Male and Female Mating Behavior is Dependent on Social Context in the Butterfly *Bicyclus anynana*

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Abstract Reproduction is often more costly to females than it is to males, leading to the evolution of ornamented or competitive males and choosy females. Reproduction costs to females, however, can be reduced through nuptial gifts provided by males. These gifts, by increasing female survival or fecundity, can promote the evolution of mutual mate choice, ornamentation, or competition in both sexes, as well as plasticity in mating behavior dependent on social context. We tested for plasticity in male and female mating behavior in a species of butterfly, *Bicyclus anynana*, where male spermatophore gifts contribute to female survival and fecundity, and where mutual mate choice and ornamentation were previously established. We examined the effect of a sexual competitor on male–female interactions by observing and comparing the behavior of male–female pairs with that of triads containing either an extra male or an extra female. In the presence of a sexual competitor both males and females copulated less than when in male–female pairs, regardless of the direction of sex-ratio skew. Active males increased their own likelihood to copulate, while active females increased their likelihood of being courted. In addition, there was an effect of social context on relative rates of male and female courting and flying. These results

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suggest that both males and females change their mating behavior in response to social context in the butterfly *Bicyclus anynana*.

Introduction

Bateman's principle suggests that mating is more costly to females than males, and therefore females should be choosy and males should compete for access to mates (Bateman 1948; Trivers 1972). This concept of a choosy sex and a competing sex has been an important component of sexual selection (Andersson 1994; Darwin 1871; Dewsbury 2005). However, in species where both sexes mate multiply within a breeding season and make significant energetic contributions to the offspring, mutual mate choice and both male and female same-sex competition may occur (Johnstone et al. 1996; Kokko and Johnstone 2002; Servedio and Lande 2006). These different types of behavior are also likely to be plastic if there is temporal (seasonal) variation in the quality and investment in reproduction exhibited by both sexes (Obara et al. 2008b; Prudic et al. 2012).

Polygynandrous lepidopterans, where both sexes are promiscuous, are good candidates for exhibiting male and female mate choice and intrasexual competition. Females produce large eggs relative to sperm, and this normally leads to male-male competition and female choosiness (Trivers 1972). However, males of many lepidopteran species give females a spermatophore that contributes significantly to reproduction, and is costly for the male to produce, making females potentially compete for access to males, and males potentially choosy (Gwynne 1981; Karlsson 1998; Prudic et al. 2012; Vahed 1998). In addition, males that choose larger females should have higher fitness because female reproductive capacity is often correlated with female size in lepidopterans (Bergstrom and Wiklund 2002; Karlsson and Wickman 1990). Finally, many lepidopterans exhibit seasonal forms adapted to seasonal environments where resources may be limited in one season but not in the other, and where the relative value (and content) of a spermatophore gift varies with the season (Brakefield and Reistma 1991; Prudic et al. 2012). This seasonality can lead to additional variation in male and female sexual behavior.

Although the polygynandrous life history of many lepidopterans makes them good candidates for exhibiting mutual mate choice and intrasexual competition, these types of behavior have rarely been studied in both sexes of a single species in this group. There is an abundance of data on male mate choice (Estrada and Gilbert 2010; McMillan et al. 1997; Obara et al. 2008b; Rutowski 1980; Rutowski et al. 1981) and male-male competition (Bergman et al. 2007, 2010; Davies 1978; Kemp and Wiklund 2001), but few published studies assessing female rejection of courting males (female choice) or female-female competition in butterflies. Recent research on the butterfly *Bicyclus anynana* showed that mutual ornamentation as well as female choice (for male wing patterns, amount of wing damage, age, and pheromones) and male choice (for female wing patterns) take place in this species (Costanzo and Monteiro 2007; Fischer et al. 2008; Nieberding et al. 2008; Prudic et al. 2012; Robertson and Monteiro 2005; Westerman et al. 2012), but the behaviors involved in intrasexual mate competition in *B. anynana* remain undescribed. In particular, it is unclear what male-male and female-female competitive signals look like in this species.

Intrasexual competition for access to mates may take a variety of different forms in lepidopterans. Pre-copulation intrasexual competition may include males competing for access to territories that females frequent, thereby increasing their likelihood to copulate (Davies 1978; Lederhouse 1982; Rosenberg and Enquist 1991), or competing for female acceptance by performing a courtship behavior to which females may respond positively or negatively (Melo et al. 2009; Nieberding et al. 2008; Obara et al. 2008a; Robertson and Monteiro 2005). Female lepidopteran intrasexual competitive behavior has rarely been documented except for the emission of attractive pheromones in moths (reviewed in Ando et al. (2004)). However, in species where females pursue males, such as *Pieris protodice* (Rutowski 1980) and the Dry Season form of *Bicyclus anynana* (Prudic et al. 2012), we might expect females also to engage in intrasexual competitive behavior, such as courting displays or bouts of female-female aerial displays.

We set out to test whether mate competition influences both male and female behavior in *B. anynana*. This African butterfly has a seasonal polyphenism in the quality of the male spermatophore and in male and female courtship displays. The spermatophore of the Dry Season (DS) male morph is of high quality as it increases the lifespan and fecundity of a mated female, and is costly to produce because male lifespan decreases after mating, while the spermatophore of the Wet Season (WS) male morph does not provide any longevity or fecundity benefits to females nor costs to males (Prudic et al. 2012). Spermatophore quality was correlated with the rate of courtship displays in both males and females. DS males courted less than WS males, and DS females courted more than WS females when butterflies were observed in even sex ratio cages containing multiple butterflies (Prudic et al. 2012). In that study, however, the relative proportions of mated versus unmated butterflies were unknown at the time of observation, and intrasexual competition was not documented. We therefore decided to observe triads of virgin butterflies to determine whether and how males and females respond to the presence of a sexual competitor, using virgin male/female pairs as a control treatment. To maximize the likelihood of a female response, we used DS butterflies for this study.

If the previously described courtship display is the primary factor determining access to a mate in intrasexual competitive environments, we expect that both sexes respond to the presence of a sexual competitor by increasing the amount of time they spend courting the opposite sex relative to that observed in both the pairs and the triads where they did not have a sexual competitor. We also expect that females increase their courting rate more than males because DS males are choosier than DS females (Prudic et al. 2012). However, if intrasexual interactions are important for accessing mates, as has been described for males in other butterfly species, we expect males and females to fly more and court less in the presence of a sexual competitor.

Methods

Study Organism

A colony of the sub-tropical African butterfly, *B. anynana*, was established in New Haven, CT in 2006, using hundreds of eggs from a lab colony in Leiden, the Netherlands. The Leiden population originated from 80 gravid females collected in

Malawi in 1988. All study animals were reared in a walk-in climate chamber at 17 °C, 80 % humidity, and a 12:12 h light:dark photoperiod. Larvae were fed young corn plants, and adults were fed moist banana slices. Butterflies were reared in a continuous system of overlapping generations, where adults emerge every day of the year.

Though *B. anynana* has been maintained in laboratory conditions for the last 25 years, and therefore may have undergone genetic bottlenecks in the transition to this environment, laboratory populations contain similar single-nucleotide polymorphism (SNP) frequencies to those of current natural populations (Beldade et al. 2006; de Jong et al. 2013), suggesting that laboratory breeding practices have maintained levels of genetic diversity similar to those found in natural populations. Furthermore, despite the absence of a description of *B. anynana* mating displays in natural conditions, observations made in large greenhouse spaces and in large climate rooms suggest that WS *B. anynana* males patrol to find females and perching WS females accept or reject males that approach them (Joron and Brakefield 2003; Nieberding et al. 2008; Prudic et al. 2012; Robertson and Monteiro 2005). The stereotypic courtship behavior exhibited by males of multiple populations in both large greenhouse enclosures and smaller rearing cages (Nieberding et al. 2008; Nieberding et al. 2012; Prudic et al. 2012; Westerman et al. 2012) suggests that the butterfly behavior observed in the experimental conditions described below is not specific to this particular breeding population, and likely contains a significant representative range of behavioral responses to intrasexual competition and mate choice.

Experimental Design

We examined whether the presence of a sexual competitor of either sex altered male–female interactions. We conducted behavioral observations of all individuals across three social contexts: 1) one male and one female (pairs); 2) two males and one female (male competition context); and 3) one male and two females (female competition context). We then analyzed the data by testing how presence/absence of a competitor influenced the behavior of male and female individuals. Fifteen trials were conducted for each treatment, and each butterfly was only used once (total $N=120$, 60 females and 60 males).

Behavior assays were conducted using cylindrical hanging net cages (30 cm×40 cm) under full spectra sun lamps (including UV wavelengths) in an observation area with east-facing windows, a behavioral arena in which both males and females are known to court, exhibit mate choice, and copulate (Prudic et al. 2012). Many hours of behavioral observations under these conditions have led us to conclude that mixed sex pairs are capable of engaging in prolonged bouts of aerial interactions that influence mating outcome in laboratory conditions (Westerman et al. 2012; Westerman and Monteiro 2013). Butterflies were separated into sex-specific cages on the day of eclosion from pupa, and visual barriers were placed between cages so that each sex could not see the other until the behavioral observation. Females in any given trial, and in both trials on a given day, were of the same age, although female age varied across observation day (range=2–8 days old, mean=3.333 days old). Males were also the same age in any given trial, and in both trials on a given day, with male age varying between observational days (range=2–10 days old, mean=3.022 days old, males are reproductive throughout this age range (Nieberding et al. 2012)). Males and females

were either 0 or 1 day apart (with males always older than females) in all but two trials of each triad treatment, in which ages varied by 3 and 5 days (with males again older than females). *Bicyclus anynana* females are sexually receptive within 24 h of emergence (personal observation), and males will court females by day two (Nieberding et al. 2012), so all the individuals used in the study were sexually mature. However, we held the age distribution constant for all treatments, and matched intrasexual competitors for age, as data suggest females find older males more attractive in *B. anynana* (Fischer et al. 2008; Nieberding et al. 2012).

Virgin females were isolated 18–22 h in advance of each behavioral assay. At the same time, one and two virgin males were isolated in cylindrical hanging cages in the observational area. While this skewed the sex that had prior experience in the observational area, it allowed us to control for the effect of introduction to a new location on activity levels, as all females were introduced to a new environment the morning of observation, and all males had approximately 24 h to acclimate to their new environment prior to observation, regardless of treatment. This suggests that any treatment effect on relative rates of male and female behavior are the result of treatment (1M:1F, 1M:2F, 2M:1F) and not the result of novel versus acclimated environment.

To begin each trial, food was removed from the male cages, and the female(s) for that trial were transferred to the cage with the male(s). For both sexes, the sexual competitors were matched in wing size, and there was no variation in wing patterning (all individuals had a DS wing pattern). After a 5-min acclimatization period, the triad was observed for 50 min from a distance of 4 m. Each individual butterfly was only used once, so we observed the first intersexual experience of virgin individuals. Triads were observed on 15 days over a 3-month period from February to April 2010, and pairs were observed on 15 days over a 3-month period from May to July 2011. (Courting and copulation are observed year round for *B. anynana* in laboratory conditions.) Two triads were observed on each day, one from each treatment, while a single pair was observed on each day. All trials occurred between 7:50 and 10:00 AM, yielding 45 total trials. A coin flip to randomize order was used to determine which triad condition would be completed first each day, with the first trial beginning at 8:00 AM (±10mins) and the second trial beginning at 9:00 AM (±10mins). Half of the trials for pairs began at 8:00 AM (±10mins), the other half at 9:00 AM (±10mins). We documented every occurrence of flying, wing fluttering, walking on the cage, courting, and mating for each individual using continuous recording. We also recorded whether courtship was directed towards other- or same-sex individuals, and the time from beginning of observation period to copulation. Courtship consists of a series of ritualized behaviors, involving *localization* (locating and approaching a female), *orientation* (male orients his body perpendicular to the posterior of the female), *flickering* (rapid fluttering of wings), a *thrust* (male touches female's wings with his head), and *attempt* (curling of the abdomen) (Nieberding et al. 2008). Successful courtship ends with genital contact and copulation. Females can perform these same behaviors towards males, and rearing temperature of the larvae and/or pupae largely determine the identity of the sex that courts most actively in large group settings (Prudic et al. 2012). While we recorded all bouts of behavior, we did not use any post-copulatory behavior in our analyses, unless otherwise stated, as we were most interested in pre-copulatory male–female interactions in the presence of a sexual competitor.

To account for the potential effect of variation in natural lighting conditions (outside weather) and of age of individuals on male and female competitive behavior, we recorded outside weather for all trials of the two competitive social context treatments, and the age of each individual (days) for all trials of all three social context treatments. Outside weather was visible through the windows lining the observation area throughout the study, which we categorized as sunny, partially cloudy, overcast, and storming. We also recorded ambient temperature for the latter two-thirds of the triads (ten of each treatment, $N=20$) and all of the pairs ($N=15$). Temperature in the observation area varied between 25 and 36 °C, though it was not variable on any given day, so the 2M:1F and 1M:2F treatments experienced the same amount of variation in temperature. Pair trials experienced a similar amount of variation in ambient temperature as the triad treatments.

Statistical Analysis

In order to determine whether there was an independent effect of adult age, outside weather conditions, or ambient temperature on male and female activity levels, we pooled the behavioral data by sex for the three treatments, and correlated general activity level (PC1 from a principal components analysis by sex containing rate of each behavior observed, see below) with age, outside weather conditions, and ambient temperature using a general linear model with a binomial distribution.

In order to evaluate the effect of sexual competitor on rates of behavior, we calculated rates of flying, fluttering, walking, and courting for each individual by dividing the number of bouts of these activities prior to copulation by the total amount of time prior to that individual's copulation event (or total observation time if no copulation took place), and averaged these rates for the two females in each 1M:2F trial and the two males in each 2M:1F trial. This allowed us to analyze rates of behavior for each sex, per behavioral context, rather than for each individual. We used these average behavioral rates for all following analyses, other than those directly testing the covariance of the behavior of the two sexual competitors in the 1M:2F and 2M:1F treatments. To determine whether there were either sex-specific or social-context-specific rates of behavior, we used a full factorial general linear model with sex and treatment as factors.

To obtain fewer measures of overall rates of behavior for each sex we performed a principal component analysis, using rate of flying, fluttering, walking, and courting for each sex (using average instead of individual rates of behavior for the two sexual competitors). In order to determine whether there was an effect of a sexual competitor on the general behavior rates of each sex, we compared average scores of the first and second principal components between treatments for each sex using ANOVA and multiple comparison Tukey-Kramer tests. To determine whether there was a treatment by age effect on the relationship between rate of behavior and time to copulation, we correlated rate of behavior and time to copulation with subject's age and treatment using a full factorial least squares model with binomial distribution.

In order to determine whether the presence of a sexual competitor influenced a) likelihood of copulation, b) the presence of male courting behavior, or c) the presence of female courting behavior, we tallied mating occurrence, i.e., presence or absence of mating, for each treatment (1M:1F, 2M:1F, 1M:2F), and for each individual in the

triads. We conducted a full factorial generalized linear model with binomial distribution on presence of courting using treatment and sex as our independent variables. We also calculated the proportion of trials in which mating occurred, and compared across the three treatments using a whole experiment χ^2 proportion test followed by pair-wise χ^2 comparisons.

In order to determine whether a higher proportion of individuals were courting/being courted than were obtaining copulations, we calculated the proportion of individuals of each sex that mated, and the proportion of individuals of each sex that courted, across all replicates of a treatment, and compared differences across the three treatments using whole experiment χ^2 proportion tests followed by pair-wise χ^2 comparisons.

In order to determine whether activity levels of one individual were predictive of the activity levels of its sexual competitor, we regressed levels of activity (PC1 and PC2) of one individual on those of the other individual for the same-sex pair in each triad trial (15 pairs of males and 15 pairs of females). Influence of activity level on likelihood to mate was estimated by regressing presence or absence of mating on rate of activity (PC1), for each of the treatments separately, using a general linear model with binomial fit. All statistical analyses were conducted in the JMP® statistical software package version 10.

Results

Principal Component Analysis

A principal component analysis (PCA) performed on the multiple behaviors scored during the observational trials identified new composite variables (PCs) that explained most variation in behavior. The resulting first PC explained 66.5 % of the variation in the data for females and 69.1 % for males, and was composed in roughly equal parts of flying, fluttering, and walking for females, and all these variables as well as courting, in equal parts, for males (Table 1). We used this first PC as a proxy for general activity level in subsequent analyses. The second PC explained 25.1 % of the variation for females and 15.9 % of the variation for males, and consisted primarily of positive loadings on courtship for females, and a contrast between courting, and fluttering and

Table 1 Principal component analysis of all behaviors observed for each of the sexes (all three treatments combined)

	Males		Females	
	PC 1	PC 2	PC 1	PC 2
Eigenvalue	2.762	0.638	2.658	1.007
Percent of variation captured	69.07 %	15.92 %	66.46 %	25.17 %
Loading: flying/min	0.530	0.084	0.565	0.053
Loading: fluttering/min	0.534	−0.413	0.595	−0.086
Loading: walking/min	0.495	−0.372	0.572	−0.016
Loading: courting/min	0.436	0.827	0.030	0.995

walking (i.e. ., whether male non-flight activity was directed specifically towards females (courting), or was primarily directed elsewhere (fluttering and walking)) for males.

Effect of Age, Temperature, and Outside Weather on Activity Levels Independent of Social Context

There was a significant general effect of age, but not outside weather conditions or ambient temperature on female activity levels (general linear model, parameter effect tests: age $F_{4,59}=4.443$, $P=0.045$; ambient temperature $F_{6,59}=0.775$, $P=0.387$; outside weather $F_{2,59}=0.673$, $P=0.519$). Females increased their activity levels with age. There was no significant effect of age, ambient temperature, or outside weather conditions on male activity levels (general linear model, parameter effect tests: age $F_{3,59}=0.454$, $P=0.507$; ambient temperature $F_{6,59}=3.313$, $P=0.081$; outside weather $F_{2,59}=2.713$, $P=0.087$). Given these results, and given the matching butterfly age distributions for the three treatments, environmental conditions and age were not included as covariates in the analyses described below unless specified.

Males Flew and Courted More Than Females, But Only Under Certain Social Contexts

Both males and females courted only the opposite sex in the three treatments. Male and female relative rates of behavior changed in response to whether they were in pairs, 1M:2F triads, or 2M:1F triads. Males had higher courting rates than females when alone with a single female or with two females, but this effect was reduced in the male competition context (GLM, treatment: $F_{2,89}=3.716$, $P=0.028$; sex: $F_{1,89}=14.148$, $P=0.0003$; treatment*sex: $F_{5,89}=3.395$, $P=0.038$) (Fig. 1a). Males also flew more often than females when in pairs, but not when in either 1M:2F triads or 2M:1F triads (GLM, treatment: $F_{2,89}=2.226$, $P=0.114$; sex: $F_{1,89}=2.916$, $P=0.913$; treatment*sex: $F_{5,89}=3.248$, $P=0.044$) (Fig. 1b). Males and females had similar rates of walking, except for when in 2M:1F triads, where females walked more than males (GLM, treatment: $F_{2,89}=3.664$, $P=0.029$; sex: $F_{1,89}=0.711$, $P=0.401$; treatment*sex: $F_{5,89}=3.856$, $P=0.025$) (Fig. 1c). Males and females had similar rates of fluttering in the three social contexts tested (GLM whole model $F=2.056$, $P=0.079$) (Fig. 1d).

Presence of a Sexual Competitor Reduces Likelihood of Courting and Copulation for Males and Females

There was an effect of both sex and treatment on likelihood to court, with males more likely to court than females in all treatments, and males less likely to court in the 2M:1F treatment than in either the 1M:1F treatment or the 1M:2F treatment (generalized linear model with binomial distribution, whole model test AICc=84.554, $\chi^2=59.615$, $P<0.0001$; effect tests: treatment: $\chi^2_{2,89}=16.177$, $P=0.0003$; sex: $\chi^2_{1,89}=27.381$, $P<0.0001$; treatment*sex: ns, $\chi^2_{5,89}=4.154$, $P=0.125$)(Fig. 2).

There was also an effect of treatment on likelihood of copulation for both males and females, with copulations more likely to occur in pairs than in either competitive scenario (Whole experiment Pearson $\chi^2_{2,44}=10.462$, $P=0.0053$; 1M:1F vs 1M:2F
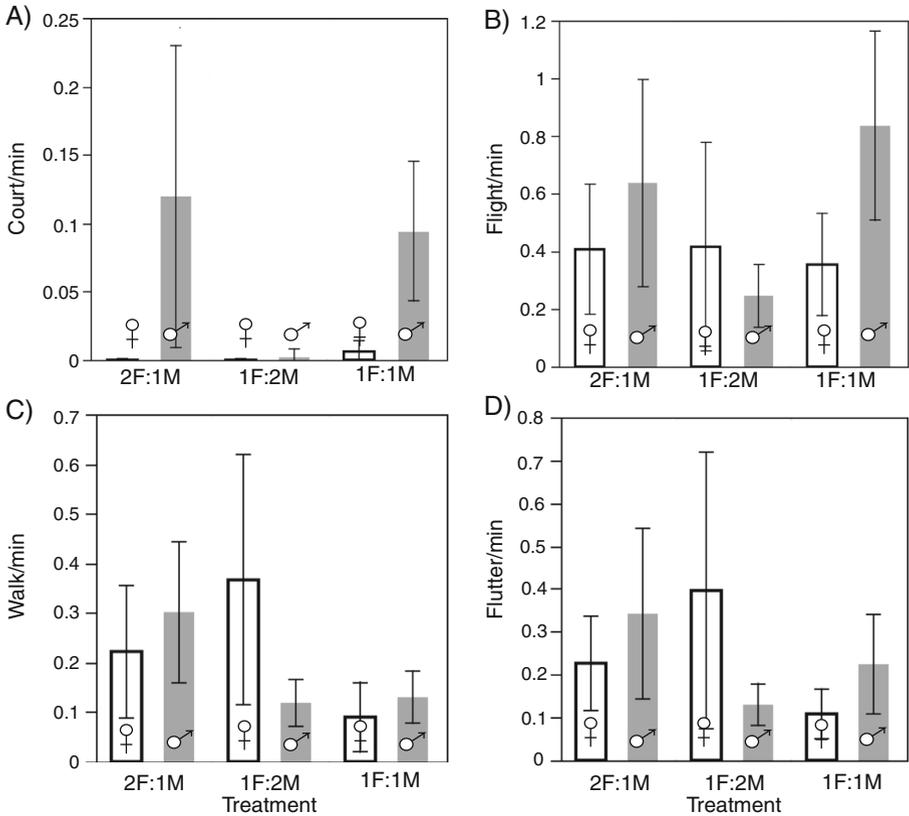
Fig. 1 Female and male relative rates of behavior vary with social group composition. Average rate of behavior compared between sexes within each treatment. In the 1M:2F triads rates of female behavior are averages between the two females, and the same applies for male behavior in the 2M:1F triads. *Error bars* represent 95 % confidence intervals. **a** Courting. **b** Flight. **c** Walks. **d** Flutters

Pearson $\chi^2_{1,29}$=5.427, P=0.019; 1M:1F vs 2M:1F Pearson $\chi^2_{1,29}$=9.378, P=0.0022; 2M:1F vs 1M:2F Pearson $\chi^2_{1,29}$=0.683, P=0.409). We report details of male and female courting behavior in the three social contexts below.

Males: Effect of Social Context on Courting

Males in the presence of a sexual competitor courted at most once during the 50-min observational period, whereas 60 % (9 of 15) of the males without a competitor and in the presence of two females, and 53 % (8 of 15) of the males in pairs, engaged in multiple courtship events. Males in the presence of two females often courted both females prior to copulation instead of simply courting a single female multiple times (Suppl. Table 1).

The presence of a male competitor significantly reduced the number of trials where courting occurred relative to trials with single males (Whole experiment Pearson $\chi^2_{2,44}$=13.591, P=0.0011; 2M:1F vs 1M:1F Pearson $\chi^2_{1,29}$=13.274, P=0.0003; 2M:1F vs 1M:2F Pearson $\chi^2_{1,29}$=5.129, P=0.0235).
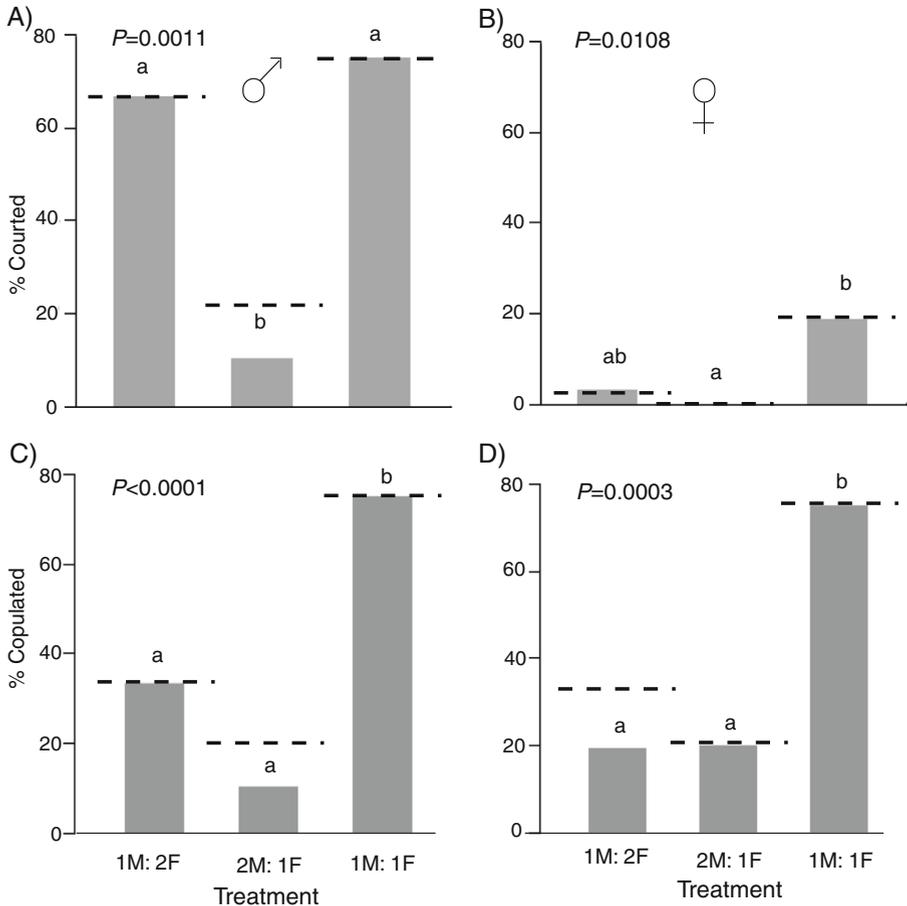
Fig. 2 Effect of sexual competitor on presence of courtship and copulation tallied across all trials for each treatment. Percentages on Y-axis represent the proportion of individuals of each sex in each treatment that courted and copulated. A greater percentage of males copulated in pairs than in triads, and male courting was depressed in the multiple male treatment. A higher percentage of females copulated in pairs than in either triad treatment. Comparison of percentage of **a** males and **b** females who courted in each treatment. Comparison of percentage of **c** males and **d** females that copulated in each treatment. Within each graph, distinct *letters* above *bars* represent statistically different copulation and courting percentages. *Dashed lines* represent percentage of trials within each treatment where a single courting (**a** and **b**) or a single copulation (**c** and **d**) event occurred. Displayed P values are for whole experiment χ^2 tests of the effect of social scenario on likelihood of courtship and copulation

Males: Effect of Social Context on Copulation

Presence of a male competitor reduced the proportion of males that copulated relative to pair trials and to trials with two females (Whole experiment Pearson $\chi^2_{2,44}=19.397$, $P<0.001$; 2M:1F vs 1M:1F Pearson $\chi^2_{1,29}=19.397$, $P<0.001$; 2M:1F vs 1M:2F, marginally non-significant: Pearson $\chi^2_{1,29}=3.725$, $P=0.054$) (Fig. 2c). Only once did a male mate with both females in the 1M:2F trials (Suppl. Table 1).

Females: Effect of Social Context on Courting

Female courting was rarely observed. Three females courted when in pairs, a single female courted when in the presence of a sexual competitor, and no females courted in the male competition social context (Fig. 1a) (Suppl. Table 1). The absence of female courting in the female competitive scenario resulted in there being a significant treatment effect on number of trials where a female courting event occurred, with females in pairs courting more often than females in competitive scenarios, but not more often than those in choice scenarios (Whole experiment Pearson $\chi^2_{2,44}=9.064$, $P=0.0108$; 1M:2F vs 1M:1F Pearson $\chi^2_{1,29}=6.209$, $P=0.0127$; 2M:1F vs 1M:1F Pearson $\chi^2_{1,29}=3.114$, $P=0.0776$) (Fig. 2b). However, given the small number of female courting events observed, we need to interpret these results with caution.

Females: Effect of Social Context on Copulation

A greater proportion of females in pair trials copulated relative to females in competitive scenarios (1M:2F triads) or choice scenarios (Whole experiment $\chi^2_{2,44}=16.289$, $P=0.0003$, 1M:1F versus 1M:2F, 75 % versus 20 %, Pearson $\chi^2_{1,29}=13.828$, $P=0.002$; 1M:1F versus 2M:1F, 75 % versus 20 %, Pearson $\chi^2_{1,29}=9.378$, $P=0.002$) (Fig. 2d).

Social Context Influenced Activity Levels for Both Males and Females

Males: Effect of Social Context on Activity Levels

The presence of a sexual competitor reduced male general activity level relative to males in choice scenarios (PC1) (ANOVA, $F_{2,44}=4.635$, $P=0.015$; Tukey-Kramer 2M:1F v 1M:1F ns, $P=0.132$; 2M:1F v 1M:2F $P=0.012$), but did not affect PC2 (consisting of inverse amounts of courtship to fluttering and walking) in males (ANOVA, ns, $F_{2,44}=1.788$, $P=0.179$), (Figs. 3 and 4b, d).

Females: Effect of Social Context on Activity Levels

In contrast to males, females did not alter their general activity level (PC1) across social context (ANOVA, ns, $F_{2,44}=1.352$, $P=0.270$) (Figs. 3b and 4c). However, females in pairs had higher PC2 scores, which were composed primarily of courtship, than females in either competitive or choice scenarios (ANOVA, $F_{2,44}=4.688$, $P=0.014$; Tukey-Kramer 1M:1F v 2M:1F, $P=0.039$; 1M:1F v 1M:2F, $P=0.024$) (Figs. 3b and 4d).

Males: Activity Rates and Competition

More active males increased their likelihood of copulation (full factorial logistic model with PC1 and treatment as factors, whole model AICc=55.421, $\chi^2=34.54$, $P<0.0001$, effect tests: treatment $\chi^2_{2,44}=12.037$, $P=0.0024$, PC1 $\chi^2=9.15$, $P=0.0019$, treatment*PC1 $\chi^2=2.331$, $P=0.312$).
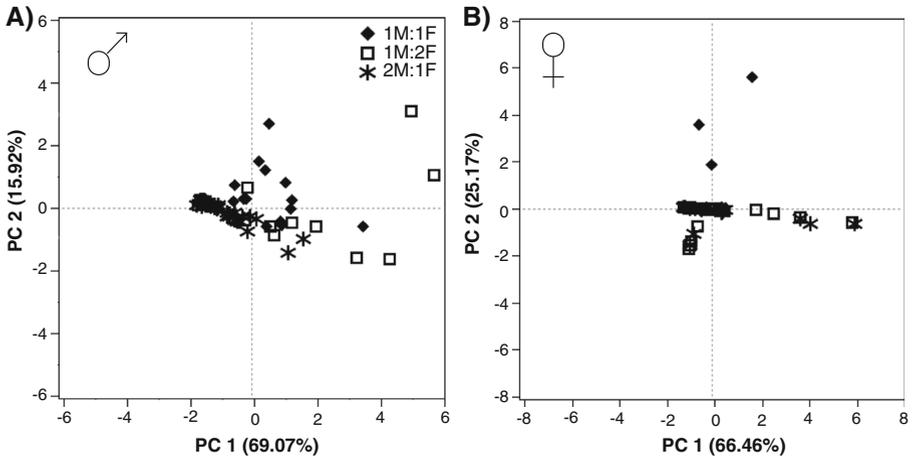
Fig. 3 Categorized PC1 vs PC2 scatter plots for **a** males and **b** females. *Numbers in parentheses* indicate percentage of variation captured by each principal component. PC1 (general activity levels) is greater for males in 1M:2F triads compared to males in 2M:1F triads. Females in pairs differ from females in both types of triads in terms of PC2 (primarily composed of courting)
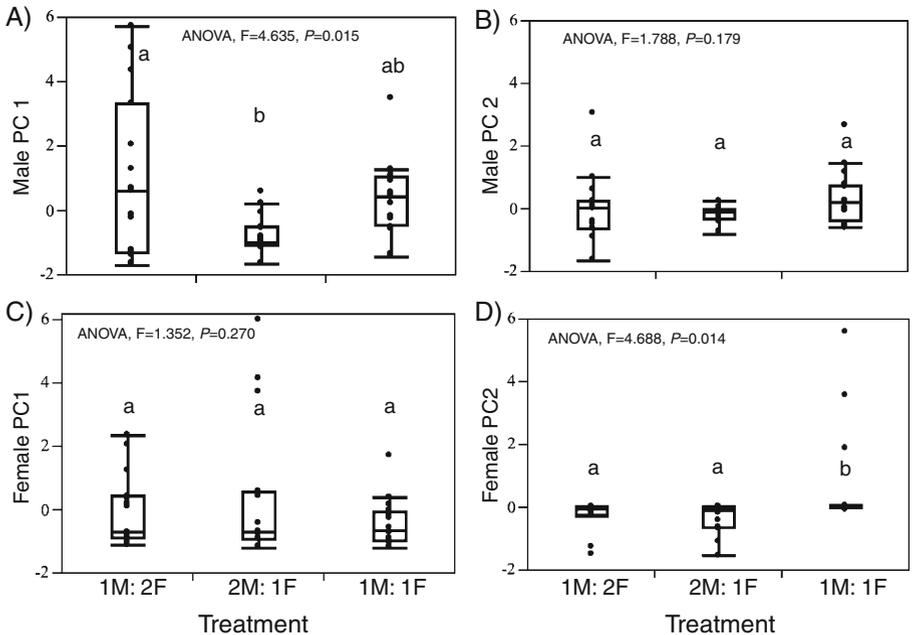
Fig. 4 Effect of a sexual competitor on male and female activity levels. Males reduce activity levels (PC1) in the presence of a sexual competitor (**a**) while females do not (**c**). Females increase their PC2 scores (primarily composed of courting) when in 1M:1F pairs (**d**), while males do not (**b**). Within each graph, *letters* represent statistically different values of PC1 or PC2. Displayed *P* values are for ANOVAs of the effect of social scenario on activity levels within each sex

Females: Activity Rates and Competition

Active females were more likely to be courted than inactive females (full factorial ordinal logistic model, whole model AICc=77.472, χ^2=16.920, P=0.0047, effect tests: treatment $\chi^2_{2,44}$=5.232, P=0.073, PC1 χ^2=8.090, P=0.004, treatment*PC1 χ^2= 5.293, P=0.071). However, unlike males, more active females did not increase their likelihood of copulation (full factorial ordinal logistic model, whole model AICc= 67.926, χ^2=24.983, P=0.0001, effect tests: treatment $\chi^2_{2,44}$=17.041, P=0.0002, PC1 χ^2=3.172, P=0.075, treatment*PC1 χ^2=0.537, P=0.764). There were no significant treatment by age effects on female rates of courting (least squares model containing treatment, age, and treatment* age, whole model F=0.149, P<0.001, parameter effect tests: treatment F=12.017, p=0.001, age F<0.001, P>0.999, treatment*age F=1.648, P=0.205), though female activity levels did increase with age (see Results reported above).

For Both Males and Females, Pairs of Sexual Competitors Matched Specific Behaviors

General activity level of the two males in triads was significantly correlated (R^2=0.501, P=0.003), while the general activity levels of the two females in triads were not (R^2< 0.001, P=0.954). Male PC2 (a contrast between courting, and walking and fluttering) was not correlated for the two males in triads (R^2=0.002, P=0.811), while female PC2 (consisting primarily of positive loadings on courtship) was positively correlated for the two females in triads (R^2=0.497, P=0.003). It should be noted, however, that PC2 values for the two females ranged from −1.7 to 0.05, reflecting the observation that female courting in the sexual competitor scenario was rare.

To summarize the results of our study: males courted less and both males and females copulated less in the presence of a sexual competitor than when isolated with a single member of the opposite sex. A decrease in courtship frequency was also observed in females when in the presence of two males, and a decrease in copulation frequency occurred when males or females were in the presence of two individuals of the opposite sex. This resulted in paired butterflies being more likely to copulate during their first hour of contact than butterflies in triads, regardless of the direction of sex-ratio skew. In the presence of a sexual competitor males also decreased their overall activity levels, and engaged in correlated bouts of activity. General activity level was positively correlated with likelihood to mate for males and likelihood to be courted for females. Males courted more than females when in pairs or in the presence of two females, but both sexes exhibited similar courting behavior when females were alone with two males.

Discussion

Our results support the hypothesis that intrasexual interactions are important for females to access mates in the Dry Season (DS), as females who were more active increased their likelihood of receiving male attention. Our results do not support the

hypothesis that the previously described courting display is the primary behavior determining access to a mate in competitive social contexts for either sex, as neither males nor females increased their courting rates in competitive social contexts relative to non-competitive contexts. While we did not observe DS females courting more than DS males, as previously reported in Prudic et al. (2012), our finding that active DS females increase their likelihood of receiving male courtship does support the Prudic et al. (2012) finding that DS females, but not DS males, compete for access to mates.

Our results suggest that the presence of a sexual competitor reduces the likelihood of copulation within an hour of contact for both male and female virgin DS *B. anynana*. The reduction of male courting and general activity levels in the presence of a sexual competitor and the correlation of activity levels between the two males in that treatment suggests that (at least in a laboratory setting) this species' male-male competition in the presence of a female is a very quiet affair where males are aware of each others' presence and moves, and become distracted from courting females, but are less active than males in female competition or mixed-sex pair social contexts. While these findings appear to contradict those describing male-male competition in field conditions for other butterflies (Davies 1978; Lederhouse 1982; Rosenberg and Enquist 1991), and those describing female preference for *B. anynana* males with less wing damage (Fischer et al. 2008), these previous studies did not examine male behavior in multiple social contexts, nor did they examine male-male interactions in a seasonal form where males are more choosy than females. So, currently it is unclear whether the low male activity levels observed in the presence of a sexual competitor is specific to DS *B. anynana* or the laboratory environment, or is a more common lepidopteran response to the presence of a sexual competitor. In addition, previous studies examined initial contact between males in the absence of females, while our study examined the behavior of males after 24 h of contact, but in the presence of a female. Previous experience with a female has been found to change the behavior of males in intrasexual interactions in the butterfly *Pararge aegeria* (Bergman et al. 2010), and it is possible that the 24 h of interaction coupled with the introduction of the virgin female in our trials significantly altered the motivation and consequent behavior of *B. anynana* males from that predicted by previous studies of male-male interactions in the absence of females (i.e. higher rates of courting and general activity levels in competitive social contexts relative to no-competition social contexts).

The positive correlation between an individual's activity level and likelihood to copulate (males) or receive courting (females) may be either the result of the most active individuals gaining increased access or becoming more visible to individuals of the opposite sex in a competitive scenario, or the result of male and female preference for more active mates. Though aerial competition for territories (Bergman et al. 2007; Lederhouse 1982; Rosenberg and Enquist 1991) has not been observed in *B. anynana* males, males of this species sporting wing damage copulate less often than males with intact wings (Fischer et al. 2008). Our finding of a positive relationship between male activity levels and likelihood to copulate, coupled with the correlated male activity level in male-male competitive interactions, suggests that *B. anynana* males may also engage in aerial competitive bouts if given more space to interact. We do not know of any descriptions of female butterflies engaging in aerial competition for territories or mates; female activity presumably solicits male attention and/or courtship, as was observed in this study. Future behavioral research in larger cages and in more natural conditions is necessary to test these hypotheses.

Our results did not fit our hypothesis that the stereotypic courtship behavior was the primary behavior determining mate acquisition, as courting decreased instead of increased for both males and females when in the presence of a sexual competitor, relative to individuals in choice and pair scenarios, while copulation rate was constant in competitive and choice scenarios. Behaviors other than the stereotypic courtship may therefore also be important for acquiring a mate in intrasexual competitive social contexts for both male and female *B. anynana* butterflies, in addition to other signals such as ornamentation (Costanzo and Monteiro 2007; Prudic et al. 2012; Robertson and Monteiro 2005; Westerman et al. 2012) and pheromones (Nieberding et al. 2008; Nieberding et al. 2012). If we had solely used the stereotypic courtship behavior as an indicator of intrasexual competition, we would have failed to document the matched activity levels in males, suggestive of male-male competitive interactions. We also would have missed both the positive relationship between female activity levels and their likelihood of being courted, and the matched activity in females, suggestive of female-female competitive interactions. Our results, therefore, highlight the importance of recording and comparing multiple behaviors, not just those that are most conspicuous, and conducting analyses unbiased by preconceived notions of which behavioral characteristics different species display during the mate acquisition process.

Our finding that DS females did not court more than DS males differs from findings reported in the Prudic et al. (2012) study, which demonstrated that female courtship was greater than male courtship in DS butterflies, and that males were engaging in mate choice while females were not. However, our result that females courted more in pairs with an even sex ratio than in triads with skewed sex ratios suggests that the presence of female courtship may be dependent on less competitive scenarios for females, as in the Prudic et al. (2012) study which contained even sex ratio groups. In addition, our result that a large proportion of males (60 %) courted but many fewer (20 %) copulated when given a choice between two females (female competition social scenario) supports the Prudic et al. (2012) result of male choice in DS butterflies. Finally, further behavioral differences between the two studies may relate to variation in virginity levels. The current study observed triads and pairs of virgin butterflies during the hour immediately following their placement in a common cage, whereas the Prudic et al. (2012) study used multiple individuals who were placed together in a cage 1 day prior to the observations, and where an unknown fraction of individuals likely mated during that period. We observed the initial social dynamics of a small group of virgins whereas the previous study observed the established social dynamics of a larger group of individuals with potentially mixed mating status. We therefore may have observed intrasexual competitive interactions used to establish dominance, which may not have been apparent after the butterflies had been interacting for over 24 h. The hypothetical implications of the virginity differences between the two studies are that mated DS males court less than virgin DS males and/or that mated DS females court more than virgin DS females. These differences in behavior between the two studies suggest that male–female interactions are plastic in *B. anynana*, and further research on the effect of mating status on male–female interactions and the effect of familiarity on intrasexual competitive behavior over time are necessary to determine the causes of this behavioral plasticity.

The finding that males in the female competition social context often courted both females prior to copulating with one female, and courted more often (but did not

copulate more) than males in the presence of a sexual competitor has implications for the way we think about and conduct research on male mate choice and sexual selection. First, our study adds to the growing body of research suggesting that mate preference of the courting sex may be important to mating outcome, particularly when the courting sex is making a significant energetic investment in reproduction (Cook and Wedell 1996; Dewsbury 1982; Engqvist and Sauer 2001; Friberg 2006; Wedell et al. 2002). Second, first court may not be a good proxy for male preference. Approach, first court, and courting frequencies have been used as proxies for male choice in a variety of mate choice studies (Dukas 2004; LeBas and Marshall 2000; Melo et al. 2009; Tompkins and Hall 1981), but courting does not always lead to mating attempts in these studies. As interest in male mating preferences increases, our results support Martel and Boivin's (2011) discussion on the importance of distinguishing between apparent choice and true choice, as one may be unrepresentative of the other. Alternatively, the male decision to court additional females may be driven by female rejection, or an undetected form of female competitive interference and not male choice. Additional research teasing apart the roles of male and female rejection and third party interference in male–female interactions that do not result in copulation are necessary to determine the relative roles of male and female choice and intrasexual competition on mating outcomes.

Conclusions

Both male and female butterflies change their behavior when in the presence of a sexual competitor. Both sexes copulate and court less, and engage in correlated bouts of activity, suggesting that they detect the presence of a competitor. While male general activity level decreases, females reduce their courting in the presence of a same-sex competitor. Further research assessing how mating behavior and intrasexual competitive interactions change over time, in triads and in populations with different sex ratios, are necessary to determine the role of male and female intrasexual competition in driving mating outcome in polygynandrous butterflies. The discrepancy between male courtship and male copulation in the female-biased treatment reminds us of the inherent pitfalls of using courtship as a proxy for mate choice in the absence of empirical data describing the relationship between the two behaviors.

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