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Assessing the use of wing ornamentation and visual display in female choice sexual selection

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ABSTRACT

Conspicuous sexual dimorphism is often ascribed to sexual selection. When the differences between the sexes are ornamental, this is thought to indicate a role for female choice. In spotted winged *Drosophila* species courtship, a male positioned in front of a female waves his wings, which have a patch of melanization on the exterior margin. In this study, we examine both female preference for wing spots and the role of vision in mating success in three species of the *suzukii* group: *Drosophila biarmipes, D. suzukii,* and *D. subpulchrella*. To assess female preference for wing spot, we removed the spot with a novel, non-invasive method, and competed spotless males with males with two spots. Phenotype did not affect mating success in any species. To eliminate the potential effect of competitive behavior on male mating success, we also ran a no-choice analysis. Mating frequency and timing was not different between phenotypes within these species. The effect of vision on mating success was assessed by comparing mating success of spotted males between light and dark conditions, both for frequency of mating, as well as timing of multiple courtship parameters. Species varied in the extent that lack of vision negatively affected mating success. Though vision is important for mating success, the spot itself may not be providing the signal that females use to make mating decisions.

1. Introduction

The presence of a conspicuous sexually dimorphic trait is often inferred to indicate the operation of sexual selection, either by intrasexual competition or mate choice preference (Lande, 1980). Evidence of sexual selection requires either demonstration that the trait confers the bearer a superior ability to compete for mates (intra-sexual selection) or a greater preference from mates (inter-sexual selection) when compared to individuals without the trait. Traits may be under both intra- and inter-sexual selection at the same time, although traits involved in competition generally have a defensive or offensive use in direct or indirect combat, whereas traits that are preferred may be ornamental and signal the quality of the individual that bears them (Zahavi, 1987). Sexual selection is not the only explanation for sexual dimorphism because different ecological constraints may also select for sex-specific trait expression (Shine, 1989).

Many males have conspicuous, sexually dimorphic morphology and behavior that they use to court females, and females assess these characteristics to choose mates (Andersson and Simmons, 2006). Proper exchange of morphological and/or behavioral signals between males and females during courtship leads to identification of the most “attractive” mates and contributes significantly to the fitness of those individuals. Though mate preference has been hypothesized to drive male sexually dimorphic display traits, assessing the effects of carrying or not carrying the traits is difficult because populations seldom are polymorphic for the presence and absence of such traits. Studies of female preference for male-limited traits may manipulate the degree of the trait expression (e.g. tail length in guppies, Bischoff et al., 1985; tail ornament size in barn swallows Kose and Møller, 1999) demonstrating the presence of runaway sexual selection for exaggerated male traits, but few studies fully remove discrete, male-limited morphological traits to test female preference. Studies examining the removal of discrete characters thought to be maintained through female preference are needed to understand how the presence of a trait influences female choice sexual selection.

Courtship communication studies using *Drosophila* are ideal for studies in female choice, particularly because simple, physiological manipulation can eliminate male signaling traits. In most species of *Drosophila*, courtship communication uses a suite of sensory modalities including olfaction and gustation for pheromones, vision for morphological and behavioral signals, and audition for courtship song (Spieth, 1974). The exchange of multiple signals across a range of modalities in both sexes during courtship makes *Drosophila* an exceptional model to study the evolution of courtship communication. Acoustic and chemical

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signals have been well documented in several *Drosophila* species (Gleason and Ritchie, 1998; Ritchie et al., 1999; Rybak et al., 2002; Veltsos et al., 2012); however, the use and importance of visual signals is understudied.

Although most Drosophilids have clear wings, some species have spots on the wings that vary among species in number, size, and degree of pigmentation. The wing spot in several subgroups of the *D. melanogaster* group is male-specific and inferred to be a sexually selected signal (Jezovit et al., 2017; Kopp and True, 2002; Prud'homme et al., 2006). Spotted winged *Drosophila* have two visual components to their visual courtship display: a frontal wing display and a wing spot. The frontal display in spotted winged *Drosophila* is a visual display in which males hold their wings out perpendicular to the body and move their wings and/or their body while positioned directly in front of females during courtship (Mazzoni et al., 2013; Revadi et al., 2015; Spieth, 1974; Yeh, 2009). The display is hypothesized to "show off" the wing spot, because the wing display is both phylogenetically correlated and genetically linked with the wing spot phenotype (Kopp and True, 2002; Prud'homme et al., 2006; Yeh and True, 2014). These flies, therefore, provide a unique opportunity to explore visual signaling.

The morphological and behavioral novelties seen in courtship within the spotted wing *Drosophila* groups imply that vision is necessary for successful courtship. The use of vision in *Drosophila* courtship is categorized into three classes (Grossfield, 1971). Class I species mate in darkness at the same frequency as in the light and are therefore light independent. Class II species are inhibited by darkness but may still achieve low mating success in the dark, whereas Class III species are not capable of mating in the dark. The class of species within a group may be conserved, whereas some species groups have high levels of variation (Jezovit et al., 2017). Species using conspicuous courtship displays, such as the spotted winged *Drosophila*, are hypothesized to be the most dependent on vision to mate (Ewing, 1983). Within the spotted winged *Drosophila*, *D. suzukii* is a Class III species (Grossfield, 1971), but no related species have been tested.

Although the genetics of spot production is well understood (Arnoult et al., 2013; Gompel et al., 2005; Koshikawa et al., 2015; Prud'homme et al., 2006; True et al., 1999; Werner et al., 2010; Yeh and True, 2014), the female behaviors associated with male spot presence are not thoroughly studied. Female preference for wing spots has limited empirical testing. When wingspots were amputed, *D. suzukii,* females had a weak preference for wing spots when females were kept in constant light (Fuyama, 1979). Normal 12:12 Light:Dark light cycle conditions resulted in amputated males being accepted at the same rate as intact males. A greater effect of the wing spot on mating was observed in *D. biarmipes*. Males with melanization on the wing were more successful; copulated faster and longer; and exhibited more vigorous courtship behavior than males without melanization (Hegde et al., 2005; Parkash et al., 2013; Singh and Chatterjee, 1987).

Variability in wing spot presence in *D. biarmipes* occurs in nature, with about 70% of males carrying spots; and 30% of males having no spots (Hegde et al., 2005). Consequently, females that mate in aggregations of spotted and spotless males mate with spotted males 70% of the time (Hegde et al., 2005). Such studies suggest that female preference has, at a minimum, maintained the spot phenotype in *D. biarmipes* (Hegde et al., 2005; Singh and Chatterjee, 1987).

In this study, we examine the use of visual signals by three species of flies that form a monophyletic group with a single origin of the wing spot, to ask if visual signals are necessary for mating, and more specifically, if the wing spot is a preferred character for female mate choice. We use two novel approaches: a non-invasive method for removing wing spots, and direct observation of mating in the dark. We hypothesize that females need to see males to accept them, and that females prefer males with spots. To explore these questions and test our hypotheses, we use choice, and no-choice mating assays to test for female preference for wing spot. We also implement experiments in which we pair the sexes in light and dark conditions to test the general importance of vision for mating. By exploring visual mating cues using multiple approaches, we find evidence that females are using visual cues to choose mates, but we find no evidence that wing spot is being used for mate choice.

2. Methods

2.1. Drosophila strains and cultures

Drosophila biarmipes (University of California San Diego *Drosophila* Stock Center: 1401.0361-11), *D. suzukii* (collected by Chris Hamm in Watson, CA), and *D. subpulchrella* (collected by the Chin Lab in Japan) were cultured and maintained on Bloomington standard formula (corn syrup, cornmeal, soy flour, yeast, and agar). Cultures were grouped in 50–100 individuals in 25 mm x 95 mm vials, and maintained at 23–24 °C with 12:12 light/dark cycle.

Virgin flies were collected under light CO_2 anesthetization within four hours of eclosion. Females were housed in groups of five and males were housed individually. All individuals were checked after 48 h for any defects on the wings including tears, and spot development in the males. Flies were aged 3–6 days before all assays, and all mating trials took place within four hours of lights on.

2.2. Male wing spot removal with CO_2

We observed that males in our stocks had fully developed spots, but males collected as virgins with CO_2 anesthesia varied in wing spot phenotype (two wing spots, one wing spot, or no wing spot). To determine that CO_2 anesthesia was the cause of spot loss, we quantified spot presence among males collected in three treatments: CO_2 anesthetization, cold anesthetization, and no anesthetization (control). Males produce wing spots within 24–48 hours post-eclosion, thus virgin collection at 4 h post eclosion results in only males lacking wingspots, which develop later. For both anesthetization treatments, flies were placed either on a block emitting CO_2 or an ice block for three minutes before being moved to individual, small, food-containing vials (16.5 mm x 95 mm). Control flies were aspirated from the collection vial to an individual vial. After 48 h, all males with two fully formed, undamaged wings were scored for the presence of spots (0 to 2) and then sorted by phenotype. In preference trials, CO_2 anesthetized males of each phenotype were used, as we saw no obvious pigmentation difference between spotted individuals that were anesthetized versus those who were not (Figure S1).

2.3. Mating arena

All behavioral assays were performed in the small vials (16.5 mm x 95 mm) with fresh food. After adding the flies by aspiration, the vial stopper (acrylic batting) was immediately pushed down into the vial to approximately 1 cm above the food for approximately 350 mm^3 of space for the flies. This was done so that the flies had a higher probability of interacting with one another.

2.4. Preference assays

To determine the effect of spot phenotype on female mate choice, choice and no-choice experiments were performed. In choice experiments one female was placed into a fresh food vial with two males (one male with two spots and one spotless male), observed for one hour, and scored from introduction for the time of courtship initiation, time of copulation, and the phenotype of the male that successfully copulated with the female. A trial was used in the analyses only if both males performed courtship. In no-choice experiments a female was placed with a male of one phenotype and the pair was scored for the time the second fly was introduced to the vial, initiation of courtship, time of courtship initiation, copulation success, time of copulation, and time when copulation was completed. Courtship latency was calculated by subtracting the introduction time from the time that courtship first occurred. Courtship duration was calculated by subtracting the time that courtship first occurred from the time that copulation started. Copulation duration was calculated by subtracting the time at which copulation started from the time at which the pair separated. If the pair did not copulate, they were not included in courtship duration or copulation duration analyses.

2.5. *Light dark assays*

To determine the effect of vision on mating success, pairs of virgin, spotted males and virgin females were placed in one of two treatments: light or dark. For 60 min after introduction, pairs were observed in either light (the control) or in the dark, under red light (wavelength, 650 nm) because *Drosophila* cannot see in red light (Hanai et al., 2008), and measured for copulation success, courtship latency, copulation latency and copulation duration. Flies observed in the light were then kept for ten days under a standard 12:12 light:dark cycle. Flies in the dark condition were kept in continuous darkness for ten days. After the ten days, vials in which both the male and the female were alive were scored for the presence of larvae. To determine if darkness is detrimental to egg laying, half of the females who were observed to mate in the light were placed in continuous darkness. The other half were kept in light conditions.

2.6. *Data analysis*

All data analysis was performed in R Studio (version 1.0.136). Comparison of two treatments for proportion/frequency data was tested for significance using a Fishers Exact Test. Comparison of two groups for proportion of individuals mating in choice tests were compared with the expectation of 50% mating with a Chi-squared test. Comparison of two treatments for timing data was tested using a Student's *t*-test Comparison of three treatments was compared using a One-Way ANOVA.

3. Results

3.1. *Male wing spot removal*

Wing spots in these species develop within 24–48 hours of eclosion (personal observation). For all three species, exposure of newly eclosed males to CO_2 for 5 min resulted in ~30% lacking the spot 48 h later and a smaller percentage having only one spot (Fig. 1). After aspiration or anesthesia on ice, all males had spots (data not shown). No significant difference in the proportion of spotless males existed between species (Supplementary Figure S1, One-Way ANOVA, $P = 0.69$). Wing spots of the males treated with CO_2 were present (Supplementary Figure S2). Male behavior was not qualitatively altered by the removal of spot because they still performed frontal wing displays for females during courtship assays and courtship initiation did not differ between spotted and spotless individuals (see below).

3.2. *Female preference and wing spots*

In choice assays, two males, one spotted and one spotless, were placed simultaneously with a female and observed until one male successfully copulated. In all species, females did not mate preferentially with males of either phenotype (Fig. 2). *Drosophila biarmipes* males were the most aggressive of the three species; spotted and spotless males alternated between courting the female and competing with the other male (displaying towards the male and pushing him with the fortarsi). Some male-male interactions were observed with *Drosophila suzukii* males. *Drosophila subpulchrella* was far less active than the other two species; courtship was minimal with very little interaction between males. In all trials in which males courted, males of both phenotypes courted females at qualitatively similar frequencies. We saw no significant difference in which male courted first (Supplementary Figure S3), and no difference in how long it took for males with two spots and males with no spots to initiate courtship (Supplementary Figure S4). The order of courtship initiation was not a predictor of mating success (Supplementary Figure S5).

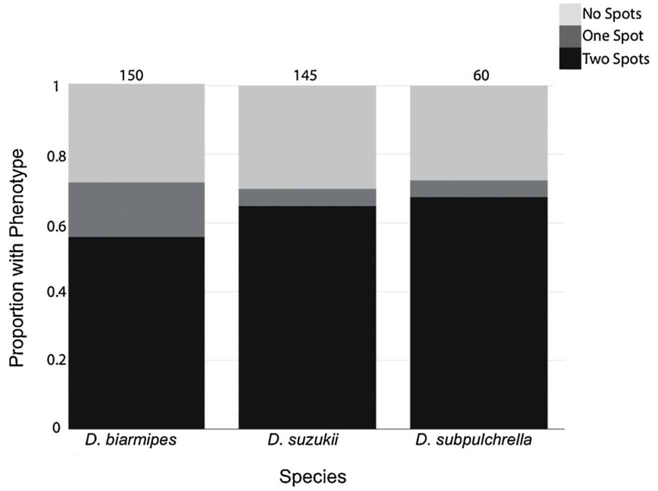

Fig. 1. Effect of CO_2 on spot development. Newly eclosed flies were anesthetized with carbon dioxide for three minutes, before placing males in vials to recover. Wing phenotype was scored after 48 h. In all species, approximately 30% of individuals did not produce wing spots. A small proportion developed only one spot. The number on top of each column is the sample size.

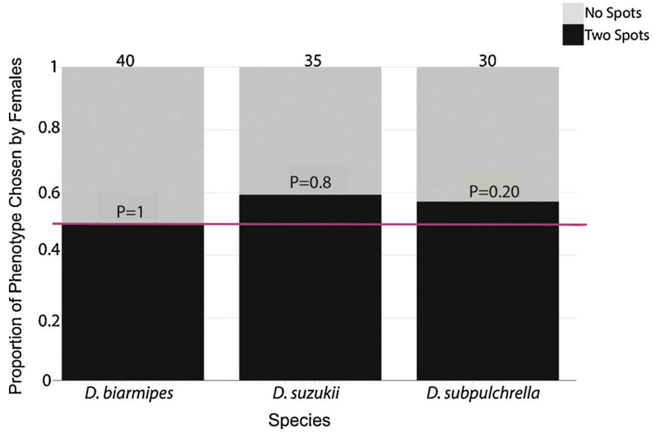

Fig. 2. Effect of spot phenotype on female choice. Mating trios (a female with a male with two spots and a male with no spots) were observed for up to 60 min, and the male that successfully mated was recorded. Phenotype preference was compared using a Chi-squared test with the null hypothesis of no choice (all $P > 0.05$). Numbers on top of each column are sample sizes. The horizonal line shows the no-preference expectation of 0.5.

In no-choice tests, a single male of one phenotype was placed with one female. Males with and without spots copulated with females at the same frequency for all species (Fig. 3). No other mating parameters were different between males of the phenotypes in no-choice experiments (Supplementary Figures S6-S7).

3.3. *Effect of light on mating success*

Pairs of single males with single females were observed both in the light and in darkness under red light. In all three species, the proportion of males who courted in the dark was the same as males in the light (Fig. 4a). Of the males that courted, *D. biarmipes* males in the dark were

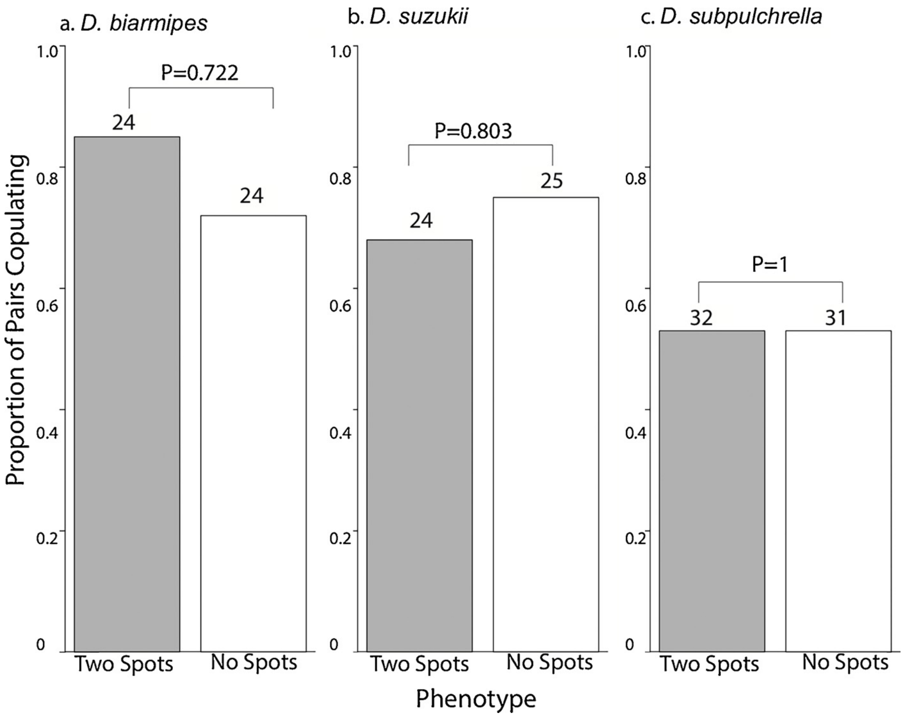

Fig. 3. Effect of phenotype on courtship success in no-choice assays. Mating pairs were observed for 60 min, and the proportion of males who successfully mated were compared between males with two spots and males with no spots using a two-tailed Fisher's Exact Test (*P* values given for each pair above brackets). Numbers on top of each column are sample sizes.

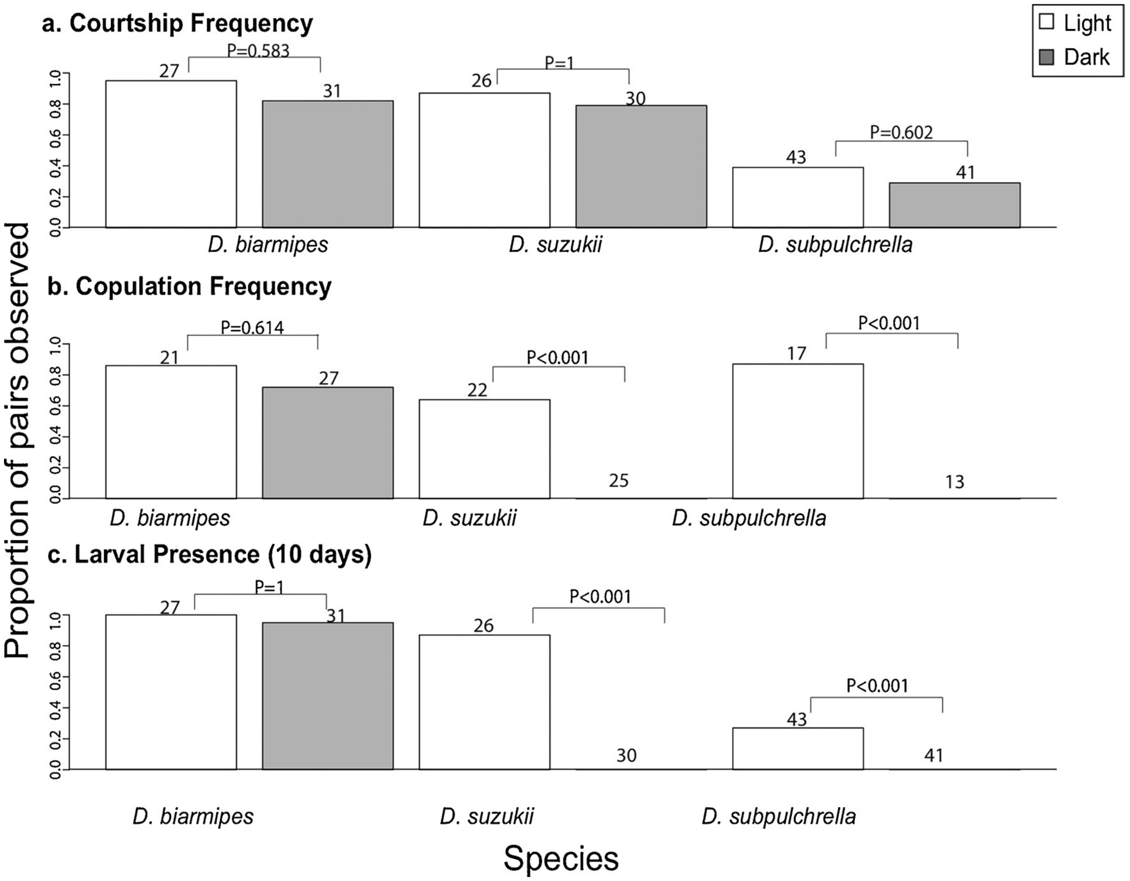

Fig. 4. Effect of light on mating. Virgin males and females were assigned to two treatments: 12:12 light: dark (normal photoperiod) or continuous darkness and observed for 60 min. All proportions were compared within a species between treatments with a two-tailed Fisher's Exact test (*P* values are given above brackets). Numbers on top of each column are sample sizes. a) The proportion of males that courted females did not differ between treatments for any of the species. b) While a proportion of all species mated in the light, only *D. biarmipes* males achieved copulation in the dark. c) Pairs were left for 10 days and scored for the presence of offspring. Both *D. suzukii* and *D. subpulchrella*, unlike *D. biarmipes,* never mated in the dark.

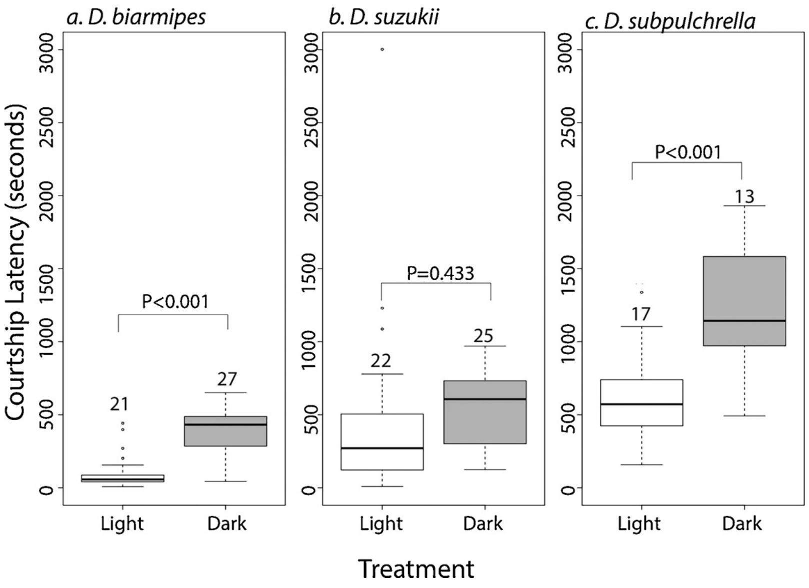

Fig. 5. Effect of light on courtship latencies. Mating pairs were observed for 60 min in either light or dark. The time from introduction to the initiation of courtship was recorded for each pair of each species. Upper and lower quartiles of the data are represented by the upper and lower boundaries of the box. Mean values are represented by the bars inside of each box, and error represented by the whiskers. Outliers are represented by dots. Significance levels are indicated by the brackets connecting the bars in comparison (*P* values from Student's t-test). Numbers on top of each column are sample sizes.

as successful at achieving copulation within 60 min as males in the light (Fig. 4b). In the dark, none of the *Drosophila suzukii* and *D. subpulchrella* males that courted mated within 60 min whereas in the light, 64% and 87%, respectively, of the males who courted achieved copulation success (Fig. 4b). In *D. biarmipes,* pairs incubated for ten days produced progeny in equal proportions in the dark and in the light (Fig. 4c). In *D. suzukii* and *D. subpulchrella*, no larvae were observed in dark treatment vials for pairs left for ten days (Fig. 4c). Larval production in the dark treatment was not reduced due to the inability to lay eggs, because larvae were observed in 100% of vials in which mated females were transferred to darkness in all three species (data not shown), thus *D. suzukii* and *D. subpulchrella* never mated in the dark, even when left for longer than the 60-minute observation period.

Of the three species observed, only *D. biarmipes* males copulated in darkness (Fig. 4b) but compared to males in the light, they took significantly longer to initiate courtship (Figure 5a). This species in general was very active in the dark, with females running around the vials and males searching for females until contact was made and courtship was initiated. *Drosophila suzukii* males did not take longer to court females in darkness as compared to males in the light (Fig. 5b), but were never accepted. Female *D. suzukii* were qualitatively less active in darkness, which allowed allow males to correctly orient themselves for courtship. Almost all *D. suzukii* courtship in the dark was directed toward the female because males used their foretarsi to locate the female's front end, and then performed their wing displays. Females were unresponsive to male displays, and rejected any copulation attempts by kicking away from the male. *Drosophila subpulchrella* took significantly longer to initiate courtship in darkness than in light (Fig. 5c). Individuals of this species were generally inactive when placed in mating vials, but even more when placed in darkness. Females rejected copulation attempts by walking away from males. Very few copulation attempts were made by *D. subpulchrella* males in either treatment (data not shown); all attempts were repelled by the females in the dark.

Because *D. biarmipes* mated in the dark, we measured additional courtship parameters. Once courtship was initiated, males had a significantly longer courtship duration in the dark compared to in the light (Fig. 6a). In the dark, males performed vigorous courtship, though not always in the correct orientation to the female. When males found themselves in close enough proximity to mount successfully after attempted copulation, they completed copulation. After copulation was initiated, copulation duration in both treatments was the same (Fig. 6b).

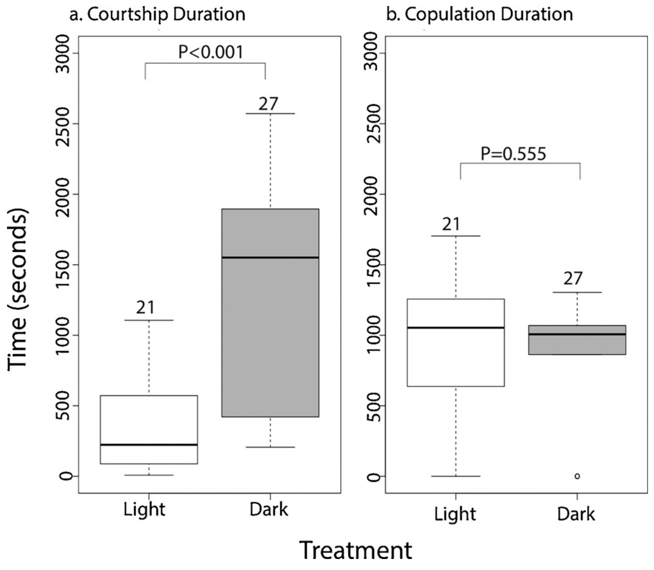

Fig. 6. Effect of light on copulation parameters in *D. biarmipes*. Mating pairs were observed for 60 min in either light or dark. The a) courtship duration was significantly longer for males in the dark but b) copulation duration did not differ between treatments. Upper and lower quartiles of the data are represented by the upper and lower boundaries of the box. Mean values are represented by the bars inside of each box, and error represented by the whiskers. Outliers are represented by dots. Significance levels are indicated by the brackets connecting the bars in comparison (*P* values from Student's t-test). Numbers on top of each column are sample sizes.

4. Discussion

4.1. Carbon dioxide treatment removes spots non-invasively

Exposure to CO_2 shortly after eclosion in these fly species prevents spot development in approximately 30% of males. Previous research on wing spots and mating success used either surgical removal of the spot via cutting (Fuyama, 1979) or natural populations of spotless flies (Hegde et al., 2005; Parkash et al., 2013; Singh and Chatterjee, 1987). Our procedure is advantageous for the study of the effect of spots on behavior because it is not invasive and does not depend upon natural variation. The genetics of spot development is well known (Arnoult et al., 2013; Gompel et al., 2005; Koshikawa et al., 2015; Prud'homme et al., 2006; True et al., 1999; Werner et al., 2010; Yeh and True, 2014; Yeh et al., 2006), so it may be possible to manipulate the spot genetically, but such an approach is considerably more difficult than ablation by carbon dioxide. The removal of the spot with CO_2 does not allow random assignment of males to treatments, thus if susceptibility to spot removal is a reflection of low fitness, males without spots should perform less well in choice and no-choice mating assays, but that is not what we found. In our assays, we observed a lack of choice by females for the two types of males, implying that spot removal does not hinder male behavior, a result found using the same light maintenance conditions for *D. suzukii* (Fuyama, 1979).

Anesthetization using either CO_2 or cold to allow for the manipulation of flies is standard procedure in *Drosophila* research. *Drosophila* generally show the same mild stress responses due to anesthetization via cold and CO_2 treatment (Barron, 2000). By our results, spot loss is not a general stress response because cold had no effect on spot production. Flies are most likely recovered from any behavioral or metabolic effects from CO_2 exposure before assays. Exposure to carbon dioxide for ten minutes affects behavior, but the effects wear off after 24 h (Colinet and Renault, 2012). The behavior of the flies in our assays was, therefore, not likely to have been affected by the CO_2 exposure because our exposure was three minutes and took place 3–5 days before the assay. Finding similar frequencies of spot loss in all three species implies a common disruption of a physiological process by CO_2. How the disruption occurs has yet to be identified.

Natural populations may lack spots innately, but documentation of the frequency of spot occurrence is poor. An estimated 30% of *Drosophila biarmipes* males in natural populations lack spots (Hegde et al., 2005), but the heritability of the spotless phenotype has not been tested and no USA stock center stocks contain naturally spotless individuals (Maxi Richmond, Drosophila Species Stock Center, personal communication). Reports of wild, invasive spotless *D. suzukii* individuals (EPPO, 2013) are anecdotal and do not account for immature males that have yet to develop the spot. Previous laboratory studies were not clear in the description of the use of natural variation in spot presence. The flies may have been collected directly from the wild and maintained in polymorphic stocks, though it is also possible that they are the result if CO_2 anesthesia (a common laboratory practice), but the methods of anesthesia were not reported (Hegde et al., 2005; Parkash et al., 2013; Singh and Chatterjee, 1987).

4.2. Females do not prefer males with wing spots

We did not find evidence for female preference for wing spots in these species of the *D. suzukii* group. This is surprising, given that conspicuous, sexually dimorphic characters typically imply sexual selection (Lande, 1980). Male characters of this nature are frequently courtship signals selected through female choice (e.g. Hill and McGraw, 2004; Robert et al., 1985; Wilkinson and Reillo, 1994) but may also result from intrasexual selection (Anderson and Vitt, 1990; Grether, 1996). In our assays we tested intersexual selection. Lack of preference did not seem to be a result in differential courtship behavior between spotted and non-spotted males. Though courtship vigor differences between the phenotypes was not quantified, both phenotypes mated at the same frequency in choice and no-choice assays. Spotted males possibly performed less courtship than the spotless males and still were able to mate, but this is unlikely because males of both types initiated courtship with the same frequency and the same latency. Thus, differences in sexual drive are not playing a role in mating success.

Though female choice sexual selection is not supported by our data, wing spots could be maintained through intrasexual selection. In competition assays, we observe male aggression, particularly in *Drosophila biarmipes* males, which were highly competitive in choice assays, displaying and physically pushing each other with their foretarsi. The spot could potentially be used by males to determine size; large males have spots further apart than small males. Differences in fighting ability should be tested, however, if one phenotype was more successful at fighting than the other, we should have seen a difference in mating success in the choice assays. Another alternative for the origin of sexual dimorphism is ecological displacement wherein different selective pressures on males and females favor different morphologies (Shine 1989), but how that might operate with these flies is not clear.

Prior experiments with *D. biarmipes* using naturally spotless flies (Hegde et al., 2005; Parkash et al., 2013; Singh and Chatterjee, 1987) used a different experimental design by measuring mate choice in large groups, with 10–15 males of both types and half that number of females. Though this scenario is thought to resemble natural conditions, multiple choice tests are difficult to standardize and may bias results towards “higher choosiness” (Murray et al., 2010). Our consistent results across all three species implies a lack of choice for wing spot. Hegde et al. (2005) found that males without wing spots took significantly longer to court and mate whereas we see no significant differences in the timing of mating in our no-choice studies in all three species providing more evidence that the wing spot is not as important for courtship decisions as previously thought.

We used both choice and no-choice tests to evaluate the potential role of wing spot in female choice sexual selection because each provides different information. Choice tests are confounded by male intrasexual competition and may not reflect only female choice. In no-choice tests females may opt for the available male in the absence of other input. Including both assays allows us to fully understand the dynamics of courtship and mating decisions in species that have dynamic courtship interactions.

4.3. Vision is important for mating success in all three species

We observed significant differences in mating success in all three species when placed in the darkness. Mating in both *D. suzukii* and *D. subpulchrella* was completely eliminated, meaning that vision is necessary for mating success. The courtship of *D. suzukii* begins with orientation to the female, followed by wing scissoring and fluttering (Revadi et al., 2015). The species is mute (Ewing, 1983), indicating that wing movements are visual and not acoustic. Without auditory signaling, communication via visual signals may be crucial to mating success. The mating behavior of *D. subpulchrella* has not been examined outside of this study, so its use of other sensory modalities during courtship is worth exploring.

Though mating was not eliminated, *D. biarmipes* males in the dark take significantly longer to mate than males in the light. Delayed mating can greatly affect fitness if other males are present; therefore, visual signals facilitate mating in this species. We know that multiple sensory modalities are used simultaneously during *Drosophila* courtship, but in some species, single signal modalities may take the role as the most important, or primary signal (Gleason et al., 2012). If the ancestral state for this group is mating that requires vision (Type III in Grossfield (1971), then *D. biarmipes,* relative to its sister species, may be losing the reliance on visual signaling and the effect of other signaling modalities on mating success should be tested. Unlike *D. suzukii*, the *D. biarmipes* male wing display has a song with multiple components (Mazzoni et al., 2013) implying a broader array of courtship signals for female assessment of male quality. More comparative work is needed among spotted *Drosophila* to understand the gain and loss of this signal.

Previous studies that placed flies in the dark measured offspring production as a proxy for the importance of vision (Grossfield, 1971; Spieth and Hsu, 1950). These studies did not identify if lack of male courtship or lack of female acceptance is the reason for not mating, which can only be determined by observing behavior. Watching courtship behavior under red light, as in this study, is crucial to identifying when courtship signaling breaks down. We find that males are able to orient towards females using tactile or other cues, but then are unable to follow females if the females move. Male displays in the dark are not qualitatively different from courtship in the light. Thus vision is not necessary for males to initiate courtship, but may be necessary for them to accurately continue courtship. Copulation is attempted by all three species but rejected by females (completely in *D. suzukii* and *D. subpulchrella*, and for an extended period of time in *D. biarmipes*). Vision is essential for female acceptance in *D. suzukii* and *D. subpulchrella*, and is important for more rapid acceptance in *D. biarmipes*.

The role of the wing display itself in courtship is difficult to separate from the wing spot, because they always occur together. Species with wing spots perform frontal wing displays during courtship, whereas related spotless species in the same species group do not (Kopp and True, 2002, personal observation). Through the dark experiments, we can determine the necessity of visual cues. The fact that the elimination of spot does not affect female mating preferences, but the loss of visual cues does, suggests that the wing movement may provide the cues necessary for mating decisions. The environmental context of the spot may change its signal efficacy, particularly if wing movement is hard to see (e.g. low light environments). Changing the environment in experiments may illuminate the potential contextual role of wing spots in the enhancement of wing displays.

5. Conclusions

We performed our tests by using two novel approaches: a non-invasive carbon dioxide treatment to remove spots, and direct observation of flies in darkness by using red light. The carbon dioxide treatment is minimally invasive, reducing behavioral side effects that occur after crude surgical procedures. The red light observations allowed us to better understand the components of courtship (from both males and females) leading to mating success or failure. We were able to directly observe, and therefore measure, where the breakdown of signal sending and receiving occurs. We suggest that future studies of visual signals in *Drosophila* species include a direct observation in darkness using either infrared or red light to understand behavioral changes that occur when species are blind.

In the *D. suzukii* group, vision is important to mating success, but the wing spot itself does not appear to affect female choice. The role of wing spots for mating may be changed in other environmental contexts, but this remains to be explored. The spot itself may not be the preferred trait if wing movement is the signal and the spot serves to enhance wing detection in more complex environments.

Our experiments were designed to have as little impact on the *Drosophila*. We chose to use standard, non-invasive procedures. Established laboratory stocks were used, and no more flies were used than necessary. There are no regulations or guidelines for *Drosophila*, however, organisms were treated with respect.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.beproc.2018.10.010.

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