

Males Become Choosier in Response to Manipulations of Female Wing Ornaments in Dry Season *Bicyclus anynana* Butterflies

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Abstract

Male investment towards reproduction is substantial in some species, and this leads to the evolution of choosy males. Male choice is often directed towards female phenotypes that are good indicators of fecundity such as body size, age, or virgin status, and often acts in the same direction as fecundity selection. In insects, only a few examples exist where male choice is directed towards female ornaments as proxies of female quality. Here, we use dry season males of the sex-role reversed butterfly species *Bicyclus anynana* to test for differences in male choosiness and investment towards females of varying attractiveness using ornament-manipulations. Male reproductive investment in this species is in the form of a nuptial gift, a spermatophore, given to females upon mating. Males were placed in cages with either wild-type ornamented females or with nonornamented females (center of the dorsal forewing eyespots painted over), and time to mating, duration of mating, and longevity of males and females after a single mating were measured. Ornament manipulations consisted of blocking the UV-reflective scales in the center of the dorsal forewing eyespots of females, a known sexual ornament. Males displayed lower latency to mate and longer mating durations with ornamented females. The longer mating duration did not, however, translate in the transfer of a nuptial gift that increased female longevity or reduced male longevity. Instead, we propose that longer mating durations with ornamented females may represent increased mate guarding behavior or increased sperm transfer.

Key words: *Bicyclus anynana*, eyespots, male investment, spermatophore

In most sexually reproducing systems analyzed so far, females, who produce large yolky gametes, or have long and expensive pregnancies, tend to invest more in reproduction than males and tend to be the choosy sex (Bateman, 1948, Trivers, 1972, Thornhill and Alcock, 1983). In exceptional systems, however, male reproductive investment is significant and males can also become choosy (Dewsbury, 1982, Johnson and Tyler, 1988).

In many insect systems, and in sex-role reversed systems in particular, there is substantial male investment in reproduction (Gwynne and Simmons, 1990, Bonduriansky, 2001). Investments are in the form of nuptial gifts that contribute to the overall energy budget of females, including their budget for egg production. For instance, in the neotropical katydid *Conocephalus ictus* Scudder, males often provide females with a large nutritious spermatophylax upon mating, which is especially valuable when food in the environment becomes scarce (Ortiz-Jimenez and Cueva Del Castillo, 2015). In these periods, females compete with other females for access to

males and males become choosy and prefer to mate with larger females (Svensson and Petersson, 1992). In many lepidopteran species, males also transfer a bolus of nutrients and sperm, a spermatophore, to females during mating, which is energetically expensive to produce (Thornhill and Alcock, 1983, Watanabe and Sato, 1993, Karlsson, 1998). For instance, in the dry season form of *Bicyclus anynana* butterflies, the transfer of male spermatophores to females increases both female longevity and fertility, and reduces male longevity (Prudic et al. 2011). This means that males that invest more in reproduction have lower amount of resources accessible for their own survival. This large male investment in reproduction contributes to the evolution of male choosiness in this system (Prudic et al. 2011).

When males become choosy they tend to select for female traits that are good indicators of female fecundity. Often, the best indicators of female fecundity in insects are body size, abdominal width, age, and virginity. These traits are good proxies for total egg

number, large fraction of unfertilized eggs, and low levels of sperm competition, which will maximize paternity (Wearing-Wilde, 1996, Bonduriansky and Brooks, 1998). Rarely do female insects use visual signals such as wing color or other ornaments to attract males, as these signals are often thought to be costly and trade-off against fecundity (Fitzpatrick et al., 1995, Kemp et al., 2008). Exceptions include the air-inflated abdomens of females of the Long-tailed Dance Fly, *Rhampomyia longicauda*, Loew a deceptive signal that tricks males into mating with them (Funk and Tallamy 2000), and large iridescent paddle-like ornaments on the midlegs of female *Sabethes cyaneus* F. mosquitoes, a signal of potential direct fecundity benefits to males (Funk and Tallamy, 2000, South and Arnqvist, 2011). Light wing melanization in female *Pieris protodice* Boisduval & Leconte butterflies is also a preferred signal by males of the species, who spend less time courting heavily melanized females (Wiernasz, 1995). While the reason for this male preference is not known, a proposed mechanism is that heavily melanized females are more likely to overheat and have shorter lifespans and reduced fecundity relative to lightly melanized females (Wiernasz, 1995). Light wing pigmentation in this species may function, thus, as an indicator of female fecundity.

Here we are interested in testing whether males of *B. anynana* butterflies invest differently in reproduction depending on the presence and absence of a wing ornament, which is a known sexual signal in this species. This African butterfly exhibits seasonal polyphenism in wing pattern and in sexual behavior that is cued by rearing temperature during larval and pupal development (Brakefield and Mazzotta, 1995, Prudic et al., 2011). In the wet season (WS), females choose their mates based on the size and brightness of the UV-reflective eyespot center found on the dorsal wings of males (Robertson and Monteiro, 2005, Costanzo and Monteiro, 2007, Prudic et al., 2011), whereas in the dry season (DS), males are the choosy sex and they prefer to mate with females displaying these same sexual ornaments rather than with females with the ornaments blocked (Prudic et al., 2011). As an evolved response to this sex-role reversal, DS females court males and display larger and brighter dorsal eyespot centers than DS males, whereas the opposite happens for WS individuals (Prudic et al., 2011, Everett et al., 2012). An open question in this system, however, is whether DS males who mate with ornament-manipulated DS females, perceive these females as being of a different quality, and invest differently in reproduction. An additional question is whether DS males can control how much of their valuable resources to give away when a mating opportunity arises.

To test whether a DS males' investment in reproduction varies with female attractiveness, we manipulated females by blocking the white centers of their dorsal wing eyespots, the sexual ornaments, with black paint. We then placed males in cages with either ornamented or nonornamented females, in a no-choice situation, and monitored the pair's sexual behavior and longevity. We hypothesized that males would invest more in reproduction when paired with ornamented females. We measured time taken to initiate copulation and mating duration as metrics for desire to invest or actual investment in reproduction. In addition, we measured longevity of males and females assigned to both treatment groups, to test for potential effects of differential male investment on male and female longevity.

Materials and Methods

Animal Husbandry

Bicyclus anynana were raised from eggs collected from a large laboratory colony (>300 individuals) maintained in Singapore. The animals

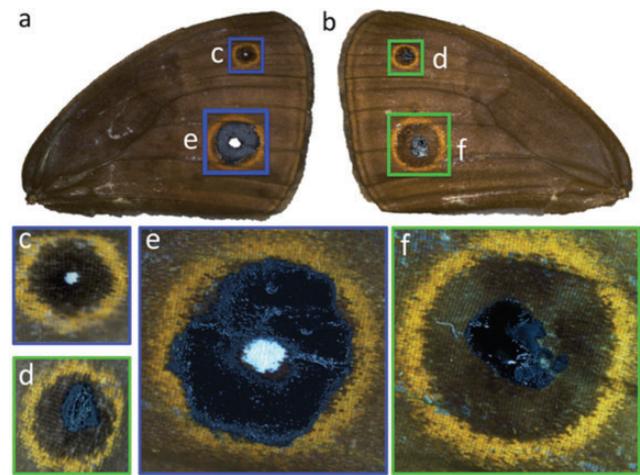


Fig. 1. Female ornament manipulations. (a) Ornamented female (OF). (b) Nonornamented female (NOF). (c and d) M1 eyespots. (e and f) Cu1 eyespots in OF and NOF, respectively. Black paint was applied to the surrounding black scales of the Cu1 eyespot of OF, whereas it completely covered the white central scales of both M1 and Cu1 eyespots of NOF.

were raised on young maize plants (*Zea mays*) in cages inside a walk-in climate room at 17°C, 65% relative humidity, and with a 12:12 (L:D) h cycle. Males and females were identified and segregated during the pupal stage. Upon eclosion, the animals were placed into individual cages according to their date of eclosion. From the day of eclosion, adults were provided with water on moist cotton but no other food.

Female Ornament Manipulations

Females, that were visually identified to be of similar body size and wing size, were randomly assigned into two groups for wing manipulation: the ornamented group and the nonornamented group. The Cu1 and M1 eyespot centers on the dorsal forewings of the nonornamented females were covered with Testors, Vernon Hills, IL, USA model enamel black paint (flat black #1149); whereas for the ornamented females, paint was applied on the black scales surrounding the dorsal forewing Cu1 eyespot center to control for paint odor (Fig. 1). The painting was done on day 1 posteclosion. Day 0 was the day of eclosion.

Mating Experiment

On day 4 posteclosion, two females from the same treatment group were introduced to two males in a cylindrical mating cage (30 cm diameter×40 cm height) in the mornings, between 9.00 am and 10.00 am, under full UV lights at 24°C. Only the first pair of individuals that mated were used in this study. The remaining two individuals were removed immediately from the cage. Individuals that failed to mate within the first 4 h were also excluded from this experiment. The time taken to initiate a mating from the moment of introduction and the mating duration were recorded. After the mating ended, the pair was separated into individual cages and transferred back to the 17°C climate room. We ran a minimum of 30 trials for each of the female treatments ($N \geq 30$).

Longevity Experiment

In order to test whether males that mated with the different female types invested differently in reproduction, we measured longevity of all mated individuals. The mated females were kept in individual cylindrical cages (30 cm diameter×40 cm height) with water and a young maize plant (*Zea mays*) for egg laying; while the mated males

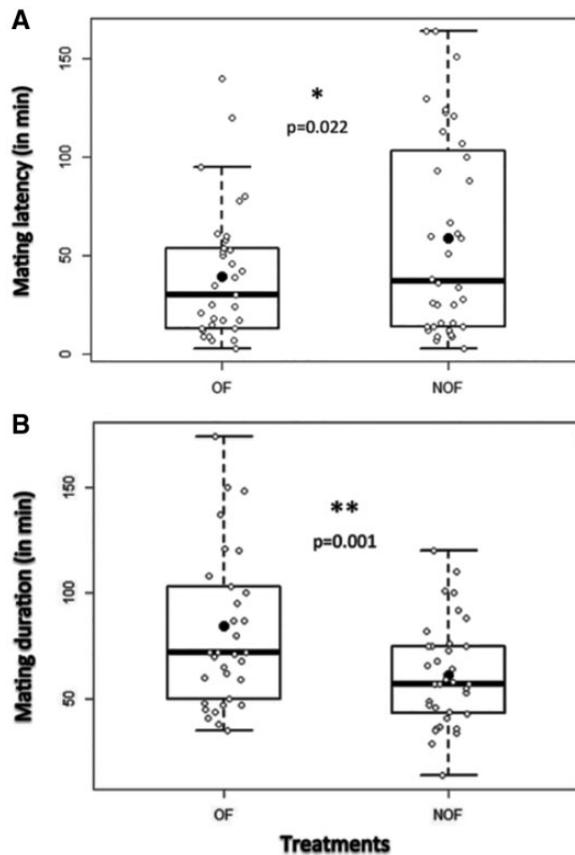


Fig. 2. Mating latency is shorter and mating duration longer with ornamented females. (a) Males took a shorter time to initiate mating with ornamented females (OF) than with nonornamented females (OF). Black line represents median and filled dot denotes mean. (b) Pairs with ornamented females mated for a longer time than pairs with nonornamented females. Error bars represent 95% CI of means. * $P < 0.05$, ** $P < 0.01$ from independent t -test (two-tailed, $\alpha = 0.05$).

were kept in individual rectangular cages (12 cm×14 cm×21 cm) with only water supplied by moist cotton. Both sexes were kept in the climate room at 17°C. There were a total of four treatments: (i) males mated with ornamented females, (ii) males mated with nonornamented females, (iii) mated ornamented females, and (iv) mated nonornamented females. We monitored animals from date of emergence until death and recorded their longevity in days.

Statistical Analysis

Data were evaluated for equality of variances using Levene's test, before statistical analyses. Raw data for mating latency and mating duration were log transformed to make variances across groups comparable. Differences in time taken to initiate mating and mating duration between treatment groups were tested with an independent sample t -test (two-tailed, $\alpha = 0.05$). Longevity data were analyzed using a two-way ANOVA with sex and female type as fixed factors. All data were analyzed with IBM SPSS Statistics Version 20 while graphs were generated using R on the back-transformed data.

Results

Males Delayed Mating With Nonornamented Females

Bicyclus anynana males took significantly longer to start mating with nonornamented females, than with ornamented females ($t(65) = 1.854$, $P = 0.022$) (Fig. 2a). The average time taken for pairs to initiate mating

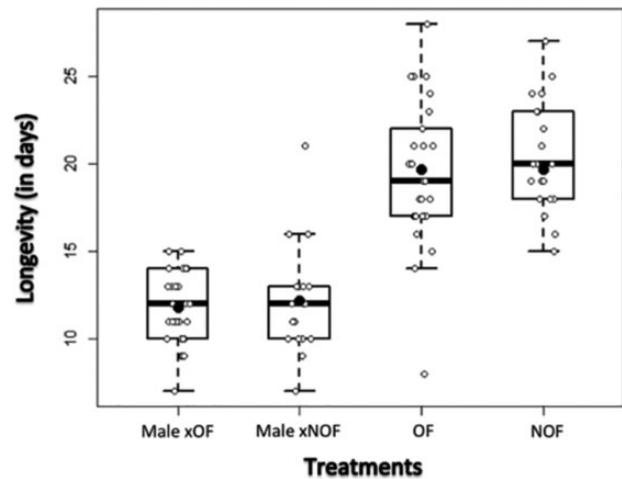


Fig. 3. Females live longer than males, but female treatment has no effect on longevity. Longevity (in days) of males mated with ornamented females (Male xOF), of males mated with nonornamented females (Male xNOF), and longevity of the respective females. Black line represents median and filled dot denotes mean. Error bars represent 95% CI of means.

with ornamented females was 39.58 min (SD = 33.77) while it was 59 min (SD = 50.99) for pairs with nonornamented females. Levene's test indicated no departure from equality of variances ($F = 0.61$, $P = 0.44$). In addition, two trials with ornamented females and four trials with nonornamented females did not lead to any mating within 4 h of introduction and were excluded from analysis.

Males Spent Significantly More Time Copulating With Ornamented Females

Males mating with ornamented females mated for significantly longer than males mating with nonornamented females ($t(65) = 2.77$, $P = 0.001$) (Fig. 2b). The average mating duration for the former pairs was 73.91 min (SD = 42.94) while it was 56.60 min (SD = 24.14) for the latter pairs. Levene's test indicated no departure from equality of variances ($F = 0.76$, $P = 0.38$).

Male and Female Longevity Was Not Altered After a Single Mating

A two-way ANOVA testing the effect of sex and female treatment on longevity showed that females had overall longer lifespans than males ($F_{(1,133)} = 125.385$, $P < 0.01$) but that female treatment had no effect on longevity ($F_{(1,133)} = 0.0623$, $P = 0.431$). (Fig. 3) In addition, there was no interaction between sex and treatment ($F_{(2,174)} = 1.124$, $P = 0.481$). The mean longevity for (i) males that mated with ornamented females was 11.77 days (SD = 2.02); and (ii) for males that mated with nonornamented females was 11.26 days (SD = 3.11). The average longevity for (i) mated ornamented females was 19.07 days (SD = 4.47); while (ii) mated nonornamented females lived for 19.07 days (SD = 4.56) (Fig. 3).

Female Fecundity

Only five mated females laid eggs during the longevity experiment. The number of eggs laid by each individual was 4 and 5 for the ornamented females, and 1, 1, and 9 for the nonornamented females.

Discussion

Our experiments showed that males are reluctant to mate with females without visible dorsal eyespot centers, the wing ornaments. The no-

choice experimental design forced males to decide whether or not to mate and when to mate, and thus allowed us to determine copulation latency across groups. We observed females courting males equally in both treatment groups, but males initiated mating attempts earlier with ornamented females, suggesting a higher reluctance to mate with nonornamented females. In addition, males invested less time mating with nonornamented females than with ornamented females.

Previous behavioral experiments in other insects showed that males displayed similar changes in copulation latency and duration towards females displaying variation in more traditional fecundity indicators. These traits included female weight in scorpionflies (*Panorpa cognata Rambur*) (Engqvist and Sauer 2003), body weight and symmetry in Indian Mealworms (*Plodia interpunctella Hübner*) (Gage 1998), body size in the African stalk-eyed fly (*Diasemopsis meigenii Westwood*) (Harley et al. 2013), body condition in the False Garden Mantis (*Pseudomantis albofimbriata Stål*) (Jayaweera and Barry 2015) and age, mating condition, and body size in the fruitfly (*Drosophila melanogaster Meigen*) (Lupold et al. 2011). Our study, however, showed that variation in these male behaviors were elicited by females varying in presence or absence of a wing ornament alone, the dorsal eyespot centers, independently of other female fecundity indicators. These results indicate that females are communicating their quality to males via these dorsal wing ornaments.

Copulation duration in *B. anynana*, however, did not affect overall male or female longevity. Copulation duration is a potential indicator of how much sperm and resources are being transferred from males to females (Svärd and Wiklund, 1986, Watanabe and Sato, 1993, Kaitala and Wiklund, 1995). Spermatophores are made up of sperm and accessory products. These accessory products contain nutrients which are able to increase female reproductive output and longevity (Boggs and Gilbert, 1979, Wiklund et al., 1993). Hence, the nutrients transferred in the ejaculates during copulation represent the male reproductive investment. Increased nutrient investment in reproduction will result in lower amount of energy left to maintain somatic demands and will result in shorter longevity (Reznick, 1985, Reznick et al., 2000, Barnes and Partridge, 2003). Our results, however, indicated that female ornamentation did not affect male or female longevity after a single mating.

These results are compatible with previous work in this species which showed that mated DS individuals had different longevities relative to virgins (Prudic et al., 2011). In particular, Prudic et al. (2011) showed that mated DS females lived significantly longer than virgin DS females, and mated DS males lived significantly shorter lives than virgin DS males. In these previous experiments, males and females were left together in the same cage throughout their lives (at a different temperature of 25 °C), allowing them to mate multiple times. The current experiment had a different purpose and design as it merely tested whether DS males were able to manipulate content of their first spermatophore in response to female levels of attractiveness, and whether this translated in longevity differences (of butterflies kept at 17 °C). We conclude that our experimental design currently does not lend support to the hypothesis that DS males manipulate the content of their first spermatophores in response to female attractiveness in a way that affects longevity of either mated partner.

There are a few possible explanations for the observed differences in copulation duration not having translated in differences in male and female longevity. One is that *B. anynana* males may be unable to regulate spermatophore content as seen in other male insects, including other male butterflies (Kaitala and Wiklund, 1995, Hughes et al., 2000, Wedell and Ritchie, 2004, Marcotte et al., 2005). In *Pieris rapae* L. butterflies, for instance, as the number of matings with older (likely

nonvirgin females) increases, males increase the number of sperm produced to overcome sperm competition with other males, but reduce the amount of nutrients in spermatophores due to lower paternity assurance (Cook and Wedell, 1996). In *Plodia interpunctella* moths, males are sensitive to female size and produce larger sperm ejaculates when mating with heavier females (Gage 1998). Another possibility is that DS males are able to manipulate content of their spermatophores in response to female attractiveness but longevity differences between female treatments may only arise upon multiple matings. This would be especially applicable to *B. anynana*, as the spermatophores in this species are relatively small compared to those of other Lepidoptera. For example, spermatophores in Monarch butterflies *Danaus plexippus* L. are 22% of their adult body in term of dry mass; while they only take up 2% of the *B. anynana* adult body (Oberhauser, 1988, Ferkau and Fischer, 2006). Therefore, the absence of differences in longevity observed across treatment groups could be explained by the relatively small reproductive investment per single mating (Ferkau and Fischer, 2006). Future studies should try to leave males and females together in the same cage throughout their lives to allow for multiple matings to take place. Finally, the longer copulations observed with ornamented females may represent investment in sperm alone, which was not investigated here. (Watanabe and Sato, 1993) showed that in another butterfly species, *Pieris rapae*, males transfer nutrients to females during the first 40 min, and sperm is only found in the body of females after this period. It is possible, thus, that *B. anynana* males mating with nonornamented females for a shorter period transferred similar amount of nutrients to these females, not impacting their longevity, but transferred fewer sperm.

A prolonged copulation, however, often has functions other than spermatophore transfer, and males may have increased copulation duration with ornamented females in order to increase these other functions. In most butterfly species, copulation duration is controlled by males (Wickman, 1986) and is heavily associated with precopulatory or postcopulatory mate guarding behaviors. Prolonged matings make females less available for other males, and hence the guarding male reduces competition of its sperm with that of potential subsequent mates (Cordero, 1990, Watanabe, 2016). In addition, in a prolonged copulation males themselves function as a guard or a mating plug, making the female unreceptive to other males (Watanabe et al., 1997). Some of these functions, thus, could explain the observed prolonged mating with ornamented females, without significantly contributing to female longevity.

In a previous longevity study (Prudic et al. 2011), DS mated females laid substantially more eggs than in the current study. Here it is important to note that the previous study was carried out at 25 °C, from mating until the day of death, while ours keep the animals at 17 °C throughout their life except during mating, when they were kept at 25 °C. For *Bicyclus anynana*, rearing temperatures >24 °C produce the wet season form, whereas rearing temperature <19 °C produce the dry season form (Brakefield et al., 1998). In the dry season, females are likely to hold their reserves to extend their own lifespan until the rains arrive, the temperature raises, and larval grasses start to grow, hence reproduction is unlikely to occur in this cold dry season. This may explain why so few females laid eggs during the course of our experiment.

Conclusion

Our study showed that male *B. anynana* of the dry season form displayed different mating behaviors when exposed to females varying in presence or absence of wing ornaments. Males exhibited higher reluctance to mate with nonornamented females and spent less time

in copula with these same females. This suggests that either fewer sperm or nuptial gifts were transferred to the female body upon copulation. However, our study was unable to find significant differences in longevity in males and females across different treatment groups. Differences in copulation duration may, instead, be related to an increased propensity for mate guarding ornamented females, or may have resulted in the transfer of fewer sperm. Further studies are needed to identify and test the consequences of the distinct mating behaviors identified in our study.

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Author contributions

Conceived and designed the experiments: S.Y.N. and A.M. Performed the experiments: S.Y.N. and S.B. Analyzed the data: S.B. and S.Y.N. Wrote the paper: S.Y.N., S.B., and A.M.

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