout & Davidowitz 2002). When there are no *a priori* reasons to assume variation in DN between individuals, FA is postulated to reflect variation in developmental stability (DS). High-quality males are likely to be developmentally stable and, therefore, to show low FA. The relationship between male mating success and FA is, however, controversial (Watson & Thornhill 1994; Møller & Thornhill 1998; Palmer 1999; Thornhill *et al.* 1999). A male of high quality may also be large and vigorous, and have large ornaments, which could be more valuable as a signal for females than FA (David *et al.* 1998; Goulson *et al.* 1999). Furthermore, FA refers to small random differences between the left and right side, up to 10% of trait size (Palmer 1996). Indeed, the differences in FA among males may be so small that it is debatable whether they can be detected by females (Swaddle 1997). To study the reliability of FA as a signal of male quality, one ideally needs to give females a choice between males that vary considerably in asymmetry, but still within the range of natural variation (Rohde *et al.* 1997; Swaddle 1997; Uetz & Smith 1999). Furthermore, FA should be uncoupled from other traits that are potentially related to male quality, such as ornament and body size, or in general from the underlying DS (McLachlan & Cant 1995; McLachlan 1997).

In the majority of butterflies, including *Bicyclus anynana*, females are able to reject courting males (Wiklund & Kaitala 1995; Brakefield & Schneider 2002). Furthermore, male *B. anynana* butterflies show a higher variance in reproductive success than females in captivity or in laboratory cultures (Brakefield *et al.* 2001). This suggests that sexual selection may be important in this species. In this paper, we examine whether female *B. anynana* choose males on the basis of the size of their dorsal eyespots, which are potential sexually selected traits (cf. Warzecha & Egelhaaf 1995), and whether eyespot symmetry provides females with any additional information on male quality.

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[*] Author for correspondence (breuker@rulsfb.leidenuniv.nl).

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Eyespots of *B. anynana* are two-dimensional matrices of overlapping pigmented scale cells on the margins of the wings. Each eyespot is made up of a white 'pupil', a black inner ring and a gold outer ring. In the late larval and early pupal stage, groups of organizing cells called foci are established that set up, probably by morphogen signalling, information gradients in the wing, leading to formation of the adult eyespots. The size of the dorsal forewing eyespots can be manipulated by using a fine needle to pierce the pupal cuticle soon after pupation, thus damaging the focal area at an early stage of signalling (Brakefield & French 1995). This makes it possible to design an experiment in which FA is uncoupled from underlying DS, and to create a large and readily detectable variation in FA. Furthermore, the two dorsal forewing eyespots can be assumed to be secondary sexual traits (cf. Warzecha & Egelhaaf 1995) because (i) the male spends part of the courtship rapidly opening and closing his wings to the female, thereby exposing the dorsal eyespots and probably spreading a plume of sex pheromone from the exposed androconia (Brakefield & Schneider 2002), and (ii) because the dorsal eyespots, unlike those on the ventral surfaces, are under no apparent selection through visual predation (Windig *et al.* 1994; Brakefield & French 1999).

In this study, we give individual female butterflies the choice between three (manipulated) males from a single stock, which vary independently in size and symmetry of their dorsal eyespots. Following the initial choice, a second female chooses between the two remaining males. Each group of three males can thus be ranked from first to third choice, and the reliability of size and FA as a signal of male quality can be examined. In addition, body size is taken into account as it may also indicate male quality (e.g. Wiklund & Kaitala 1995).

2. MATERIAL AND METHODS

(a) *Experimental animals*

Butterflies were derived from a laboratory line labelled real-Low. *Bicyclus anynana* shows phenotypic plasticity for the ventral eyespots, but not for the dorsal eyespots. Unselected butterflies reared at 20 °C lack ventral eyespots (dry-season phenotype), whereas those reared at 27 °C have conspicuous ventral eyespots (wet-season phenotype) (Brakefield 1997; Brakefield *et al.* 1996). Butterflies of the real-Low line have a long history of artificial selection and now yield the dry-season form at all temperatures, including 27 °C (Brakefield *et al.* 1998). These butterflies were used for the experiment as they lack ventral eyespots, but still possess the dorsal eyespots (Brakefield *et al.* 1998). This avoids the potential problem that females, partly at least, choose males on the basis of ventral eyespots. The rearing and mate-choice experiments could thus be carried out under similar conditions (more details below).

A cohort of about 800 larvae was raised without food stress on young maize plants in a climate cabinet at 27 °C, 12 L : 12 D cycle and high relative humidity. Males and females were separated at pupation. The first and last animals to pupate were discarded. To increase the variance in eyespot size and asymmetry, male pupae were damaged with a fine sharp needle 3 h after pupation, either in (+) or away from (−) the focal area of both dorsal forewing eyespots. Pupae were allocated at random to one of three treatments: group 1, males were damaged away from the focal area on both left and right side (−/−); group 2, focal damage on one side and non-focal on the other (+/− or −/+); and group 3, focal damage on both sides (+/+). The phenotypic effect of the focal-cell damage varied from complete to a partial reduction of 30% in size. Overall, the treatments resulted in increased variance in both eyespot size and asymmetry (more details below). Damage outside the focal area produced no phenotypic effect.

Unmated females of 3–5 days old were given the choice between three males, one from each group, in a net cage (13 cm × 21 cm × 29 cm). Males were also unmated and 2–5 days old. After the female had mated, the pair was removed, and another female was given the choice between the two remaining males on the following day. The three males can then be ranked for mating order from first (1) to third (3) choice. The first male is thus the successful one of three, whilst the second male is then the winner of the remaining two. Any trials in which no mating occurred within 4.5 h were excluded from the analysis. All trials were started just after lights on and at the same time each day. They were run in the standard environmental conditions used for rearing. A total of 88 trials, of which 76 were fully successful, were conducted over 9 days. We thus observed 152 (2 × 76) matings.

(b) *Measurements*

The left and right dorsal surface of the wings of all males were digitally photographed twice on separate days (i.e. replicate photographs) with a Leica DC 2000 camera mounted on a Wild binocular microscope (magnification × 8.82). The order of photography and measurements was random with respect to mating order, day and trial. In addition, measurements were carried out 'blind', without knowledge of male identity. Measurements of the size (in mm^2) of the black inner ring of the anterior (A) and posterior (P) eyespots (figure 1) were carried out using SCION IMAGE (freeware from the National Institutes of Health, USA, 1998). The distance between the pupils of A and P was used as an index of wing size (WS) and male size (figure 1). Repeatability of the measurements was high (99.2–99.5%).

(c) *Statistical analyses*

FA analyses were carried out according to Palmer & Strobeck (2002). The sequential Bonferroni procedure was applied to each set of related tests to avoid making type I errors.

The focal damage treatments ensured that many males had some very small eyespots (figure 2*a*). The treatments had no effect on WS (ANOVA: $F_{1,302} = 0.62$, $p = 0.43$) (figure 2*b*). The damage substantially increased the variance in eyespot asymmetry in the total sample of 228 males compared with group 1 males with no damage to the foci (signed asymmetry values, VAR(A), total = 1.13; VAR(A), group 1 = 0.46; VAR(P), total = 1.34; VAR(P), group 1 = 0.72; Levene's heterogeneity of variance tests, p-values $\ll 0.001$) (figure 2*c*). Furthermore, for the total sample of males, both eyespots showed ideal FA (A: skew = 0.11 n.s., kurtosis = 0.59 n.s.; P: skew = 0.16 n.s., kurtosis = 0.37 n.s.) and no directional asymmetry (t-tests: p-values > 0.05). We had thus created a large population of males that varied considerably in eyespot size and asymmetry from which the females could choose.

Overall, within the males, the size of an eyespot did not significantly correlate with its FA (r^2, 0.1–0.2%), even though in 19.8% of the butterflies these two traits were strongly associated. Males in which all four dorsal eyespot foci were effectively damaged, resulting in the disappearance of these eyespots, had zero size and zero asymmetry. Part of the variation in FA could, how-

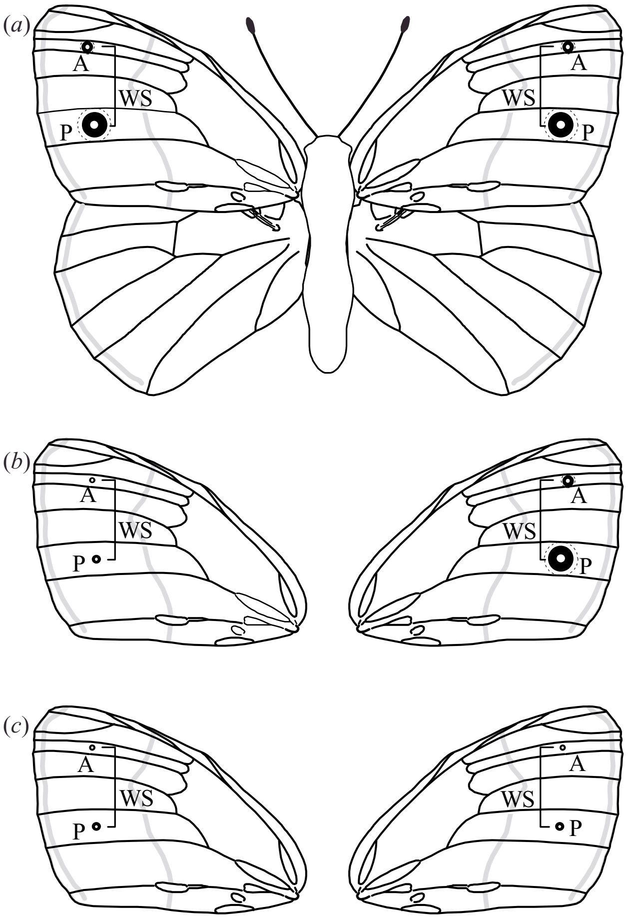

Figure 1. Dorsal wing surface of representative males of each group of manipulated male *Bicyclus anynana* butterflies. (*a*) Males that were damaged away from the focal area on both left and right side (group 1); (*b*) focal damage on one side (left or right) and non-focal on the other (group 2); and (*c*) focal damage on both sides (group 3). The ventral wing surface has no eyespots. The two dorsal eyespots, A and P, and the interfocal distance as an index of wing size (WS) are indicated.

ever, be explained by size differences among the two dorsal eyespots, A and P. We therefore used the mean value of the two replicate measurements of R–L divided by the mean trait size as a measure of individual FA of a trait (FA3 of Palmer & Strobeck 2002). As they are half-normally distributed, these values were transformed by a Box–Cox power transformation of the form $(\mathrm{FA3} + 0.000\,001)^{0.24}$ prior to applying parametric tests that assume normality (cf. Breuker & Brakefield 2002*b*). The sum of the FA3 values of both eyespots was used as a measure of individual asymmetry. This is a multiple FA3 (CFA) index and has more explanatory power than that of a single trait (cf. Leung *et al.* 2000; Gangestad *et al.* 2001).

After testing and measuring, the three males per trial were ranked from 1 to 3 (from high to low) on the basis of each trait: total size of the eyespots, CFA and WS. We used a stepwise ordinal logistic regression with a logit link function to examine if the ranks of any of these three traits explained significant variation in the observed mating order (1–3). Odds ratios and their 95% confidence intervals (CI) were calculated. Odds ratios less than one indicate a positive relationship, with the coefficients of the model being the log values of the odds ratios.

In addition, we performed a principal component analysis (PCA) on the five male variables that may covary: the size of each individual eyespot and WS. This enabled us to analyse more rigorously uncorrelated indices of each of the main variables that we wished to examine for their potential role in female mate choice, namely male eyespot size and symmetry, and WS. The different principal component (PC) axes in the PCA-space explain different aspects of size variation (table 1). PC1 explained 51.8% of the total variance and corresponded to variation in eyespot size with a similar contribution from each eyespot. PC2 explained 20.4% and was mainly due to WS. PC3 explained 17% and corresponded to variation in eyespot asymmetry. PC4 explained 7.3% and was associated with A relative to P eyespot size. PC5 did not explain a significant proportion of overall variance. Thus, four out of the five orthogonal PCs were readily attributable to biological features of the variation. As the correlation among the three traits (FA, eyespot size and WS) was minimal and non-significant, ordinal logistic regression analyses with the ranks of the scores of PC1–PC3 are expected to yield similar results to those based on the trait values themselves. We included PC4 in our analyses as this was not reflected by any of our untransformed traits.

In addition, during the mate-choice, experiments we collected information on the onset of, and time spent, *in copula* of each of the observed matings (76 × 2). We examined whether any of the above traits explain variation in these two variables.

Significance is at the 0.05 level. All analyses were carried out with the statistical software package MINITAB 12.22.

3. RESULTS

Table 2 shows the data on mating order in relation to ranking of total eyespot size, CFA and WS. There was a significant bias of female choice in favour of males with a higher total size of the two dorsal eyespots (table 3*a*, $G = 10.15$, d.f. $= 2$, $p = 0.006$). Successive fitting of the factors ranked WS and CFA, two-way interactions (e.g. WS × CFA) and the three-way interaction (WS × total eyespot size × CFA) did not improve the ordinal logistic regression model explaining the mating order (the ΔG in each change of model was not significant; $p > 0.05$). The values for the odds ratios decrease (table 3*a*) indicating that it is increasingly less likely that a male with smaller eyespots mates first. The odds ratio for males with the smallest eyespots (rank 3) is significant, but not for those ranked second (rank 2). This indicates that males with intermediate eyespot size still had some chance of mating first, but not those with the smallest eyespots.

The same interpretations follow when the ranks based on the PC1–PC4 scores are analysed (table 3*b*). The odds ratios were higher than one. This is because high PC1 scores correspond to males with smaller eyespots. Therefore, these results also indicate that males with large eyespots mated first and those with small eyespots last. The odds ratios in table 3*b* are very similar to the inverse values of those of table 3*a*. The correspondence between the ordinal logistic regression results based on PCA-transformed and on untransformed data indicates that the different variables that may covary were successfully uncoupled. Furthermore, PC4, contrasting the size of the A and P eyespots, did not explain any of the variation in mating success.

There was no indication that females preferred larger males as mating partners. However, overall they seemed

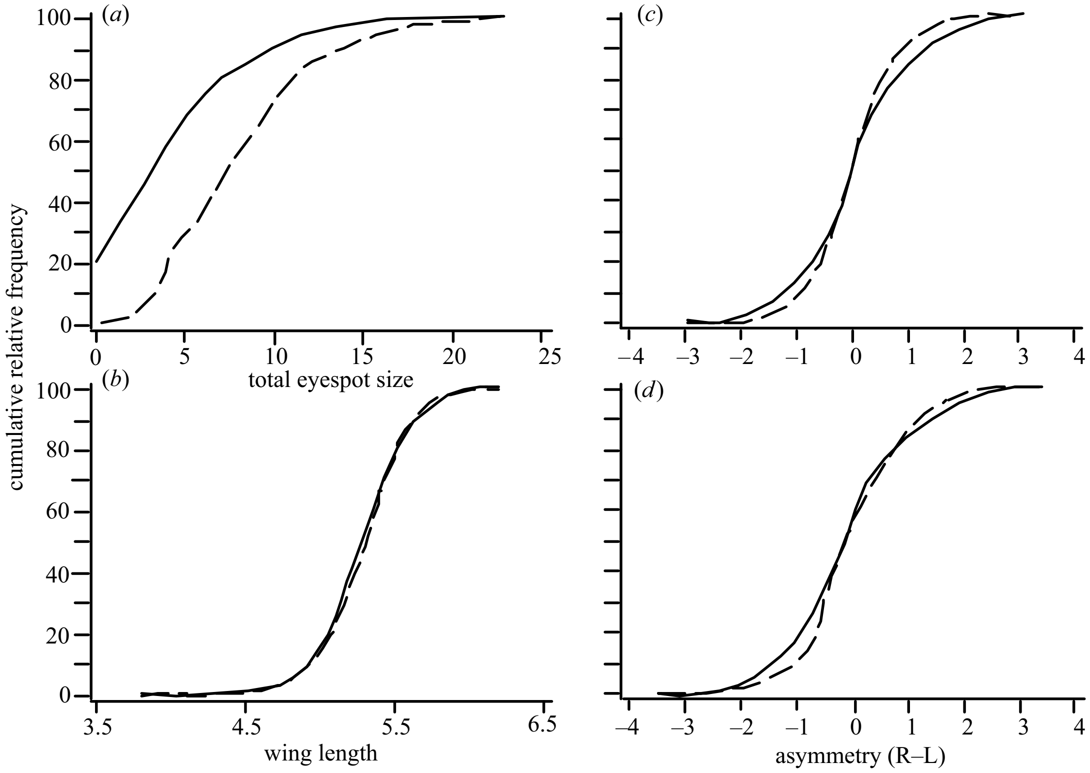

Figure 2. Cumulative relative frequency (×100%) distributions of (*a*) total eyespot size (i.e. size A + size P), (*b*) interfocal distance (WS), (*c*) asymmetry values (R–L) of A eyespots, and (*d*) asymmetry values (R–L) of P eyespots. Solid lines refer to the total dataset of males ($n = 228$), dashed lines refer to control males in which eyespot size was not manipulated ($n = 76$).

Table 1. PCA analysis on five traits in *Bicyclus anynana* males. The five traits are the size of each individual wing eyespot and interfocal distance (WS).
(The proportion of variation explained by each PC is indicated. The coefficients indicate the contribution of each variable to the PC. Coefficients of equal sign and size indicate equal contributions to the PC.)

	PC1	PC2	PC3	PC4	PC5
eigenvalue	2.59	1.02	0.87	0.37	0.15
proportion	0.52	0.20	0.18	0.07	0.03
cumulative	0.52	0.72	0.90	0.97	1.00
variable					coefficients
WS	−0.10	−0.87	0.50	−0.04	0.03
A left	−0.48	−0.24	−0.53	−0.44	−0.49
P left	−0.52	−0.16	−0.39	0.53	0.52
A right	−0.50	0.30	0.37	−0.57	0.45
P right	−0.50	0.27	0.45	0.44	−0.53

to mate with medium-sized males when choosing among two males (χ^2-analysis: $\chi^2 = 22.7$, d.f. = 4, $p = 0.000$, table 2).

Total eyespot size, CFA and WS did not explain any variation in time spent *in copula* (range 10–200 min, with a mean of 32 min ± 1.8) (logistic regression *p*-values $\gg 0.05$). The onset of mating ranged from 1 to 240 min after the start of the trial (average of 62 min ± 4.2, $n = 152$).

4. DISCUSSION

Female *B. anynana* choose males on the basis of the total size, but not asymmetry, of their dorsal eyespots. The absence of any effect of asymmetry is striking, as this result was obtained even though our manipulations of eyespot formation yielded many males with exaggerated levels of FA relative to controls (figure 2*c*). The size of the male had no role in mating success in our experiments. There are two, not mutually exclusive, possible explanations for our results: (i) females were able to reliably detect differences in size, but not in FA, of the dorsal eyespots in males, and (ii) FA is not used as a measure of male quality as it does not reliably reflect the quality of an individual.

(a) *Detecting differences in eyespot size*

Females choose males on the basis of the size of the dorsal eyespots. Because there were no overall differences

Table 2. Classifications of animals used in the 76 successful trials of the mate-choice experiment.
(Mating order: 1, first to mate; 3, last to mate. FA, total eyespot size and WS of each male were classified from low to high. A low FA (rank 3) corresponds with the more symmetrical males, a high FA (rank 1) corresponds with the least symmetrical males. Values indicate the numbers of males of each phenotype category that were successful at each stage of the mating trial. (asymm., asymmetrical; int., intermediate; symm., symmetrical).)

	eyespot size			FA			WS		
	small	int.	large	symm.	int.	asymm.	small	int.	large
mating order	3	2	1	3	2	1	3	2	1
1	18	26	32	20	26	30	26	21	29
2	24	24	28	26	30	20	15	40	21
3	34	26	16	30	20	26	35	15	26

Table 3. Ordinal logistic regression models, with a logit link function, for two separate analyses of the mating experiments. In both, the dependent variable is mating order. The predictor, which is a factor not a covariate, is in (*a*) ranked total eyespot size, and (*b*) ranked PC1 score. Interpretation of results is explained in the text.

						95% CI	
predictor	coefficient	s.d.	Z	p	odds ratio	lower	upper
(*a*)							
const(1)	−0.25	0.22	−1.14	0.255			
const(2)	1.19	0.23	5.08	0.000			
size(2)	−0.47	0.30	−1.56	0.118	0.63	0.35	1.13
size(3)	−0.96	0.31	−3.14	0.002	0.38	0.21	0.70
(*b*)							
const(1)	−1.15	0.23	−4.94	0.000			
const(2)	0.28	0.22	1.28	0.202			
size(2)	0.41	0.30	1.35	0.177	1.50	0.83	2.70
size(3)	0.89	0.30	2.91	0.004	2.43	1.34	4.40

in genetic variation across our three groups of males, we conclude that females were able to detect size differences in eyespot size between males.

The courtship of male *B. anynana* butterflies consists of several steps (Brakefield & Schneider 2002). After the male locates the female, he orients and positions himself at an angle to her side. He opens and closes his wings at high speed for some time, in a flickering behaviour, thereby exposing the dorsal surface of his wings. She is apparently then able to observe the eyespots. It is, however, doubtful whether she would be able to reliably assess differences in size between the left and right side with the male flickering at high speed and given that he is at an angle to her. After flickering, the male repositions himself and attempts to make genital contact. Observations made in this study indicated that many males attempted to court and that on the frequent occasions when a female rejected a male, she was likely to do so in this final phase of the courtship (cf. Brakefield & Schneider 2002). We, therefore, believe that females probably actively chose males and tended to make their choice after observing the dorsal eyespots (intersexual selection). In the majority of other butterflies, females are also able to reject courting males and hence choose mating partners (Wiklund & Kaitala 1995). We also observed rather few examples of males actively competing with each other for a female (intra-sexual selection).

(b) *Fluctuating asymmetry of sexually selected eyespots*

The alternative explanation of why female *B. anynana* butterflies might only use the size of the eyespots, but not asymmetry, as a signal of male quality is that, assuming females can assess asymmetry, FA does not reliably reflect quality. This can be examined by testing three hypotheses. According to the handicap principle (Zahavi 1975), high-quality individuals are able to produce larger symmetrical ornaments at a low cost compared with lower-quality individuals. It has, therefore, been proposed that the FA of sexually selected traits should (i) correlate negatively with trait size (but, see Hosken 2001). However, due to strong, recent directional selection, the DS of these sexually selected traits is likely to have become reduced. Sexually selected traits are thus postulated to (ii) show higher FA than homologous nonsexual traits, and (iii) to be more sensitive to stress than other traits (experimental evidence reviewed by Bjorksten *et al.* (2000)).

In this study, we could not test the hypothesis that FA and trait size are negatively correlated as we artificially manipulated the size and asymmetry of the eyespots. However, in other studies on *B. anynana* with unmanipulated males the relationship between size and FA for the two dorsal forewing eyespots was never significant (r^2, 0.0–8.2%) or negative (58 datasets, A: or 30−, 28+, one-proportion test, $p = 0.90$; P: 25−, 33+, one-proportion test, $p = 0.36$) (Brakefield & Breuker 1996; Breuker & Brakefield 2002*a*,*b*). FA of the sexually selected dorsal eyespots is thus not consistent with the handicap hypothesis concerning FA. Therefore, eyespot FA is unlikely to provide information on male quality to female *B. anynana*.

Previous studies on *B. anynana* have found that the FA and the coefficient of variation of FA of the dorsal forewing eyespots is consistently higher than that for the ventral (non-sexual) eyespots, and that males show higher FA than females in all eyespots (Brakefield & Breuker 1996; Breuker & Brakefield 2002*a*,*b*). The coefficient of variation for the size and FA of the dorsal eyespots is 30–40, which is indicative of a trait under (very) strong directional selection, as is expected of secondary sexual traits (Gangestad & Thornhill 1999; Breuker & Brakefield 2002*a*). A high FA and variation in FA is consistent with the hypothesis that the development of sexual ornaments is weakly stabilized, due to their recent history of directional selection.

Sexually selected traits are also postulated to depend strongly on the condition of an individual and thus to be more likely to show higher levels of FA. It follows that sexual traits are more likely to respond to stress than homologous non-sexual traits (but see Bjorksten *et al.* 2000). However, in experiments in which heat shocks were applied to pupae of *B. anynana* during the sensitive period for eyespot development, the dorsal eyespots were no more sensitive than those on the ventral surface (Breuker & Brakefield 2002*a*,*b*). Thus, taken together, our observations support the notion that eyespot FA is not a reliable index of male quality in *B. anynana*, and that females do not make any choice of males on the basis of differences in eyespot size between left and right wings of the male.

(c) *Sexual selection of the dorsal eyespots*

The relative dorsal eyespot size (size divided by wing length) is not higher in male than female *B. anynana* (Brakefield & Breuker 1996; Breuker & Brakefield 2002*a*,*b*). Although females choose males with larger dorsal eyespots, there is no sexual dimorphism in eyespot size. This contrasts with many sexually selected traits, such as tail feathers of birds and eye stalks of stalk-eyed flies (David *et al.* 1998; Fitzpatrick 1998). Artificial selection experiments on dorsal eyespot size in *B. anynana* have also demonstrated substantial additive genetic variation with rapid responses to selection producing novel phenotypes with large (or small) eyespots after a few generations (Monteiro *et al.* 1994). Thus, if sexual selection favours males with large dorsal eyespots, why is there no apparent tendency to evolve such phenotypes or sexual size dimorphism in eyespots either in natural populations or in our laboratory stocks? Our experiments in this study, however, manipulated eyespot size only in the direction of reduced size. We need now to explore the consequences for mate choice of butterflies with larger eyespots than represented in this study. Perhaps there are, as yet, undetected negative effects of larger dorsal eyespots on fitness.

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