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Research

Male *Bicyclus anynana* Butterflies Choose Females on the Basis of Their Ventral UV-Reflective Eyespot Centers

Manizah Huq,[1] Shivam Bhardwaj,[1,] and Antónia Monteiro[1,2,3,]

[1]Department of Biological Sciences, National University of Singapore, 14 Science Drive 4, Singapore, [2]Science Division, Yale-NUS College, 10 College Avenue West, Singapore, and [3]Corresponding author, e-mail: antonia.monteiro@nus.edu.sg

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Abstract

Butterflies often use their dorsal and ventral wing color patterns for distinct signaling functions. Color patterns on hidden dorsal wing surfaces are often used in sexual signaling, while exposed ventral patterns are often used to ward off predator attacks. At rest, however, part of the ventral forewings are often hidden by the hindwings, allowing individuals to also use the patterns on this wing surface for sexual signaling. Here, we test this hypothesis in *Bicyclus anynana* (Butler, Lepidoptera, Nymphalidae) butterflies by first determining the degree of sexual dimorphism in ventral forewing patterns, focusing on the eyespots, from both wet and dry season forms, and then testing the role of the larger ventral forewing eyespots of dry season females in male mate choice. We also test male investment in reproduction. We show that ventral forewing UV-reflective eyespot centers, in addition to dorsal forewing eyespot centers previously examined in this species, play a role in sexual signaling as males preferentially mated with females with their ventral eyespot centers intact instead of blocked with black paint. This male preference, however, did not translate into a detectable higher reproductive investment via a single mating toward ornamented females. This study provides an example of how ventral forewing patterns, often hidden by hindwings, are used in sexual communication, in this case by females to attract males.

Key words: ventral wing pattern, male choice, eyespot, UV signal, *Bicyclus anynana*

Butterfly species often evolve distinct colors and patterns on each of their wing surfaces. By doing so they are presumably optimizing signals on different parts of their body to serve different ecological functions. A widespread view is that signals on exposed ventral surfaces function in predator avoidance (Stevens 2005, Rutowski et al. 2010, Olofsson et al. 2013, Prudic et al. 2015, Ho et al. 2016) and perhaps also in species recognition (Obara and Hidaka 1968, Obara 1970, Fordyce et al. 2002), whereas signals on hidden dorsal wing surfaces, including UV signals, are thought to function in mate signaling (Robertson and Monteiro 2005, Kemp 2008, Rutowski et al. 2010, Tuomaala et al. 2012, Prudic et al. 2011, Allen et al. 2011, Oliver et al. 2009).

Experimental validation of the role of butterfly wing patterns in mate signaling has been examined in a few species focusing primarily on the role of dorsal wing patterns in female choice. *Pieris rapae* (Linnaeus, Lepidoptera, Pieridae) females showed clear preference for males with more chromatic dorsal wing coloration that were brighter in long wavelengths, darker in UV wavelengths, and had larger quantities of pterin pigment (Morehouse et al. 2007, Morehouse and Rutowski 2010). When pterins were removed from male wings, males were less successful in acquiring mates (Morehouse and Rutowski 2010). In another study, female *Hypolimnas bolina* (Linnaeus, Lepidoptera, Nymphalidae) disliked males with their dorsal UV-reflective iridescent blue color scales removed, as duration of courtship of these males was prolonged and mating success was reduced (Kemp 2008). Similarly, wet season female *Bicyclus anynana* preferentially mated with males with unblocked UV-reflective white eyespot centers (Robertson and Monteiro 2005). Thus, manipulative experiments have shown that dorsal wing patterns function in sexual signaling in a few pierid and nymphalid butterflies.

Aside from hidden dorsal patterns, the patterns on the ventral forewing surface can also be largely hidden from conspecifics and predators when the butterfly is at rest because this area on the forewing can be conditionally covered by the hindwing and could play a role in sexual signaling. No study, however, has directly tested this hypothesis. Previous fieldwork on two closely species of *Lycaeides* butterflies showed that paper models printed with the ventral wing spots and orange markings typical of each of the species were used by male *Lycaeides idas* (Linnaeus, Lepidoptera, Lycaenidae) to distinguish and initiate courtship with conspecifics more often than with heterospecifics (Fordyce et al. 2002). This suggests that markings on the ventral surface are used by males in directing their approach to conspecifics. However, the role that these ventral patterns played in predicting mating outcomes with live butterflies, when female choice was also at play, was not examined in this study, nor was the role of the hidden ventral forewing patterns in species recognition or sexual

signaling. Here we set out to test the hypothesis that hidden ventral forewing patterns may also function in sexual signaling in *B. anynana*, a species that has been previously investigating for both male and female mate choice.

Prior research in *B. anynana* showed that the UV-reflective centers of dorsal eyespots in both males and females have a role in sexual signaling (Robertson and Monteiro 2005, Prudic et al. 2015, Ng et al. 2017). These dorsal eyespots are concealed when the butterfly is at rest but become visible during male or female courtship (Breuker and Brakefield 2002, Nieberding et al. 2008, Prudic et al. 2015). *Bicyclus anynana* is a polyphenic butterfly with two seasonal forms, a dry season (DS) and a wet season (WS) form. Both males and females can court each other but male courtship appears to be predominant in the WS form, whereas female courtship is more frequent in the DS form, when male investment in reproduction is higher (Westerman et al. 2014, Prudic et al. 2015, Ng et al. 2017). WS females are choosy and prefer to mate with males with their dorsal eyespots intact (Robertson and Monteiro 2005, Prudic 2015), whereas DS males are choosy and prefer to mate with females with their dorsal eyespots intact (Prudic et al. 2015, Ng et al. 2017). In addition, these DS males mate more readily and stay in copula longer with females with intact dorsal eyespot centers than with females where the centers have been blocked with black paint (Ng et al. 2017).

In order to examine the role of ventral forewing eyespots in sexual signaling we first explored whether the centers of these eyespots were UV-reflective and sexually dimorphic in size in either of the seasonal forms. This allowed us to perform mate choice experiments with the seasonal form and the sex most likely to care about these eyespots, i.e., we assumed that mate choice might be taking place in the form that displayed higher levels of sexual dimorphism toward the sex that displayed the largest eyespot centers. In *B. anynana*, the white center of the more posterior Cu1 eyespot on the ventral forewing (Fig. 1) is normally fully or partially concealed by the hindwing when the butterfly is at rest, but the M1 eyespot is normally fully exposed. So, in this study, we examined size sexual dimorphism for both eyespots in both WS and DS butterflies before proceeding to mate choice experiments using choosy DS males and females with blocked and intact eyespot centers.

In addition to testing whether DS males care about the presence of the eyespot centers in females, we also tested whether these males contribute differentially to reproduction when mating with an ornamented or a non-ornamented female (OF or NOF; with or without ventral eyespot centers blocked). Previous work in this species established that DS males can significantly increase a female's longevity and fecundity, as well as reduce their own longevity, by providing females with a nuptial gift upon mating (Prudic et al. 2011). Previous work also examined whether choosy DS males, who discriminate between females with and without dorsal eyespot centers, are able to manipulate the content of their nuptial gifts. The ability of males to reduce gift quality toward NOFs and/or increase gift quality toward OFs was measured indirectly via scoring female and male longevity and female fecundity upon mating (Ng et al. 2017). While results from these experiments did not provide evidence for a males' ability to manipulate gift size in response to female attractiveness, here we repeat these experiments but examine the same males' response to presence or absence of ventral, instead of dorsal, eyespot center ornaments in females.

Materials and Methods

Experimental Animals

The lab colony of *B. anynana* butterflies was fed and grown on young maize plants (*Zea mays*) at 27°C with a 12:12 light to dark cycle and 60% humidity that normally generates the WS form. Fourth instar larvae were transferred to a climate room at 17°C and 12:12 light to dark cycle and 60% relative humidity in order to generate the DS form of the butterfly. Pupae were sexed under a microscope and separated to ensure that the adults used in the experiments were virgins. Adults of the same sex who emerged on the same day were placed together in rectangular cages (12 cm × 14 cm × 21 cm) and kept separate from adults of different ages.

Eyespot and Eyespot Center Size Measurements and UV Photography

Wings from 20 males and 20 females from each seasonal form were dissected and imaged using a Leica Stereo Microscope. Only the left forewing of all specimens were measured. Area measurements for ventral forewings, individual anterior M1 and posterior Cu1 eyespots, and white centers were calculated using ImageJ (NIH, v1.45s), as described previously (Monteiro et al. 2015, Bhardwaj et al. 2018). To detect UV pattern reflectivity, males and females of both wet and dry seasons were photographed using a Nikon D7000 digital camera with a 2-inch Baader U-Filter (Model No. 2458291 from Baader Planetarium). The Baader U-Filter has a transmission peak of 80% at CWL 350 nm wavelength of light, and a bandwidth of 60 nm (320–380 nm). The wings were illuminated using an Iwasaki Eye Color Arc lamp that provided UV light. The camera exposure was ISO400 and aperture F5 was used. The average shutter speed for the photographs was 20 s.

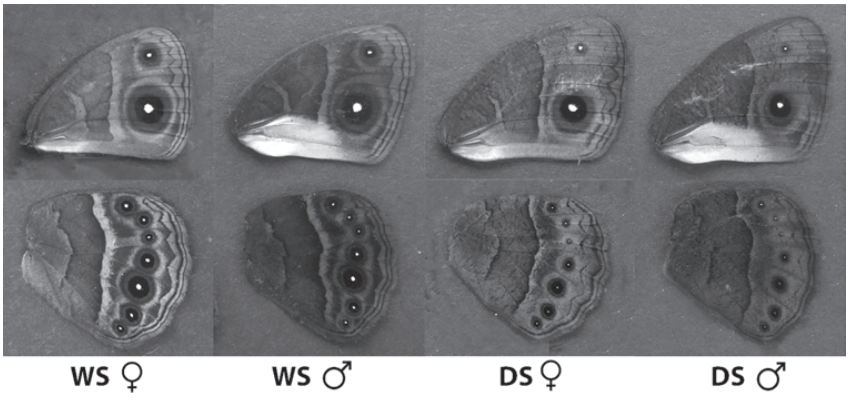

Fig. 1. Photographs of *Bicyclus anynana* ventral wing patterns reflecting in the ultraviolet (bright areas). WS: wet season form; DS: dry season form.

Wing Manipulations

Females were randomly assigned into either the OF or NOF group for wing manipulations. A Robert Simmons Expression detail Spotter brush no. 5 was used to precisely apply paint onto the wings. NOFs had either 1) the anterior small M1 eyespot; 2) the large posterior Cu1 eyespot; or 3) both the M1 and Cu1 eyespot centers on their ventral forewings covered with Testors enamel black paint (flat black no. 1149). A single dot of black paint was applied to the inner black ring of the Cu1 eyespot in all the OFs to control for odor and color of paint (Fig. 1). No black dots of paint were applied to OFs around the M1 eyespots due to the small size of this pattern element.

Mate Choice Experiments

Mate choice experiments were carried out to determine whether DS males could discriminate between ornamented and non-ornamented DS females (i.e., with or without their eyespot centers intact). Animals used in the mating experiments were starved from the day of eclosion by providing only moist cotton with a drop of orange dye. The dye served as a guide for the butterflies to locate the source of water. The animals used in the experiments ranged from 2 to 6 d old. The two females in each trial were of the same age and were chosen to be of relatively similar size. The females were placed in a cylindrical hanging net cage (30 cm × 40 cm) and the male's genitalia was dusted with a fluorescent powder to help identify its choice of mate should the mating not take place within the duration of observation. A mating trial started once the male was released into the cage, and took place from 0900 to 1200 hours at 25°C. After a mating occurred, the pair was identified and all three butterflies were sacrificed and kept in the freezer for later analysis. A total of 46 trials were conducted for the joint M1 and Cu1 eyespot manipulations, 40 trials were conducted for the Cu1 manipulations, and 31 trials were conducted for the M1 eyespot manipulations. If mating did not occur within the duration of observation, butterflies were left to mate overnight. Trials in which no mating occurred or males mated with both females were excluded. In the trial where both the M1 and Cu1 eyespots were manipulated, time to initiate copulation and duration of mating were also recorded. Female wing area was measured for all females, and female age and male age were also recorded for each trial.

Longevity Experiments

This experiment was conducted to evaluate the differential investment made by DS males toward ornamented and non-ornamented DS females as well as the costs and benefits associated with this investment in no-choice mating trails. The longevity and fitness of mated pairs were compared between males mated with OFs and males mated with NOFs. There were 31 trials for each treatment. Once adults emerged, they were kept with only moist cotton so that any changes in longevity would be mostly due to the transfer of spermatophores and not due to differences in adult feeding behavior. A single female and a single male were introduced to each other, 4 d post-eclosion, under full spectrum lights and left together in order to allow for multiple matings. The experiment was conducted in black cylindrical cages (30 cm × 40 cm) and the pair was kept with a young maize plant and water. Throughout the duration of the experiment, the animals were kept under White (Plantmax 54W 46″ T5 HO Daylight White Plant Grow Fluorescent Tube) and UV lights (Arcadia Marine Blue actinic T5 46″). All experiments were conducted at 25°C in front of a window. The longevity of the animals was monitored daily until their death (day 0 is the day of eclosion) and the number of eggs laid was recorded.

Statistical Analysis

Eyespot size and eyespot center size was compared across seasonal forms and sexes using analyses of covariance (ANCOVA), where seasonal form, sex, and the interaction between sex and seasonal form were used as fixed factors, and wing area was used as a covariate. These analyses used the generalized linear model (GLM) procedure in SPSS Statistics (version 19). Data were log 10 transformed to linearize the allometric relationship between eyespot size and wing size and also to meet homogeneity of variance criteria (as determined by a Levene's test). Graphs were made in Microsoft Excel (version 14.6.5 for the Mac) and Adobe Photoshop 3 (Adobe Systems) using reverse transformed data (when applicable). Chi-square tests were carried out for mating outcome data using Social Science Statistics (online calculator www.socscistatistics.com). The effect of male or female age, as well as type of eyespot manipulation, on mating outcome across trials was estimated using a GLM for binomial data with a logit-link function. A mating with the OF was coded 1, whereas a mating with the NOF was coded 0. We tested the significance of the factors via likelihood ratio tests (LRT). This analysis was performed in the R statistical framework (R Development Core Team 2013, RStudio Team 2016), with the packages lme4 (Bates et al. 2015), car (Fox and Weisberg 2011) and rcompanion (Mangiafico 2016). Time to copula and mating duration was compared between experimental groups with a Mann–Whitney *U* test in SPSS. Differences in wing area of paired females in mate choice trials were examined via a paired t-test (two-tailed) using Excel commands. Variation in time to copula or mating duration across trials was examined via a linear model with female age, male age, wing area for each of the females, as well as total female wing area, as potential explanatory variables. Models were examined via the LRT criterion. Time to copula was log10 transformed to make it normally distributed. Longevity of OF and NOF was compared via t-tests (two-tailed) using Excel commands. A Linear Model was also used to test whether wing manipulations (OF vs NOF), female wing size, and their interaction, affected female longevity. The linear model was done in R with the package car (Fox and Weisberg 2011).

Results

Ventral Forewing Eyespots Display UV Signals and Sexual Dimorphism in Overall Size and Center Size in DS Forms

Both DS and WS forms have a strong UV signal in the center of their forewing eyespots (Fig. 1). Hindwing eyespot centers of DS forms have lower levels of UV compared to WS forms, as previously shown with spectral measurements (Monteiro et al. 2015) (Fig. 1).

Measurements of the size of M1 and Cu1 ventral forewings eyespots revealed interesting patterns of sexual dimorphism and plasticity. While the responses of eyespot size to temperature (slopes of the blue and red lines; Fig. 2b and d) have been previously partially discussed in the literature (Brakefield and Reitsma 1991, Monteiro et al. 2015), here we focus on the observed patterns of sexual dimorphism observed within each seasonal form. ANCOVAs on eyespot size identified multiple instances of size differences across the sexes as well as sex by season interactions. We observe that aside from M1 eyespots, which are not sexually dimorphic in overall size (Table 1; Fig. 2b), and where males and females respond similarly to temperature (Table 1), Cu1 eyespots display significant sex by season interactions, i.e., DS individuals display more dimorphism than WS individuals, and females display the larger eyespots (Fig. 2b). Sexual dimorphism is more overt in the size of the white UV-reflective

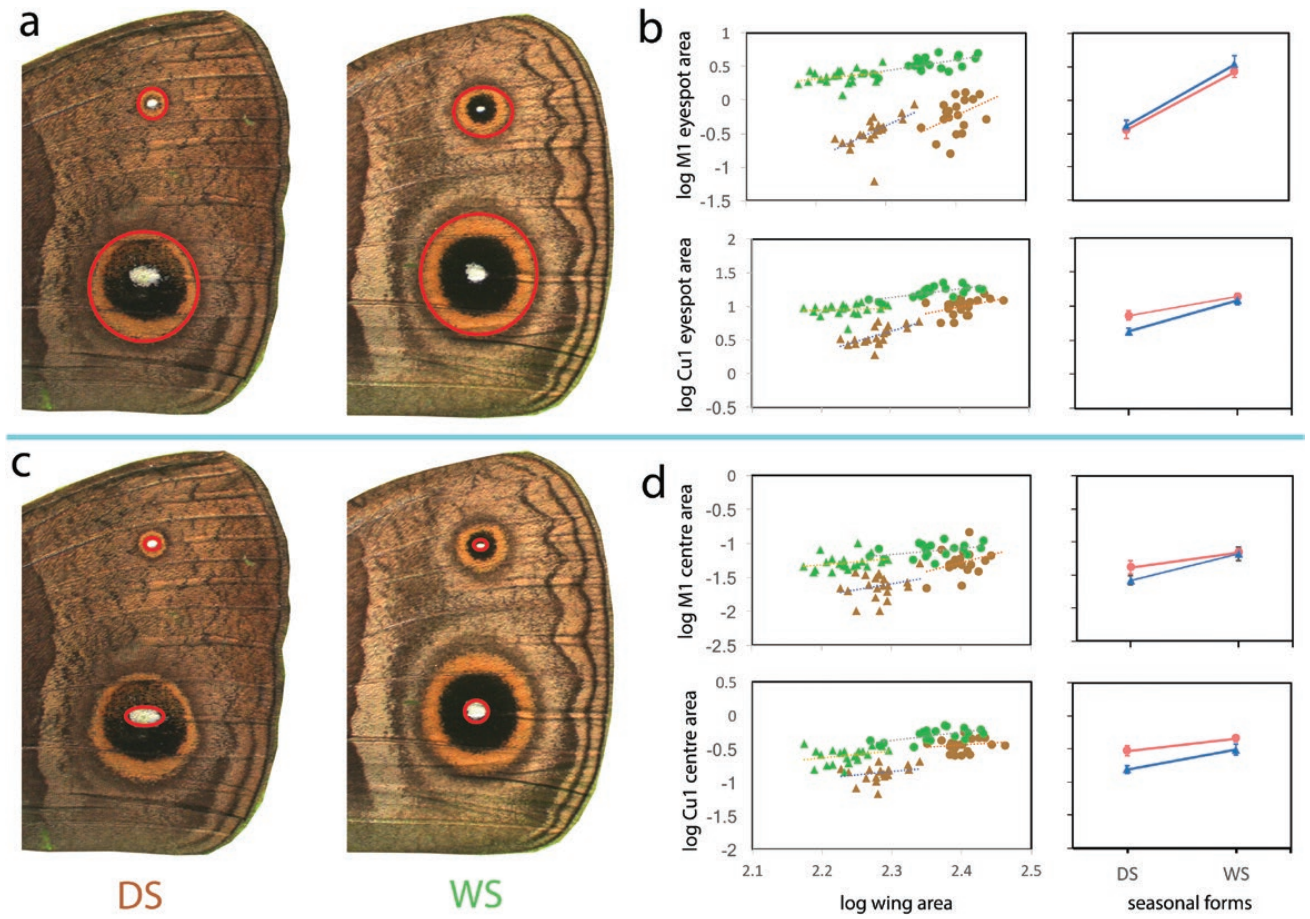

Fig. 2. Sexual dimorphism and plasticity in M1 and Cu1 eyespots. (a, b) Ventral forewing eyespot area and (c, d) Eyespot center area measurements performed in this study. Graphs on the left plot log 10 eyespot (or eyespot center) area on log 10 wing area for males (triangular symbols) and females (round symbols) and for wet season (green symbols) and dry season forms (brown symbols). Lines of best fit are blue for males and red for females. Graphs on the right represent the estimated marginal means for each sex and seasonal form evaluated at a wing size of 208 mm^2, showing that sexual dimorphism is stronger in the dry season and ornaments are larger in females. Error bars represent 95% CI means. Table 1 contains test statistics.

centers of both M1 and Cu1 eyespots where again a stronger dimorphism is observed in DS forms (Table 1). This suggests that ventral eyespot size, and in particular, size of the white centers of Cu1 eyespots, may be used by DS females as ornaments to attract DS males.

Males Mated Preferentially With OFs but Took a Similar Time to Mate With Either Female Type

In a few cases, when animals were left together overnight, DS males mated with more than one female, and these trials were excluded from analysis. For trials with M1 manipulations, a single trial was excluded, for trials with Cu1 manipulations, 11 trials were excluded and for trials with joint M1 and Cu1 manipulations, 11 trials were also excluded. When both M1 and Cu1 eyespots were blocked (Fig. 3a), there was a clear mating basis. Out of 35 trials, males mated with OFs in 28 of those trials but with NOFs in only seven trials (χ^2 = 25.2, P < 0.001) (Fig. 3b). When only the Cu1 center was blocked, there was also a significant mating bias. In a total of 29 trial, males mated with OF in 20 trials and with NOF in only nine trials (χ^2 = 8.3, P = 0.003) (Fig. 3c). However, when only the M1 center was blocked, there was no longer a significant mating bias. Out of 30 trials, males mated with OF in 18 trials and with NOF in 12 (χ^2 = 2.4, P = 0.121) (Fig. 3d). There was no difference in female wing size between OF and NOF across trials (Paired t-test, for M1+Cu1 trials: t = 0.239, P = 0.813, n = 33; Paired t-test for Cu1 trials: t = −1.736, P = 0.093, n = 29; Paired t-test for M1 trials: t = 1.894, P = 0.068, n = 30). Male age, female age, and experiment type (e.g., manipulations of M1+Cu1 eyespots, Cu1 eyespots, or M1 eyespots alone) did not significantly impact mating outcome, and there were no significant interactions between male age or female age and experiment type (GLM, Experiment type: χ^2 = 1.168, P = 0.558, df = 2; Male age: χ^2=0.018, P = 0.894, df = 1; Female age χ^2=0.953, P = 0.329, df = 1; Experiment type by Male age: χ^2=3.107, P = 0.212, df = 2; Experiment type by Female age: χ^2 = 2.523, P = 0.283, df = 2).

Out of the 35 trials where the NOF had both the M1 and Cu1 eyespots blocked, 29 matings occurred during the 3-h observation period and were used to test for differences in average time to mating and mating duration. No significant differences, however, were found between the time males took to mate with OF or NOF (Mann–Whitney U = 57, N_{OF} = 15, N_{NOF} = 6; P = 0.328) (Fig. 4a). The median time for males to mate with OF was 25.5 min (Min = 2.5, Max = 141) and 35 min to mate with NOF (Min = 16, Max = 80). Mating duration was not significantly different across female types (Mann–Whitney U = 51, P = 0.769). The median mating duration of males with OF was 55mins (Min = 27.5, Max = 90), and that of males with NOF was 60 min (Min = 30, Max = 66) (Fig. 4b). Time to mating decreased with female age across trials (LM: $F_{1,27}$ = 9.267, P = 0.005), but neither age nor wing size variables helped explain mating duration variation across trials (LM: $F_{1,19}$ = 3.964, P = 0.061).

Table 1. *F* statistics and *P* values from analysis of covariance on eyespot size and eyespot center size

Eyespot	Factors used in ANCOVA	*F* values	*P* values	df (Factor, Error)
M1 eyespot	Sex	1.01	0.319	1, 75
	Season	355.72	***<0.001***	1, 75
	Sex by Season	0.46	*0.501*	1, 75
Cu1 eyespot	Sex	9.65	***0.003***	1, 75
	Season	201.70	***<0.001***	1, 75
	Sex by Season	171.43	***<0.001***	1, 75
M1 Center	Sex	2.62	0.110	1, 75
	Season	65.70	***<0.001***	1, 75
	Sex by Season	7.45	***0.008***	1, 75
Cu1 Center	Sex	19.57	***<0.001***	1, 75
	Season	70.76	***<0.001***	1, 75
	Sex by Season	5.25	***0.025***	1, 75

Sex, seasonal form, and the interaction between sex and seasonal form, are fixed factors and wing size is the covariate. All data was log 10 transformed. *P*-values in bold-italic indicate that factor(s) have a significant effect in explaining size differences.

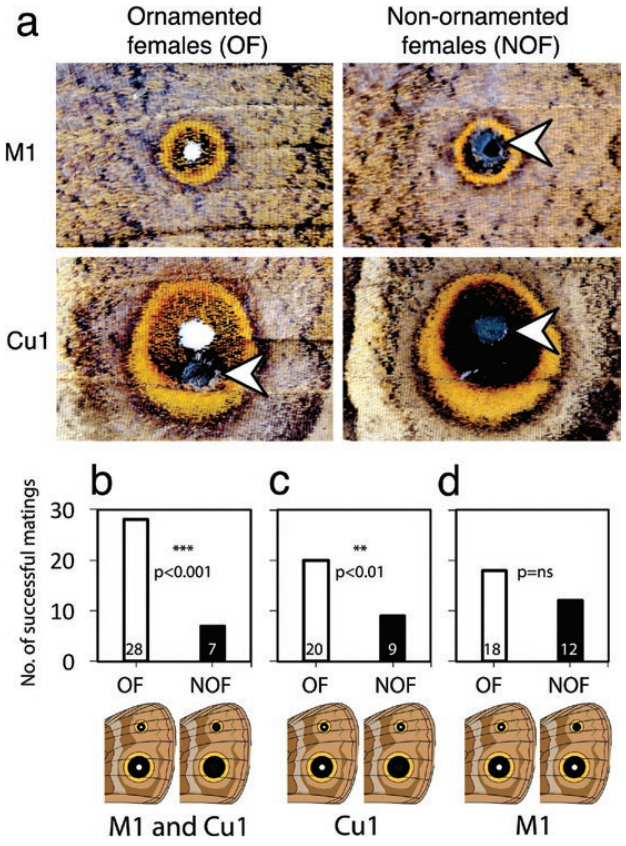

Fig. 3. Experimental manipulations on the ventral forewing and results of mating trials. (a) The Cu1 eyespots of females were either control painted (white arrowheads) outside the white center (OFs) or the white centers of their M1, Cu1, or both eyespots were blocked with a dab of black paint (NOFs). Females mated more often with OF than with NOF when both the M1 and Cu1 eyespots were blocked (b) or when Cu1 eyespots alone were blocked (c). (d) Mating outcomes were not significantly different when only the M1 eyespots were blocked.

Both Female Types had Similar Longevity

Longevity of either sex was also not altered in no-choice experiments where single males were mated with either a OF or a NOF (with both M1 and Cu1 centers blocked) (Independent sample *t*-test, two-tailed, $\alpha = 0.05$, $t = 0.664$, $P = 0.509$ males; $t = 0.553$, $P = 0.582$ females) (Fig. 4c and d). Average longevity for males that mated with OF was 6.52 d (SD = 0.77, $n = 31$) while the average longevity for males who mated with NOF was 6.39 d (SD = 0.76, $n = 31$). The average longevity for OF was 9.06 d (SD = 1.69, $n = 31$) while the average longevity for NOF females was 8.81 d (SD = 1.97, $n = 31$). Levene's test indicated equal variances across groups ($F = 1.30$, $P > 0.05$ for males; $F = 1.30$, $P > 0.05$ for females). Neither female ornamentation, female wing size, nor their interaction, explained variation in female longevity across all trials (LM, Wing ornamentation: $F_{1,58} = 0.207$, $P = 0.651$; Female wing size: $F_{1,58} = 1.631$, $P = 0.207$; Wing ornamentation by Female wing size $F_{1,58} = 0.001$, $P = 0.978$). A single mated NOF from the longevity experiment laid 10 eggs until it died.

Spermatophore Number was Similar in the Bursa of OFs and NOFs

Dissections carried out in the bodies of females, after their natural death, showed that there were no significant differences in the number of spermatophores transferred to either OF or NOF (Fig. 5). We did not find any female with more than one spermatophore in their bursa, suggesting that despite the males being present throughout the experiment, no matings or single matings took place. For OF ($n = 31$), we were able to recover a single spermatophore from 27 females, while for NOF ($n = 31$) 26 females were found to have a single spermatophore each. There was no significant differences in the number of mated and unmated females across treatment groups ($\chi^2 = 0.13$, $P = 0.718$).

Discussion

The current study demonstrates that the UV-reflective white eyespot centers in the most posterior part of the forewing (the part that can be conditionally hidden by the hindwing) show strong sexual dimorphism in DS individuals, where females display the largest trait size. This sexual dimorphism is reduced in WS individuals. Females used these Cu1 eyespot centers in mate signaling which, when blocked, led to changes in mating patterns. There was a strong bias for males to mate with females with unblocked eyespot centers in the mate choice experiments involving Cu1 only and both M1 and Cu1 eyespot center manipulations, but not M1 eyespot manipulations alone. All other parameters such as age and body size of females were kept constant and were, thus, unlikely to have contributed to the observed mating biases.

Previous behavioral experiments with multiple butterfly species (Morehouse et al. 2007, Kemp, 2008, Morehouse and Rutowski

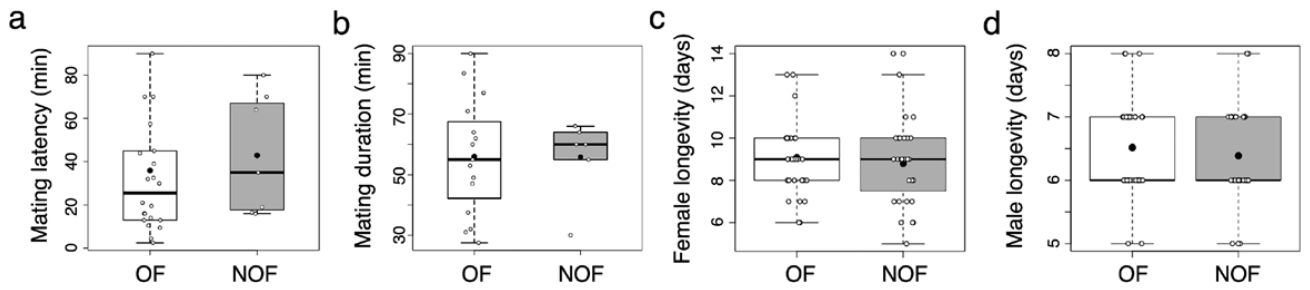

Fig. 4. Box and whiskers plot of mating latency, mating duration, and longevity of OF and NOF and their male mating partners. There were no significant differences in mating latency (a) and mating duration (b) across treatment groups. Female (c) and male longevity (d) also did not differ across treatment groups. Black horizontal line represents median and filled dot represents mean of respective samples. Error bars represent min and max values (no further than 1.5 x the inter-quartile range), and ends of the boxes represent the lower and upper quartiles.

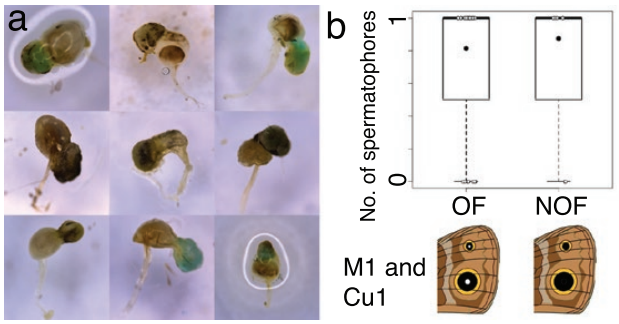

Fig. 5. Spermatophores transferred to females during the longevity experiment. (a) Some of the spermatophores transferred to females in the course of the experiment. A spermatophore is a two-lobed structure with the first lobe containing nutrients and accessory glands (darker/greener color) and the second lobe, attached to the ductus bursae, holding sperm. (b) Spermatophore count across treatment groups did not vary. OF (n = 31) and NOF (n = 31).

2010), as well as with *B. anynana* (Robertson and Monteiro 2005, Costanzo and Monteiro 2007, Prudic et al. 2011, Westerman et al. 2012, Westerman et al. 2014), discovered sexual signaling roles for multiple pattern elements on the hidden, dorsal surfaces of the wings of these species, but no study had yet examined the role of conditionally hidden ventral wing patterns in mate signaling. This study showed that choosy DS males notice these white UV-reflective pattern elements and mate with females that have them more readily than with females where these patterns have been blocked. This mating preference may explain why sexual dimorphism is mostly visible in the posterior eyespots of the DS form, with females having the larger trait size as compared to males.

The original study that focused on the role of UV-reflective eyespot centers in *B. anynana* (Robertson and Monteiro 2005), found that WS females preferred males with intact UV-reflective eyespot centers on their dorsal surfaces. Subsequent studies found that DS males also preferred females with intact UV-reflective eyespot centers on their dorsal surfaces (Prudic et al. 2011), mated more quickly with these females, and also spent more time in copula with them (Ng et al. 2017). The current findings supplement these previous results by showing that *ventral forewing eyespots* are also sexual ornaments used by DS females to attract DS males.

Despite clear mating biases, the time males took to initiate a mating and mating duration did not differ between female treatments. These results differ from those of Ng et al. (2017) where both time to mating and mating duration were shorter, and longer, respectively for OF as compared to NOF with their dorsal eyespot centers blocked. We initially hypothesized that variation in the age of animals used across both experiments could explain the different results. We used 2–6 d old animals in our experiments (but always females of the same age in each cage) while Ng et al. (2017) only used 4 d old animals. In the current study, variation in female age did help explain variation in time to mating across trials, with older females mating more readily, but no measured variable helped explain variation in mating duration across trials. Male age also did not impact time to mating nor mating duration across trials. We propose that DS males ultimately care more about the presence of dorsal eyespots than ventral eyespots in females, and change their behavior accordingly. A direct experiment to test this hypothesis should be performed in future.

Our work, however, similar to that of Ng et al. (2017), showed that DS males do not appear to be able to modulate the contents of their first spermatophore in order to give a larger nuptial gift toward OFs and a smaller one toward NOFs. In addition, in a small proportion of individuals used for the longevity experiments, we could not recover a spermatophore, and no individual was found with two spermatophores. As previously argued (Ng et al. 2017), despite no evidence for males being able to manipulate the content of their first spermatophores in response to female ornamentation, they may be able to do so relative to second spermatophores, but that remains to be explored.

One possible explanation for males mating preferentially with females with intact ventral forewing UV signals could be that these signals function in species recognition at close range, when the signals are actually displayed. In Lepidoptera, UV signals are important for initial mate attraction (Obara 1970, Scott 1973, Obara and Majerus 2000), and exposed ventral wing patterns can be directly assessed by males who search for, and court females sitting in vegetation with their wings closed, and thereby function in species recognition. Evidence for this function was found in *Lycaeides idas*, where patrolling males distinguish heterospecific *L. melissa* females from conspecifics, to whom they directed most of their courtship, based on their exposed ventral patterns (Fordyce et al. 2002). In *B. anynana*, while the ventral forewing eyespots are often partially or fully hidden by the ventral hindwing when the female is at rest, DS females often court males where they can fully display both ventral and dorsal eyespots. So, the mating biases observed in *B. anynana* may have derived from males recognizing and preferentially mating with females with the presence of the ventral UV signal, after they had a chance to see them.

Other possibilities for why males prefer OFs could be that UV signals are simply attractive to males or might function as indicators of female quality. In the first scenario, males might prefer OFs because their female offspring will also carry the ornament, and be preferred by

males in the subsequent DS. This would be a Fisherian system favoring sexy daughters. The benefit of having large UV-reflective ornaments would simply be because males like them, and this trait alone gives individuals a competitive edge (Huk and Winkel 2008). In the second scenario, males care about the presence of these UV signals in females because they are costly to produce and perhaps function as an indicator trait of female fecundity (Doutrelant et al. 2012, Henderson et al. 2013, Barry et al. 2015). Most research on indicator traits in butterflies, and in particular UV-reflective traits, has been conducted on male signals and has suggested that UV traits may be costly to produce due to the resources required for the assembly of complex nanoscale structures (Fitzpatrick 1998, Kemp and Rutowski 2007, Kemp et al. 2011) which may indicate male quality (Kemp and Rutowski 2007, Papke et al. 2007) or spermatophore quality (Rajyaguru et al. 2013). Research on indicator traits in females is more sparse, as this sex is often the choosy sex, but when males exert choice they often focus on female body size, a clear indicator of female fecundity (Svensson and Petersson 1992). Future work in this area might investigate whether enlarging the size of these ventral white eyespot centers in females might be favored by males, or whether the size of ventral or dorsal eyespots are honest indicators of female fecundity by testing for a correlation among both traits in a population.

Our results from the two longevity experiments indicate that males do not appear to be able to impact female longevity via their nuptial gifts in response to female attractiveness. These results parallel the longevity results obtained for similar manipulations of dorsal eyespot centers (Ng et al. 2017) and indicate either that males cannot manipulate the content of their first spermatophores, once these are made, in response to female attractiveness, or, alternatively that they can do so, but a single spermatophore transfer is insufficient to alter female longevity, especially in species with small spermatophores, as is the case with *B. anynana* (Wedell and Karlsson 2003, Ng et al. 2017).

During the longevity experiment, a single female laid 10 eggs until its demise so no analyses were done with these data. Previous work on *B. anynana* (Fischer et al. 2004), reared at 27°C, and where mated adults were either only given water or banana, showed that oviposition did not take place in the water-fed individuals, contrary to the banana fed ones. Oviposition only started in the water-fed individuals 1 or 2 d after females were fed banana. In a different experiment, where butterflies were also reared at 17°C, and also placed at 25°C for egg laying in the presence of a single male (as in the current experiment), females did lay eggs even without food, but average egg number was low (less than two eggs per female per day; Prudic et al. 2011). It is unclear why the current experiment, with similar settings to those used by Prudic et al. (2011) apart from the room and type of lights used, led to so few females laying any eggs at all.

Follow up longevity experiments could improve on experimental design on several fronts. One variation would be to perform group matings instead of single pair matings. Introducing competition as a factor in the cages would encourage males as well as females to acquire more mates and invest more in reproduction (Wedell and Cook 1999). One way to monitor both male as well as female investment would be to feed male and female larvae on radioactively labeled food with different labels. This would allow us to track their respective reproductive investments later as adults. An additional improvement would be to feed adult males daily to enable them to replenish their sperm reserves and allow for multiple matings to take place.

Conclusion

This study provides the first evidence that wing pattern elements found on the conditionally displayed ventral forewing surface of butterfly wings can function as sexual signals. Our study demonstrated that the ventral forewing eyespots of DS females of *B. anynana* play a key role in attracting DS males. Males were clearly able to discriminate between OFs and NOFs, where the UV-reflective white centers were blocked, and mated more frequently with the former. Males that mated with OFs, however, did not significantly increase female longevity.

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