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RESEARCH

Cryptic Mate Preference in Male *Bicyclus anynana* Butterflies

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Abstract While male mate choice has received sparse attention in comparison to female choice, it occurs often in insects. In addition, male insects may preferentially allocate sperm and ejaculate in response to female quality. Previous research indicates that male *Bicyclus anynana* butterflies can learn mate preference through prior exposure to females, though naïve males mate randomly. It is unclear whether this preference learning may also influence male sperm and ejaculate allocation after mate selection, or whether males have cryptic mate preference for female wing patterns independent of preference learning. Here we test whether *B. anynana* males adjust their sperm and ejaculate allocation in response to a learned preference. We also assess whether males exhibit an innate cryptic preference and adjust their sperm and ejaculate in response to female wing pattern. We compared number of eggs laid by females and spermatophore (male butterfly ejaculate) weight in four no-choice treatments: naïve male butterflies (having no prior exposure to females), paired with a 2 or 0-spot female, and experienced male butterflies (having a previous three-hour interaction with a 0-spot female), paired with a 2 or 0-spot female. All females used were naturally 2-spot females, 0-spot females had artificially blocked spots. We found that 0-spot females laid significantly more eggs than 2-spot females, independent of male experience. There was no effect of female phenotype or male experience on spermatophore weight. Our findings suggest that male *B. anynana* have an innate cryptic preference for 0-spot females, which has been shown in other studies to only be seen as a pre-copulatory preference when enhanced by early experience.

Keywords Cryptic choice · sexual selection · Lepidoptera · mating behavior · visual signaling

Introduction

Mate selection is a diverse evolutionary characteristic across the Metazoa. Animals do not mate randomly, but instead carefully choose a mate based on certain traits (Andersson 1994). While male mate choice has received sparse attention in comparison to female choice, the energetic cost of both spermatophores (a protein capsule containing sperm and nutrients) and courting behavior makes male mate choice advantageous and likely common among insects and other animals (Dukas et al. 2006). These male mate preferences can be innate and independent of early experience, or learned (ten Cate and Mug 1984; ten Cate

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and Vos 1999; Verzijden et al. 2012). Learned preferences are often developed through social experience while the animal is a juvenile. These learned preferences can develop through exposure to a parental phenotype (parental sexual imprinting), or through exposure to a non-parental adult phenotype (oblique imprinting) (Tramm and Servedio 2008; Verzijden et al. 2012; Westerman et al. 2012).

While learned preferences are often thought to be independent of innate preferences, innate preferences and sensory biases can also be enhanced through learning (ten Cate and Bateson 1989). Preference learning has the potential to enhance the speed of selection by strengthening innate preferences and reducing the likelihood that individuals will accept mates with less-preferred phenotypes (Servedio and Noor 2003). Both sexes can display innate and learned mate preferences through either pre-copulatory or post-copulatory mechanisms (Andres and Rivera 2000; Dukas et al. 2006; Eberhard 2009; Lüpold et al. 2011; Westerman et al. 2012). Pre-copulatory male mate choice can be considered the frequency with which males choose to mate with certain females. If males possess a phenotypic preference, they court this phenotype more frequently than other phenotypes. Post-copulatory mechanisms include “cryptic” male mate preference – adjusting the composition of their reproductive nutrients (such as spermatophores in many insects) in response to the phenotype of the female they copulate with (Bonduriansky 2001). This mechanism is termed “cryptic” because male preference is not detectible until after copulation occurs. When males exhibit cryptic preference, they may successfully mate with multiple female phenotypes, but exert their preference by manipulating the amount or quality of nutrients they allocate to a given female during copulation. This manipulation of resource allocation is one way a learned mate preference could manifest in male insects, including butterflies.

Previous research demonstrated that male butterflies can learn mate preferences (Westerman et al. 2014). In *Bicyclus anynana* butterflies, males previously exposed to females with 0 spots on their dorsal hindwing preferentially mate with 0-spot females in later choice assays. Naïve males, who are kept in sex-specific cages isolated from females until mating trials, mate equally often with 0-spot and 2-spot females in choice assays. This learned preference is apparent in the trained males’ decision to mate with the previously observed female phenotype (pre-copulatory mechanisms); however, whether this preference results in differences in their physical reproductive contribution as well (post-copulatory mechanisms) has not yet been explored. It is also unknown whether naïve *B. anynana* males exhibit cryptic preferences (differentially allocate resources during copulation in response to female appearance, age, or mating status).

Various insects undergo reproduction through male transfer of a spermatophore to a female. The spermatophore consists of a soft, inner matrix and a hard, outer envelope. Proteins in these differing sections impart distinct functions following copulation (Meslin et al. 2017). Males transfer a spermatophore to the spermatheca of the female via the bursa copulatrix, a female organ that stores the spermatophore until degradation. Following copulation, the contents of the spermatophore travel to different locations in the female reproductive tract. Sperm travels to the spermatheca, while nutritional content, such as protein, is absorbed and digested by the bursa copulatrix (Meslin et al. 2017).

The spermatophore provides many important functions besides the simple transfer of sperm. The complex protein capsule contains various nutrients vital to the production of offspring, as well as factors that manipulate the reproductive physiology of the accepting female (Tram and Wolfner 1998). These seminal fluid proteins (SFPs) are key players in fertilization, regulate female reproductive physiology, and can have either negative or highly beneficial effects on females (Walters and Harrison 2010). Males can transfer substances that decrease the attractiveness of the female, thereby decreasing her chances of mating multiple times and increasing the odds that his genes get passed to the next generation (South and Lewis 2011). Males might also include substances that are attractive to the female but do not provide the female with any nutritional benefits. This is termed the “Candymaker” hypothesis (Warwick et al. 2009). Though spermatophore manipulation was previously believed to be an activity strictly carried out by males, recent research has shown that females are not just passive recipients of the given spermatophore. By utilizing proteases, they can manipulate their absorption of spermatophore nutrients and factors (Meslin et al. 2017). Therefore, there may be sexual conflict regarding the effects of the spermatophore on the female. In addition, males in many insect species often provide significantly more sperm during their first mating than subsequent transfers of spermatophores (Lewis and Wedell 2007).

While it is known that previously mated males transfer smaller spermatophores than virgin males in many insect species, it is unknown whether prior social experience influences spermatophore content in virgin males. *Drosophila* males exhibit preferential ejaculate allocation based on female quality and mating history (Lüpold et al. 2011). Males transfer significantly more sperm to mated females than to virgin females. Males also deliver 15–18% more sperm to young or large females than to old or small females, respectively (Lüpold et al. 2011). These results suggest male insects may modify their spermatophore in response to their perception of the female they copulate with, and that males tend to invest more in copulations with females who appear to be more fecund.

Since male insects can exhibit preferential allocation of ejaculate based on female quality, and are capable of learning mate preferences, the question of whether these learned preferences lead to differences in spermatophore allocation presents itself. Here, we first assess whether naïve males exhibit a cryptic preference and alter their reproductive contribution in response to female wing pattern. We then assess whether virgin males alter their reproductive contribution in response to prior social experience, using spermatophore weight and female fecundity (egg production). As previous studies indicate prior social experience impacts pre-copulatory mate choice mechanisms in male *B. anynana* butterflies, here we seek to determine the effect of the same prior social experience on post-copulatory mate choice mechanisms.

If males alter their spermatophores or ejaculate in response to prior social experience, we expect 0-spot females mated to males trained with 0-spot females to lay more eggs than either 0-spot females mated to naïve males, or 2-spot females mated to males trained with 0-spot females, since males prefer 0-spot females after training (Westerman et al. 2014). Since naïve males lack the prior experience with 0-spot females and have previously been shown to mate randomly when given a choice between 0- and 2-spot females (Westerman et al. 2014), we expect females mated to naïve males to lay similar numbers of eggs, regardless of whether they have 0- or 2- UV reflective hindwing spots. This means 0-spot females mated with experienced males should exhibit the highest fecundity (measured here as post-copulatory egg count). Alternatively, males may have an innate cryptic preference for either 0- or 2- spotted females. If this is the case, we would expect 0- or 2-spotted females (depending on the direction of male innate cryptic preference) to lay more eggs, independent of male pre-mating social experience. In this scenario, a pre-existing cryptic preference for 0-spotted females seems most likely, given that males learn pre-copulatory preferences for females with 0- but not 2- dorsal hindwing spots. If variation in female fecundity is directly associated with variation in male spermatophore size, we would expect spermatophore weight and egg counts to have a direct relationship, and hypothesize that larger spermatophores would mean more nutrients, higher fecundity, and more eggs.

Materials and Methods

Study Animal and Husbandry

Bicyclus anynana, commonly known as the “squinting bush-brown,” is a species of butterfly within the large, diverse family Nymphalidae. *Bicyclus anynana* is native to the tropics of eastern Africa, and supplements its adult nectar diet with rotting fruit (Brakefield and Reistma 1991). Useable protein is obtained from yeasts within this rotting fruit (Fischer et al. 2004). Its habitat is characterized by woodlands and savannah (Brakefield and French 1993; Brakefield and Reistma 1991). The species’ small size and short generation time make it ideal for research, and it has been utilized heavily in the fields of evolutionary and ecological genetics (Brakefield et al. 2009; Monteiro 2017; Monteiro et al. 1994). The species has been reared in the lab since 1988. Our population of *B. anynana* was started in Spring of 2017 from a few thousand eggs obtained from a population in Singapore. Butterflies were reared in a climate-controlled greenhouse, maintained at 27 °C, 70–80% humidity, and a 13:11 light: dark cycle (lights on a 0700 h, off at 2000 h), with supplemental full spectrum Sun Blaze T5 high output 120 V fluorescent light fixtures (containing ultraviolet, UV, wavelengths) in addition to natural light. These rearing conditions ensure the production of wet season butterflies in this seasonally polyphenic species, which is the seasonal form previously used to identify male preference learning in this species (Bear and Monteiro 2013; Prudic et al. 2011; Westerman and Monteiro 2016; Westerman et al. 2014).

We utilized popup, transparent cages and opaque corrugated plastic barriers to separate butterflies from one another throughout the greenhouse. All mating, egg laying, and rearing took place in these cages. Virgin males and females were reared in cages with individuals of common sex and age. For this experiment, mating was housed in small cages (0.3 × 0.3 × 0.3 m) and egg laying housed in larger cages (0.6 × 0.6 × 1.2 m). To mitigate the variable of time on data, the time of mating, training, and dissection was kept constant throughout; with only minor differences in when these activities took place. (See below for details.) Caterpillars were reared on corn (*Zea mays*) plants grown from Jolly Time popcorn kernels, and egg laying took place on similar corn plants. Adults were fed banana.

All experiments and treatments ran concurrently, from September 2018-May 2019, so weather, sunlight, and other time-sensitive variables were experienced equally by all treatments and are presumed to have no substantial effect on experiment outcomes. Throughout the experiment size of corn plants available for egg laying, the amount of banana supplied as a food source, the time of training for experienced treatments, and time of mating for all treatments were kept constant.

Experimental Design

This project focuses on the effect of two variables: the presence or absence of spots on female hindwings, and pre-mating social experience in males; on number of eggs laid and male spermatophore size. For the variable of spots, 3-day-old, 2-spot females were painted one day prior to use. These females were painted with black paint (Testors enamel gloss black 1147) to manipulate the number of dorsal hindwing UV reflective eye-spots, so they have either 0 or 2 spots on their dorsal hind wings (Fig. 1B and C). All females used were naturally 2-spot females. 0-Spot females were 2-spot females with artificially blocked spots, while 2-spot females were females painted with two black spots adjacent to their UV reflective spots, to account for any effect of black paint on male preference. Previous studies have shown that these butterflies can both see these UV reflective spots and make mating decisions based on the presence, absence, and number of dorsal UV reflective spots (Robertson and Monteiro 2005; Westerman et al. 2012, 2014).

There were four treatments: naive males paired with 2-spot females, naïve males paired with 0-spot females, trained (experienced) males paired with 2-spot females, and trained (experienced) males paired with 0-spot females, for each experiment. These treatments are described in detail below. For the training of males, naive 1-day-old males were isolated with a 3-day-old, sexually mature, 0-spot female. Like previous studies (Westerman et al. 2012, 2014), this study's brief social interaction consisted of a three-hour training session. The 1-day-old males were not mature enough to copulate, and thus after the training they were socially experienced, yet still virgins.

"Naïve" treatments utilize naïve, untrained 3-day-old males, who were kept in sex-specific cages,

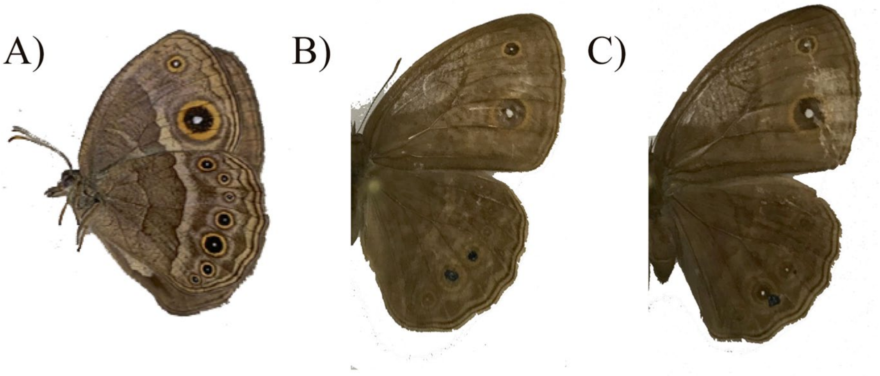

Fig. 1 *Bicyclus anynana*. **A** ventral wing surface of a wet season female. **B** Dorsal wing surface of a 2-spot female painted to have 0 dorsal hindwing spots. **C** Dorsal wing surface of a 2-spot female with 2 control black spots on her dorsal hindwings

isolated from females, until being paired with either a 2-spot female or a 0-spot female on day 3. Since previous studies have found that female behavior during the training period does not influence male choice for female dorsal hindwing spot number in *B. anynana*, (Westerman et al. 2014) and male latency to court and courting persistence does not influence female choice (Robertson et al. 2020) we did not record butterfly behavior during either the training period or the mating period for these experiments.

We measured variation in male reproductive contribution two ways and thus all female butterflies in each of the four treatments were destined for one of two fates, egg laying or dissection. The first measurement consisted of recording the number of eggs laid by a mated female after 7 days alone in a cage with a corn plant. These mated females were provided with a corn plant as means of laying eggs and a banana slice for nutrition (n = 15 per treatment, except for naïve mated 2-spot, which had an n of 16). The second measurement consisted of weighing the transferred spermatophore's mass after dissection and removal. These mated females were dissected and the transferred spermatophore isolated and weighed (n = 15 per treatment, except for naïve mated 2-spot, which had an n of 16).

Behavioral Assays

Naïve Treatments

At approximately 8 AM, a previously painted 2 or 0-spot 3-day-old female was introduced to a 3-day-old male. A separate 0.3 × 0.3 × 0.3 m cube cage housed each mating pair. After approximately three hours of interaction (including copulation, which generally ranges from 18 to 45 min for wet season virgin male *B. anynana* paired with virgin females (Westerman and Monteiro 2016)), males were ethically disposed of, placed in individual glassine envelopes, and frozen. Half of the mated females were ethically disposed of, placed in individual glassine envelopes, and taken to the laboratory for spermatophore extraction. The transferred spermatophore was then isolated and weighed (balance Model: VWR-224AC, Max: 220 g, Min: 0.1 mg), see below for details. The other half of the mated females were given a corn plant and banana, and isolated in a large cage (0.6 × 0.6 × 1.2 m) for egg laying. See below for details.

Experienced Treatments

At approximately 8 AM, a previously painted 3-day-old 0-spot female was introduced to a 1-day-old male in a large (0.6 × 0.6 × 1.2 m) cage. After approximately three hours of interaction, the female was ethically disposed of, placed in individual glassine envelopes, and frozen. The trained male was kept in their individual cage, given banana on cotton for a food source, and isolated for approximately 48 h. After this two-day isolation period, the trained males were mature and capable of mating. At approximately 8 AM, half of the trained males were paired with 0-spot females and half were paired with 2-spot females (all of whom are 3-days-old). After approximately three hours of interaction (including copulation), males were ethically disposed of, placed in individual glassine envelopes, and frozen. Half of the mated females were given a corn plant and banana, and isolated in a large cage for egg laying. The other half of the mated females from each treatment were ethically euthanized, placed in individual glassine envelopes, and taken to the laboratory for spermatophore extraction. The transferred spermatophore was then isolated and weighed.

Egg Production

Half of the females from each treatment were isolated for egg laying. These females were placed in a large cage upon mating and provided a one-month-old corn plant for egg laying and a slice of banana on moist cotton for a food source (Supplemental Fig. 1B). These females were isolated for 7 days, during which banana and water was provided *ad libitum*. After seven days, the females were ethically disposed of, placed in individual glassine envelopes, and frozen. Eggs were removed from the corn plant, placed in a small Petri dish, and counted under a dissecting microscope using a fine paint brush.

Spermatophore Extraction

Upon mating, the female's ostium bursa displayed obvious discoloration and disorder. Females were then placed in a cold environment for two to three minutes, after which dissection occurred. After removal of fat bodies, the ovaries and bursa copulatrix were isolated, with the transferred spermatophore bound inside. The spermatophore is a smooth, spherical, white capsule, which releases white fluid upon puncture (Supplemental Fig. 1A). Individual spermatophores were placed in a small, white weigh boat and weighed one at a time on an analytical lab balance (Model: VWR-224AC, Max: 220 g, Min: 0.1 mg).

Butterfly Size

We used two methods to control for any effect of butterfly size on egg production or spermatophore size across treatment. First, butterflies were matched in size across treatment by eye prior to each assay. Second, butterfly forewings were measured and compared across treatments, as a proxy for butterfly size. After experiments were completed and butterflies frozen, intact forewings were gently clipped from each butterfly, photographed under a Zeiss dissecting microscope, and length, width, and surface area calculated using ImageJ. Since not all butterflies had intact wings at the end of the experiment, there is a smaller sample size for wing measurements than for behavioral assays; however sample sizes are similar across all treatments. N = 13 females, 24 males for naïve mate 0sp; N = 8 females, 19 males for naïve mate 2sp; N = 7 females, 15 males for experienced mated 0sp; N = 7 females, 19 males for experienced mated 2sp.

Statistical Analyses

N = 61 per experiment (egg laying and spermatophore weight) (15 naïve mated 0-spot, 16 naïve mated 2-spot, 15 experienced mated 0-spot, 15 experienced mated 2-spot for both the spermatophore and egg count experiments). Forewing length, max height, and surface area were compared across treatment for males and females, using ANOVA. Eggs laid and spermatophore mass were compared across female phenotype and male experience, using an ANOVA, as our data were normally distributed. A general linear model was run to test the effect of female phenotype, male experience, and the interaction between female phenotype and male experience on number of eggs laid and transferred spermatophore size. All statistical analyses were conducted in JMP v 13.

Ethical Note

Butterflies were reared and disposed of in accordance with USDA-APHIS permit # P526P-17-00343. All caterpillars used in this study were fed corn *ad libitum*, and all butterflies used were fed banana *ad libitum*, housed in spacious cages (0.6 × 0.6 × 1.2 m), and provided moist cotton for obtaining water. All butterflies used in this study were disposed of humanely, either by natural death or by quick decapitation. Butterflies killed by decapitation were placed in individual glassine envelopes and placed in a -30 °C freezer after dissection.

Results

Females and males were similarly sized in all four treatments (Supplemental Fig. 2) and thus any effect of treatment was not due to relative differences in butterfly size.

We found a significant effect of female dorsal hindwing spot number on number of eggs laid (N = 61, F ratio = 4.555, p = 0.037, 0-spot mean = 45.70 ± 3.21, 2-spot mean = 36.10 ± 3.15). Females artificially modified to have 0-dorsal hindwing spots laid more eggs than 2-spot females (0-spot mean = 45.70 ± 3.21, 2-spot mean = 36.10 ± 3.16) (Fig. 2A). However, we did not find a statistically significant difference between the number of eggs laid by females mated to experienced males versus naïve males (N = 61, F ratio = 1.440, p = 0.235 experienced mean = 43.63 ± 3.29, naïve mean = 38.10 ± 3.24), and there was not a significant interaction effect of male experience and female spot number on number of eggs laid (general linear model effect tests: female spot number F ratio = 4.414, p = 0.040, male experience (naïve versus trained) F ratio = 1.40 p = 0.241, spot number x male experience F ratio = 0.334 p = 0.565, N = 61).

We did not find a statistically significant difference between the spermatophore weight transferred to 0-spot versus 2-spot females (N = 61, F ratio = 0.554, p = 0.460, 0-spot mean = 0.273 mg ± 0.017, 2-spot mean = 0.255 mg ± 0.017) (Fig. 2B).

Nor did we find a statistically significant effect of male experience on the spermatophore weight transferred to females (N = 61, F ratio = 2.760, p = 0.102, experienced mean = 0.243 mg ± 0.017;

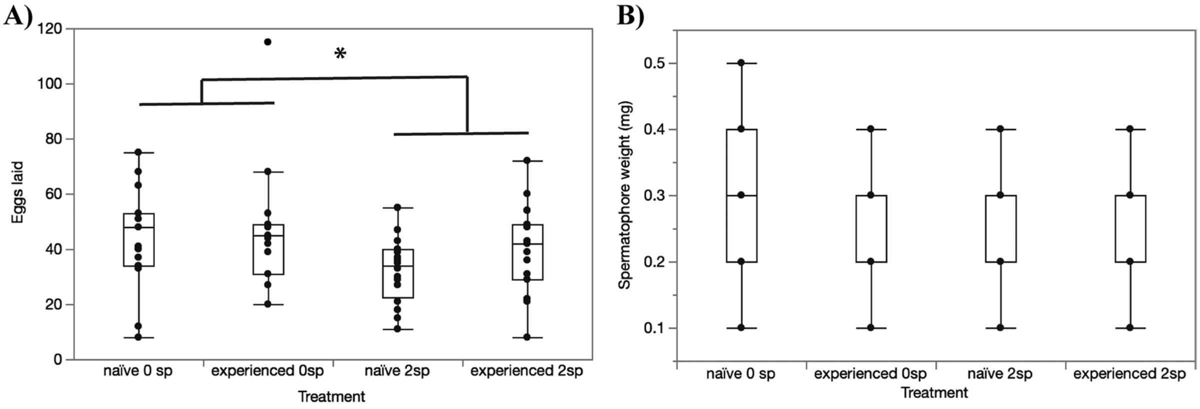

Fig. 2 Effect of female wing pattern and male experience on male resource allocation. **A** 0-spot females laid more eggs than 2-spot females, but there was no effect of male experience on female egg production. **B** No effect of female wing pattern or male experience on spermatophore weight. Horizontal lines within the boxes denote median. Upper and lower bounds of the boxes indicate the 25th and 75th percentiles, with whiskers extending to 1.5 the interquartile range (the length of the box, or difference between the 25th and 75th percentiles), or to the minimum and maximum data values, whichever is smaller. For panel B, median = 25th percentile for experienced 0sp and experienced 2sp; median = 75th percentile for naïve 2sp. N = 61. * = statistically significant effect

naïve mean = 0.284 mg ± 0.017). There was not a significant interaction effect of male experience and female spot number on transferred spermatophore size (general linear model effect tests: female spot number F ratio = 0.602, p = 0.441, male experience F ratio = 2.732, p = 0.104, spot number x male experience F ratio = 0.001, p = 0.973, N = 61). Our spermatophore weights were similar to those reported for well-fed, healthy males in other studies of *B. anynana* spermatophores (0.33 mg ± 0.14) (Lewis and Wedell 2007), however, our sample size was relatively small, and would only allow us to detect large treatment effects on spermatophore size, especially given the sensitivity of our balance. That being said, we would have needed 971 experimental trials for the observed 0.02 mg larger spermatophore given to 0 spot females to be deemed statistically significant, and 189 trials for the observed 0.04 mg larger spermatophore given to naïve females to be deemed significant (see post hoc power analyses in Supplemental Table 1) and therefore if there is an effect of female phenotype or male experience on male spermatophore weight it may be a small one.

Discussion

We found that females artificially modified to have 0 dorsal hindwing spots laid, on average, 26% more eggs than females with 2 dorsal hindwing spots, independent of whether they were paired with a socially experienced or naïve male. This was unexpected, since naïve wet season males mate randomly when given a choice between 0- and 2-spotted females (Westerman et al. 2014), and wet season *B. anynana* males were not thought to have dorsal wing-pattern based naïve preferences (Bear and Monteiro 2013; Prudic et al. 2011). Our results suggest that naïve wet season *B. anynana* males do have a dorsal wing-pattern based preference, but it is cryptic and post-copulatory. While this cryptic preference may not be associated with a large difference in the weight of the transferred spermatophore, wet season and dry season *B. anynana* males produce spermatophores that differ in nutrient content but not weight (Prudic et al. 2011), and something similar may be occurring in wet season males paired with either 0- or 2- spot females. The difference in egg production of 0- and 2- spot females is similar in proportion to the difference previously detected in females paired with wet and dry season males, (Prudic et al. 2011), suggesting this cryptic male preference may have a similarly significant effect on realized female fecundity.

While male mate choice has received sparse attention in comparison to female choice, evidence posits that it is common among insects (Dukas et al. 2006). Males often incur significant costs from ejaculate production, rejecting the common “sperm is cheap” theory (Lüpold et al. 2011). Since their ejaculate is costly, one might expect males to preferentially allocate their reproductive nutrients in response to female quality. Post-copulatory mechanisms have been extensively researched in females, as their role in sexual selection continues well after copulation with sperm manipulation and egg laying (cryptic female choice) (Eberhard 2009). However, post-copulatory mechanisms in males are less explored, as male manipulation outside of mating decisions is difficult to observe. Our findings indicate the presence of cryptic preference in males’ resource allocation. Socially naïve males may not display their preference for 0 spot females via mate choice (Westerman et al. 2014), but may instead display their preference via the composition of their spermatophore and ejaculate.

The increase in egg production in 0-spot females could be the result of an increase in reproductive resource allocation by males, an increase in reproductive resource allocation by females in response to a difference in male behavior, or a combination of the two. Since we did not compare male behavior during the mating assays, it is difficult to untangle these hypotheses at this time. However, a previous study found that neither male latency to court nor male courting persistence influence female mate selection in choice assays in *B. anynana* (Robertson et al. 2020). Thus if male courting behavior does influence female reproductive resource allocation, this would also be an example of a cryptic preference, albeit a female one. Furthermore, newly emerged *B. anynana* males behave similarly around sexually mature 0 and 2-spot females (Westerman et al. 2014). Future research should examine whether sexually mature males also behave similarly around 0 and 2-spot females in an effort to assess the extent of this cryptic preference, as well as the relative contributions of males and females to the 26% increase in egg production of females artificially manipulated to have 0 dorsal hindwing spots.

The limited research in male post-copulatory mate choice mechanisms, and the connection between cryptic choice and learned pre-copulatory preferences, makes our finding that naïve males exhibit a post-copulatory preference similar to the pre-copulatory preference of experienced males particularly intriguing. This finding suggests that the “learned” male preference of 0 over 2-spot females is already present, but the three-hour exposure to 0-spot females strengthens that preference so that it manifests as a pre-copulatory preference in addition to a post-copulatory preference (Westerman et al. 2014). This transition from a post-copulatory preference to a pre-copulatory preference in response to a learning event is one way that learning could enhance reproductive isolation and speciation. `.

Learning has often been hypothesized to either increase or decrease the rate of sexual selection and thus of speciation (Kawecki 2013; Lachlan and Servedio 2004). In birds, sexual selection for mating songs diverges more rapidly in learning models versus non-learning models (Lachlan and Servedio 2004). Thus, learning is hypothesized to reduce the waiting time for speciation to occur and is predicted to accelerate the rate of speciation. When taken in concert with the results of Westerman et al. 2014, our results provide an example of a mechanism for learning to cause an increase in the rate of speciation: an experience-induced transition from a post-copulatory preference to a pre-copulatory preference.

While we documented a relatively large (26%) effect of perceived female wing pattern on female fecundity, it is worth noting that *B. anynana* females can live approximately a month when fully healthy (Molleman et al. 2009), and we only collected eggs for the first week post-copulation, not the lifetime of the female. It is possible that the frequency of egg laying could differ after this week period, which would lead to different counts for egg production. However, prior studies on the effect of spermatophores on female butterfly reproductive output show that variation in spermatophore quality tends to exhibit an increased effect on female fecundity over time (Karlsson 1998). This suggests that our finding that 0-spot females lay more eggs than 2-spot females would likely be bolstered if females were allowed to lay eggs for more than seven days.

While we did not find an effect of either female wing pattern or male experience on spermatophore size, the effect of artificial female wing pattern on female fecundity suggests that future research should explore the effect of male preference on spermatophore content in *B. anynana* and other butterflies. In addition, it suggests that male cryptic choice may be more prevalent than previously thought, particularly in species where males make energetically costly contributions to reproduction, such as through spermatophores.

Conclusion

We found that male *B. anynana* butterflies preferentially allocate spermatophore or ejaculate content to females based upon female wing pattern. 2-Spot females manipulated to appear to have 0-spots laid significantly more eggs than 2-spot females with 2 visible spots, irrespective of whether mated to an experienced or naïve male. This suggests male *B. anynana* have a post-copulatory, cryptic preference for 0-spot females. These results support previous studies that indicate male insects can manipulate the contents of their spermatophore or ejaculate. The combination of this innate cryptic preference for 0-spot females and male *B. anynana*'s learned pre-copulatory preference for 0-spot females (Westerman et al. 2014) provide a pathway for how mate preference learning could act on existing preferences and enhance sexual selection and reproductive isolation.

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Author Contribution J.P.S. and E.L.W. designed the study, J.P.S., D.N.R., and T.L.T. conducted experiments, J.P.S., T.L.T, and E.L.W. analyzed the data, all authors wrote and reviewed the manuscript.

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Declarations

Conflict of Interest The authors declare no conflict of interest.

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