Dancing in the Dark: The evolution of visually mediated courtship behaviors and sexual dimorphisms in spotted winged *Drosophila*

By

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Dancing in the Dark: The evolution of visually mediated courtship behaviors and sexual dimorphisms in spotted winged *Drosophila*

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Abstract

I employed a multidisciplinary approach to examine the function and evolution of sexual dimorphisms, male wing spots and male wing displays, across four species subgroups of *Drosophila*. In chapter one, I explored the function of wing spots in three species of the *D. suzukii* group using female choice tests by placing mating pairs in darkness. I found that females do not show a statistical preference for spotted males in choice experiments, but that vision is either required for or facilitates mating success. The wing spot may not be as important for mating decisions as previously hypothesized but could enhance courtship displays in more complex environments. In chapter two, I used whole genome sequence data to build a phylogeny of the spotted winged *Drosophila* in the *D. melanogaster* group, identified the likelihood of ancestral states of the wing spot character, and tested for conservation of the morphology. The rate of evolution is too rapid to determine if wing spots are ancestral and subsequently gained or lost throughout the subgroups, but multiple convergent events were identified. The wing spot did not have a phylogenetic signal and was correlated with frontal courtship display behaviors, suggesting that wing spots are likely a rapidly fluctuating sexually selected character. In chapter three, I measured visually mediated behaviors of 13 species of spotted and non-spotted *Drosophila* and mapped behavior to the phylogeny to identify associations between behavior and wing spot morphology that could explain the observed evolutionary patterns. Courtship behavior is labile, and visually mediated long-term copulation acceptance is associated with wing spot morphology. The results in total suggest that species delimitation could be driven by female choice sexual selection on male dimorphisms. In chapter four, I detailed potential functions of the wing spot. This study lays the groundwork for further study on the function of various male displays observed in species where males have sexual dimorphisms.

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Table of Contents

List of Tables

List of Figures

List of Appendices

Introduction

A signal is any act or structure that alters the behavior of another organism (the receiver) and has evolved in response to feedback on the signal from the receiver, which is simultaneously evolving (Stevens 2013). Signals are a product of sensory system evolution and are very diverse across (and sometimes within) species due to their universal importance for fitness (Stevens 2013). Courtship signaling operates to communicate information about mate identity and status (Smith and Harper 2003), thus plays a role in sexual isolation and can contribute to species delimitation (Coyne and Orr 2004).

We observe many instances of behavior accompanying dimorphic morphology in courtship (reviewed in West-Eberhard 2003), however, few studies examine correlated traits from both functional and evolutionary perspectives to understand how behavior and morphology may influence one another (e.g. Ord and Martins 2006). How and why particular behavioral traits and their accompanying morphologies appear and diversify to build complex phenotypes among lineages has long been questioned (see Hinde and Tinbergen 1958; Tinbergen 1959; Lorenz 1986) and is still central to many research questions in evolutionary ethology.

I took interest in several species subgroups of the *Drosophila melanogaster* group, known as the spotted winged *Drosophila* (Figure I.1), in which males of some species have conspicuous sexual dimorphisms with behavioral and morphological components (Figure I.1). These males have melanization on the wing known as a "wing spots", which is always paired with a frontal courtship display (Kopp and True 2002b), in which males orient themselves in front of the female and wave their wings in a species-specific manner. Within the spotted winged *Drosophila* subgroups there are also species with monomorphic wing morphology (both sexes have clear wings, see Figure I.1). The wing display has been presumed to function as a way to

show females their spots, and that females choose mates based on wing spots (Kopp and True 2002b). The function of wing spot has not been studied extensively, and the studies of the role of

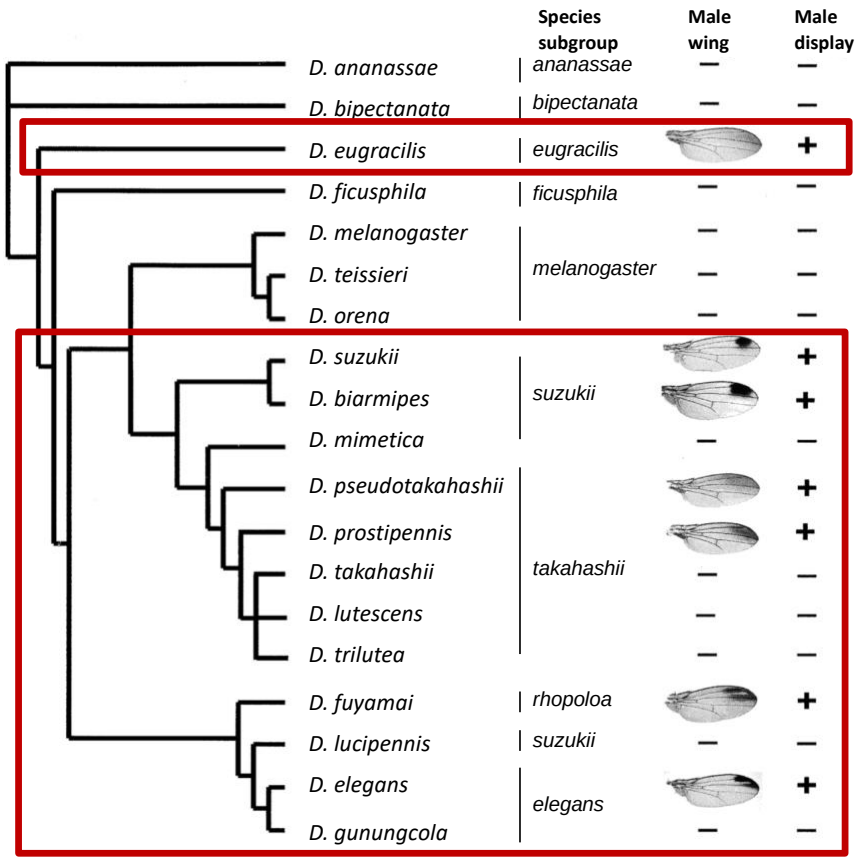


Figure I.1 The spotted winged *Drosophila* groups, showing variation in male wing spot pattern and wing display (adapted from Kopp and True, 2002b). A "-" denotes no wing spot or wing display observed. A "+" denotes wing display. The "spotted winged *Drosophila*" referenced in this study include the species subgroups enclosed in the red boxes. Within the spotted winged *Drosophila* subgroups, there are dimorphic and monomorphic species.

wing spots in female choice sexual selection have contradictory or unclear results. A study of *D. suzukii* found a weak preference for wing spots, but only when females were placed in constant light from the time of eclosion (emergence from pupa) until mating (males of both phenotypes had similar mating success when mated to females kept in standard 12:12 light-dark conditions, Fuyama 1979). Several studies on *D. biarmipes* found that spotted males are preferred by females significantly more than spotless males (Singh and Chatterjee 1987; Hegde et al. 2005;

Parkash et al. 2013). No other spotted species have been studied for spot preferences. If wing spots are sexually selected, we would expect that females would prefer to mate with spotted males and need to see the spots to accept mating.

Regardless of preference for wing spots, the frontal courtship display component observed in spotted species alludes to the significance of visual signals for mating success. Species without melanization lack a wing display (Kopp and True 2002b), suggesting that wing spot and wing display traits are correlated and therefore selected together because they provide visual signals communicating necessary information for mating success. We would expect that mating success is affected by removal of vision in dimorphic species, but not in monomorphic ones, suggestive of sexual selection operating on male phenotype differentiation. If there is no association between mating success and phenotype then dimorphic phenotypes may either result from other selective forces (natural selection) or stochastic forces (drift).

We can use phylogenetic analyses (ancestral character state reconstruction, phylogenetic signal, and correlated trait evolution) to test hypotheses on the potential causes of wing spot, wing display, and courtship behavior evolution. The spotted winged *Drosophila* species are an ideal system to use for comparative analyses of mating behaviors in relation to visual reliance because we observe an appreciable level of differentiation of wing spot presence among the related species available to study comparatively (eight species with spots and five without spots). Transitions between dimorphic and monomorphic species are seen multiple times between closely related species within subgroups (Figure I.1), suggesting that there have been recent and potentially rapid fluctuations to or from wing spots. After mapping male morphologies to the phylogeny, we can map courtship behavior traits and test for associations between visually mediated courtship outcomes and sexual dimorphisms. From such analyses we can infer whether

specific visual signals expressed as conspicuous sexual dimorphisms could operate as premating isolators in the spotted winged *Drosophila* using comparative behavior methods.

My dissertation implemented behavioral and phylogenetic methods to explore the function and evolution of dimorphic male characters. The overarching goal of my work was to explore the function of one element of a complex phenotype (the wing spot) that has been under-explored, and then compare the use of one sensory modality between dimorphic and monomorphic species to paint a broad picture of what forces may have caused variation in dimorphic phenotypes across the spotted winged *Drosophila* groups. Specifically, I tested the following hypotheses:

1. Wing spots are maintained by direct female preferences.
2. Wing spots and wing displays are correlated male traits.
3. The ancestral state to the spotted winged subgroups is spotted, and there have been multiple independent losses of wing spots.
4. Species that have wing spots are dependent on vision for mating success.
5. Visual reliance on mating success is correlated with sexual dimorphisms independent from relatedness.

In chapter one I asked if wing spots specifically function as a signal for female choice. I addressed the question by testing for female choice in a subset of three spotted species from the *D. suzukii* group by competing spotted and spotless males for female choice, as well as presenting each phenotype to females separately to identify differences between spotted and spotless males in stimulating females during courtship. I also tested for reliance on vision for mating success by placing mating pairs in the light and the dark and measuring their mating success. I found that though visual signals are important for mating success, females do not show

a preference for wing spots and males without wing spots mate as quickly. The results suggest that spots may not, *per se*, function as a direct signal for female choice. The spots could still be sexually selected because they increase visibility of other displays in alternative environments or have a role in intrasexual selection.

In chapter two I addressed questions regarding the evolution of male dimorphic traits. No existing phylogeny was suitable for my analysis, so I sequenced the genomes of eight species and used the available whole genome sequences of 13 additional species to construct a new tree. From whole genome sequences, I was able to produce a well-supported species tree. I reconstructed the ancestry of wing spot morphology and measured the phylogenetic signal of the wing spot to infer potential modes of evolution. My analysis of the morphology shows that the evolution of wing spots is too rapid to infer the ancestral states, so we cannot determine if traits have been gained or lost. My results are consistent with the hypothesis that fluctuating modes of sexual selection are likely shaping the pattern of male sexual dimorphisms observed along the tree because wing spots are evolving rapidly, lack phylogenetic signal (are not conserved) and are likely coevolving with wing display behaviors.

In chapter three I addressed questions regarding the association of male dimorphisms with visual reliance on mating success. I assessed the reliance of 13 species of spotted winged *Drosophila* on vision for mating success by placing pairs in the light and the dark and measuring both courtship and copulation outcomes. To test for trait correlations independent of evolutionary history, I used phylogenetic independent contrasts (PIC) methods to measure the association between courtship behavior response and the presence of sexual dimorphism. I found that courtship behaviors are not conserved, and that long-term mating success is strongly associated with sexually dimorphic phenotypes (spotted species mating is significantly

suppressed in darkness, while spotless species do not experience significant suppression). The results provide support for rapidly fluctuating sexual selection playing a role in differentiation in the spotted winged *Drosophila*.

Given the importance of visual signals in dimorphic species, but the lack of evidence for female preference for wing spots, I was left with the question of why dimorphic males have wing spots. In chapter four I discussed potential functions of the spot. I outlined the potential reasons for the maintenance of spot that are alternative to direct female choice: shared genetic architecture with wing displays, signal efficacy through increased visibility, natural selection on more thermally tolerant males, and intrasexual selection for access to females. The discussion of potential spot function highlights prospective future directions of study in the spotted winged *Drosophila* system.

My research used an integrative approach to place signal-receiver relationships in a phylogenetic context to examine the mode of evolution of novel sexual dimorphisms involved in courtship signaling, and to understand the function of the wing spots in female mate choice. My results provide support that conspicuous dimorphisms can predict the role of sexual selection as a driver of isolation and speciation, however, not all signals previously hypothesized to function for direct female choice may do so in a strong manner. Many exciting questions remain to be answered about spotted winged *Drosophila* that make it a promising system for the study of courtship behavior and correlated courtship trait evolution.

Chapter 1: Assessing the use of wing ornamentation and visual display in female choice sexual selection*

*Adapted from: Roy, P.R. and J.M. Gleason (2019). Assessing the use of wing ornamentation and visual display in female choice sexual selection. *Behavioural Processes(158), 89-96*.

Abstract

Conspicuous sexual dimorphism is often ascribed to sexual selection. When differences between the sexes are ornamental, the characteristics are 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 patches 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* subgroup: *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 spots on each wing. 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 affects mating success, the spot itself may not be providing the primary signal that females use to make mating decisions.

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). Sexual selection is not the only explanation for sexual dimorphism because different ecological constraints for each sex may also select for sex-specific trait expression (Shine 1989). Evidence of sexual selection requires greater mating success for the bearer of a trait compared to an individual without the trait either through a superior ability to directly compete for mates (intra-sexual selection) or a higher mating success through choice from mates (inter-sexual selection). Traits may be under both intra- and inter-sexual selection, 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).

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 (e.g. Siva-Jothy 1999; Ng and Kopp 2008). 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 of 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 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 (Kopp and True 2002b; Prud'homme et al. 2006; Jezovit et al. 2017). 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 (Spieth 1974; Yeh 2009; Mazzoni et al. 2013; Revadi et al. 2015). 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 2002b; Prud'homme et al. 2006; Yeh 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 may be 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. 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. Classification of addition species will increase our understanding of how much visual signaling matters in mating success in species with sexual dimorphisms versus those without them.

Although the genetics of spot production is well-understood (True et al. 1999; Gompel et al. 2005; Prud'homme et al. 2006; Werner et al. 2010; Arnoult et al. 2013; Yeh and True 2014; Koshikawa et al. 2015), female response behaviors associated with male spot presence are not thoroughly studied. Female preference for wing spots has limited empirical testing. When wing spots were amputated by clipping out the melanized areas, *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 (Singh and Chatterjee 1987; Hegde et al. 2005; Parkash et al. 2013). Variability in wing spot presence in *D. biarmipes* has been reported 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 choose spotted males 70% of the time, significantly more than spotted males (Hegde et al. 2005). Such studies suggest that female preference has, at a minimum, maintained the spot phenotype in *D. biarmipes* (Singh and Chatterjee 1987; Hegde et al. 2005).

In this study, we examine the use of visual signals by three species of *Drosophila* that form a monophyletic group with a single origin of the wing spot (Kopp and True 2002b; Prud'homme et al. 2006), 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 both 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.

Methods

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 established by 50-100 individuals in 25 mm x 95 mm vials and maintained at 23-24°C with a 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 in 16.5 x 95mm vials. All individuals were checked after 48 hours 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.

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 hours post eclosion results in only males lacking wing spots, 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 95mm). Control flies were aspirated from the collection vial to an individual vial. After 48 hours, 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 S1.1).

Mating Arena

All behavioral assays were performed in small vials (16.5 mm x 95mm) 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.

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.

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 minutes after introduction, pairs were observed in either light (the control) or in the dark, under red light (wavelength, 650nm) because *Drosophila* cannot see in red light (Hanai et al. 2008), and measured for copulation success, courtship latency, courtship duration 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.

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. We placed 95% confidence intervals on the estimated proportion of females that favor spotted males in choice experiments to infer how far away from the null hypothesis our estimate could be in truth with our sample size.

Results

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 minutes resulted in ~30% lacking the spot 48 hours later and a smaller percentage having only one spot (Figure 1.1). 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 S1.1, One-Way ANOVA, $P = 0.69$). Wing spots of the males treated with CO_2 were present (Supplementary Figure S1.2). 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).

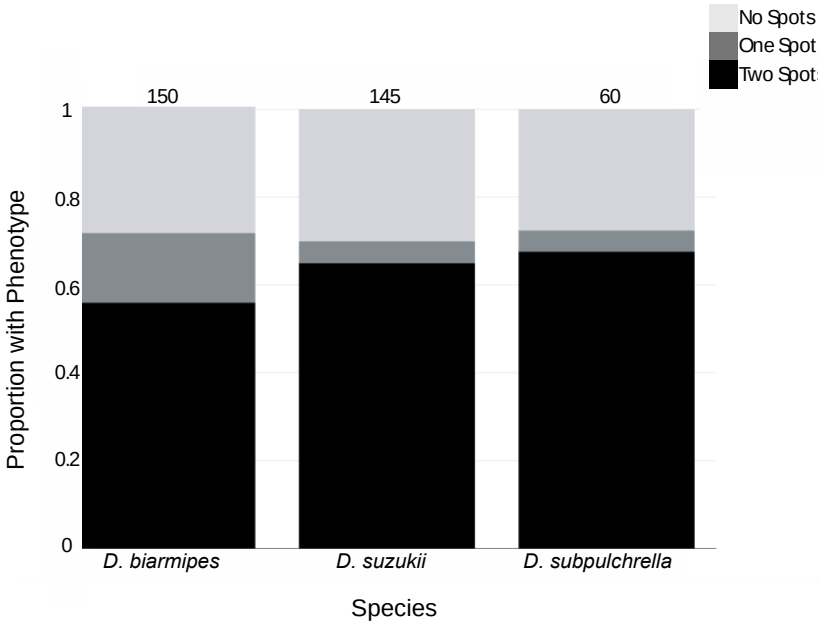


Figure 1.1 Effect of CO_2 on spot development. Newly eclosed flies were anesthetized with carbon dioxide for three minutes, before placing males in vials to recover. Wing phenotype was scored after 48 hours. In all species, approximately 30% of individuals did not produce wing spots. A small proportion developed only one spot. The number on top of each column is the sample size.

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 or 60 minutes had elapsed. For all species, females did not mate preferentially with males of either phenotype (Figure 1.2). The range of proportion values for spot preference was measured using 95% confidence intervals (Figure 1.2).

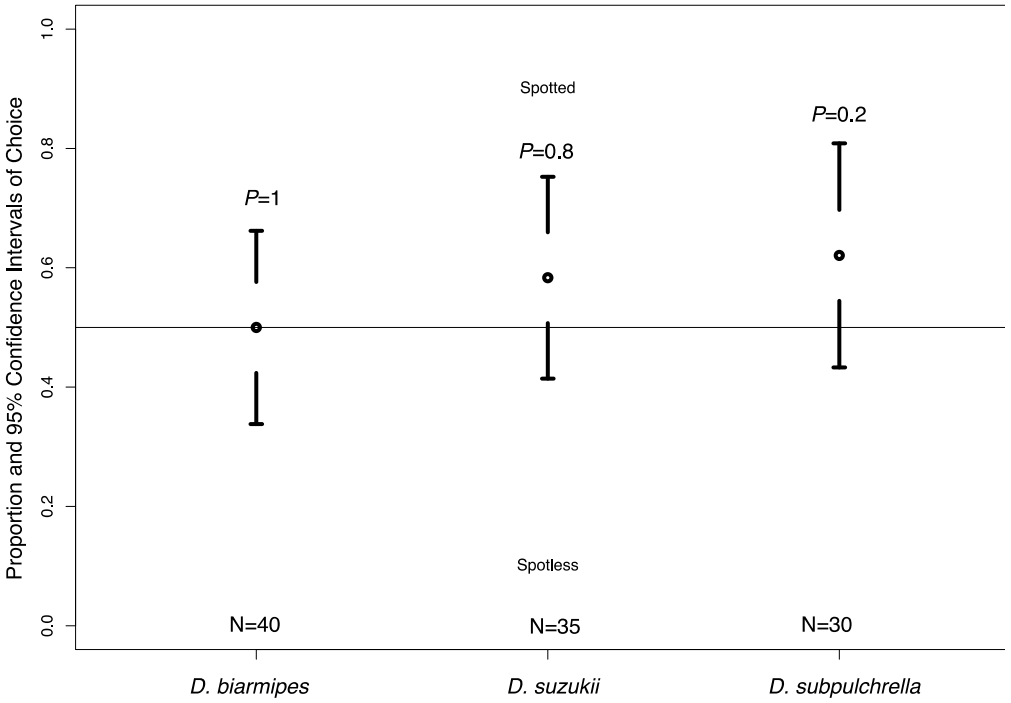


Figure 1.2. Effect of spot phenotype on female choice. Mating trios (a female with a male with two spots and a male with no spots) were observed for up to 60 minutes or until mating occurred, and the male that successfully mated was recorded. If no mating occurred, the trials were not included in the analysis. Phenotype preference was compared using a Chi-squared test with the null hypothesis of no choice (all $P > 0.05$). The dot represents the proportion of spotted males chosen by females and the whiskers represent the 95% confidence intervals for the proportions. Numbers on the bottom of each dot and whisker are sample sizes. The horizonal line shows the no-preference expectation of 0.5.

There were notable male interactions during the choice experiments. *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 foretarsi). 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 S1.3), and no difference in how long it took for males with two spots and males with no spots to initiate courtship (Supplementary Figure S1.4). The order of courtship initiation was not a predictor of mating success (Supplementary Figure S1.5).

In no-choice tests, a single male of one phenotype was placed with one female. The frequency at which males with and without spots courted was not statistically significantly different in any of the species (Figure 1.3). No other mating parameters were significantly different between males spotted and spotless males in the no-choice experiments (Supplementary Figures S1.6-S1.7).

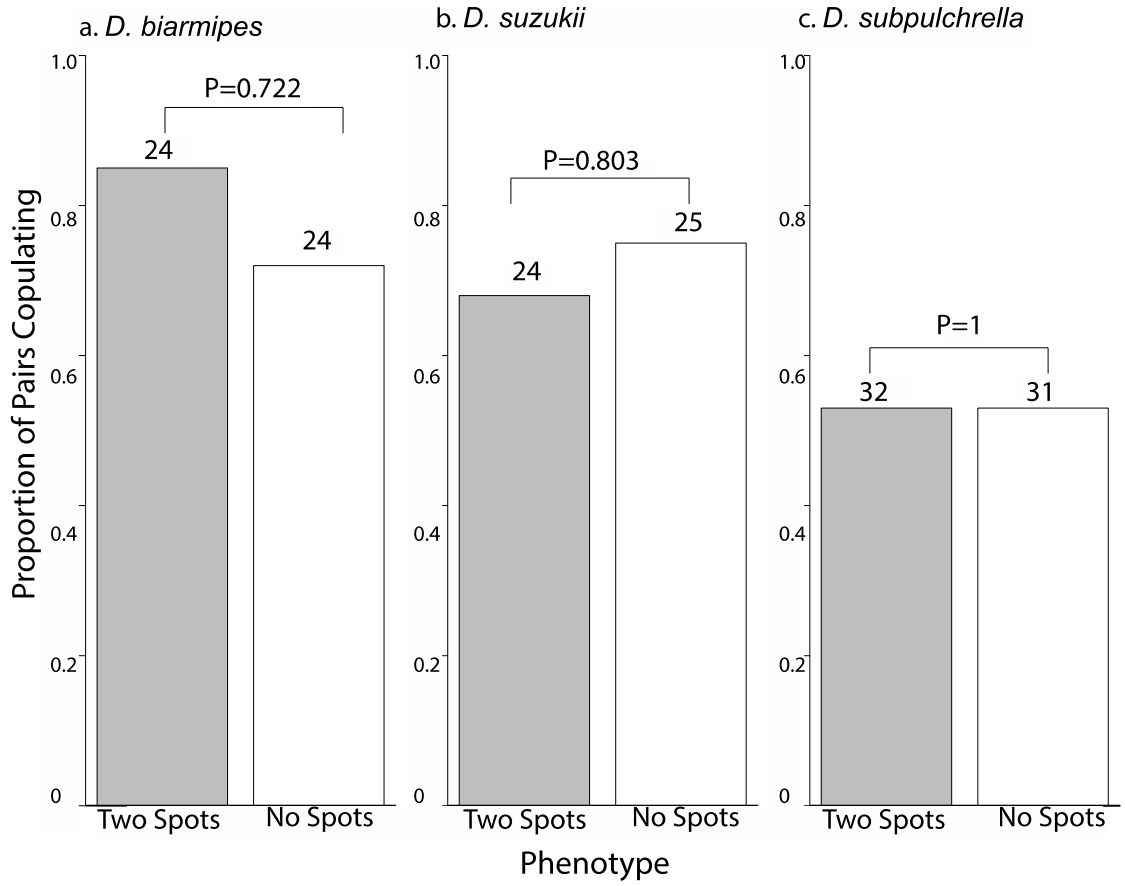


Figure 1.3. Effect of phenotype on courtship success in no-choice assays. Mating pairs were observed for 60 minutes, and the proportion of males who successfully mated were compared between males with two spots and males with no spots using a two-tailed Fisher's Exact Test (*P* values given for each pair above brackets). Numbers on top of each column are sample sizes.

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 not statistically different from males in the light (Figure 1.4a). Of the males that courted, *D. biarmipes* males in the dark were similarly as successful at achieving copulation within 60 minutes as males in the light (Figure 1.4b). In the dark, none of the *Drosophila suzukii* and *D. subpulchrella* males that courted mated within 60 minutes whereas in the light, 64% and 87%,

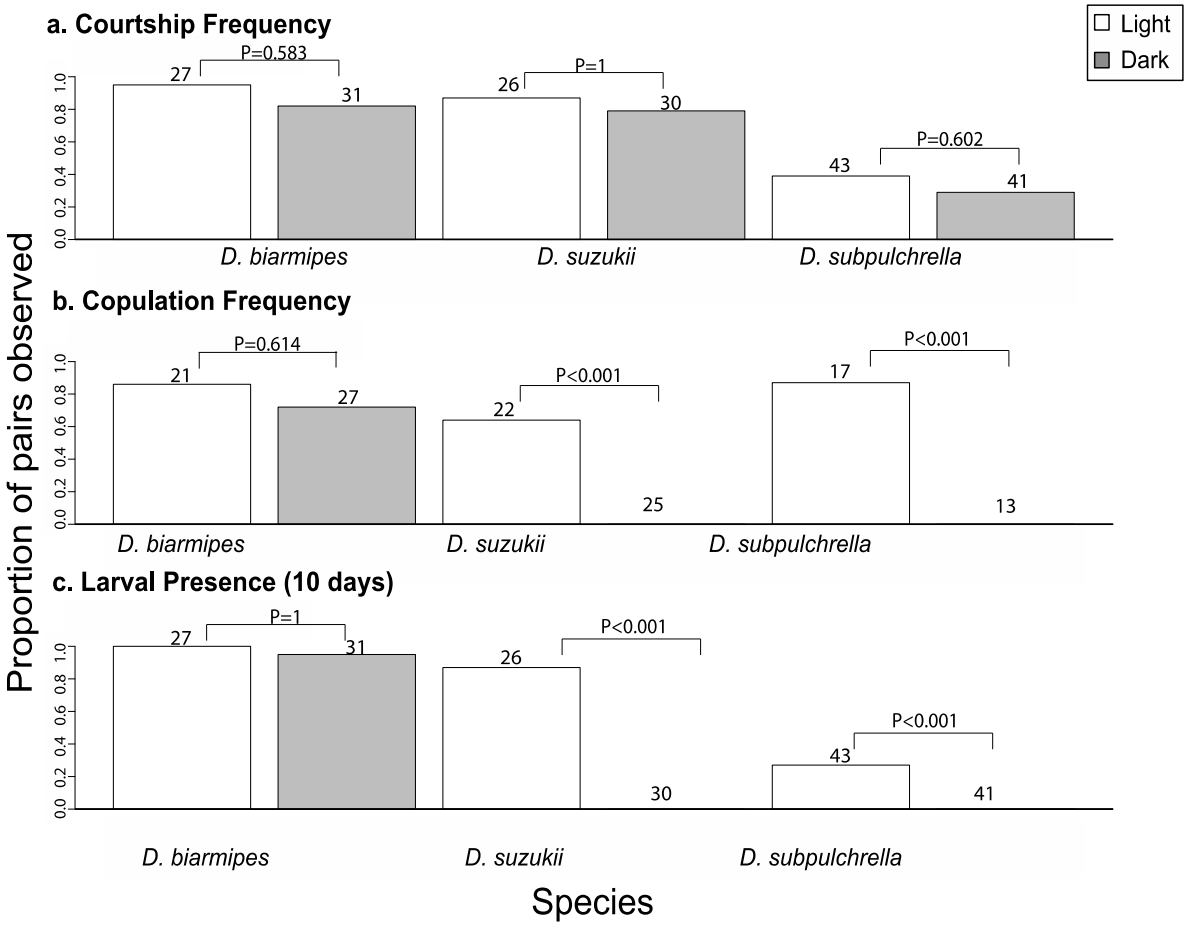


Figure 1.4. Effect of light on mating. Virgin males and females were assigned to two treatments: 12:12 light: dark (normal photoperiod) or continuous darkness and observed for 60 minutes. All proportions were compared within a species between treatments with a two-tailed Fisher's Exact test (*P* values are given above brackets). Numbers on top of each column are sample sizes. a) The proportion of males that courted females did not differ significantly 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.

respectively, of the males who courted achieved copulation success (Figure 1.4b). In *D. biarmipes*, pairs incubated for ten days produced progeny in equal proportions in the dark and in the light (Figure 1.4c). In *D. suzukii* and *D. subpulchrella*, no larvae were observed in dark treatment vials for pairs left for ten days (Figure 1.4c). 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 (*D. biarmipes*, N=9; *D. suzukii*, n=8; *D. subpulchrella*, N=7), 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 (Figure 1.4b) but compared to males in the light, they took significantly longer to initiate courtship (t-test, $P < 0.001$; Figure 1.5a). 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 courted females at a similar frequency in darkness as compared to in the light (Figure 1.5b) but never mated. 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 anterior, and then performed their wing displays. Females were unresponsive to male displays in darkness, and rejected any copulation attempts by kicking away from the male. *Drosophila subpulchrella* took significantly longer to initiate courtship in darkness than in light (t-test, $P < 0.001$; Figure 1.5c). Individuals of this species were generally inactive when placed in mating vials, and even less active 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 (fewer than ten); all attempts were repelled by the females in the dark.

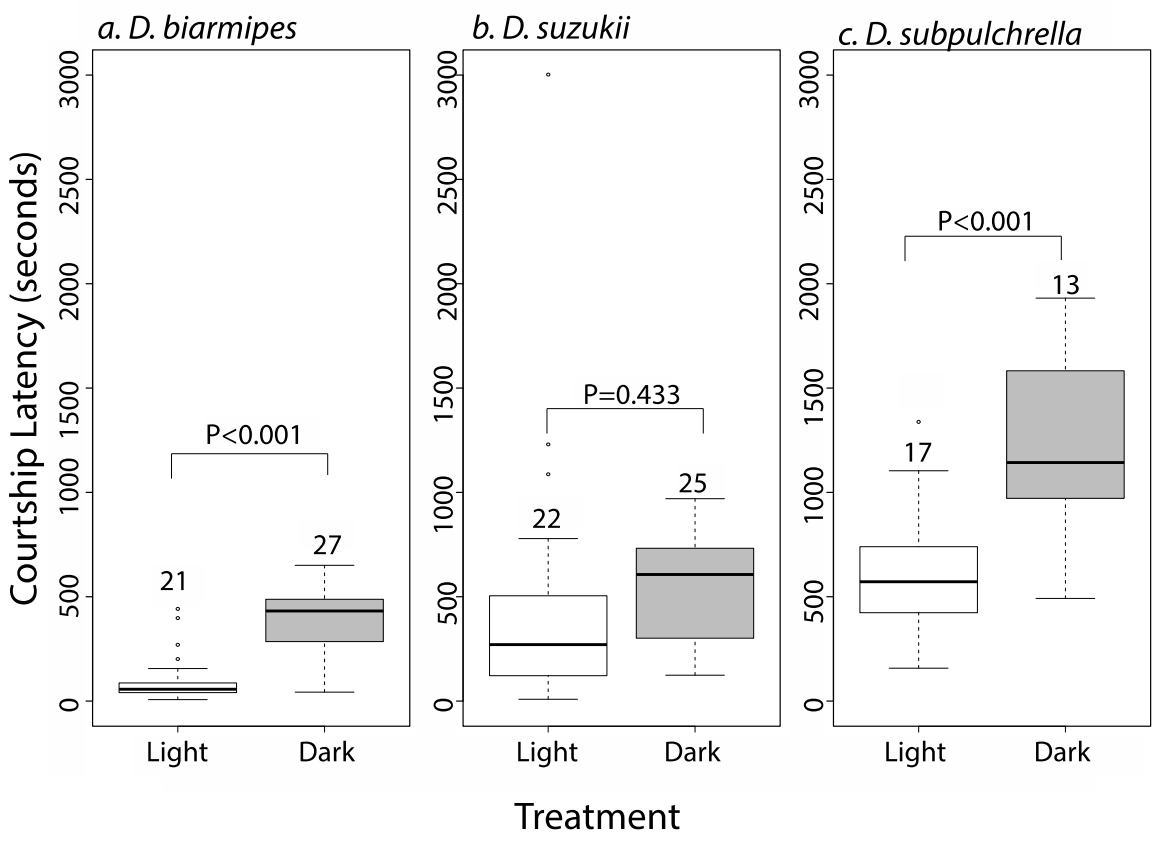


Figure 1.5. Effect of light on courtship latencies. Mating pairs were observed for 60 minutes in either light or dark. The time from introduction to the initiation of courtship was recorded for each pair of each species. Upper and lower quartiles of the data are represented by the upper and lower boundaries 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.

Because *D. biarmipes* mated in the dark, we measured additional courtship parameters. Once courtship was initiated, males had a statistically significantly longer courtship duration in the dark compared to in the light (t-test, $P < 0.001$; Figure 1.6a). 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 similar (Figure 1.6b).

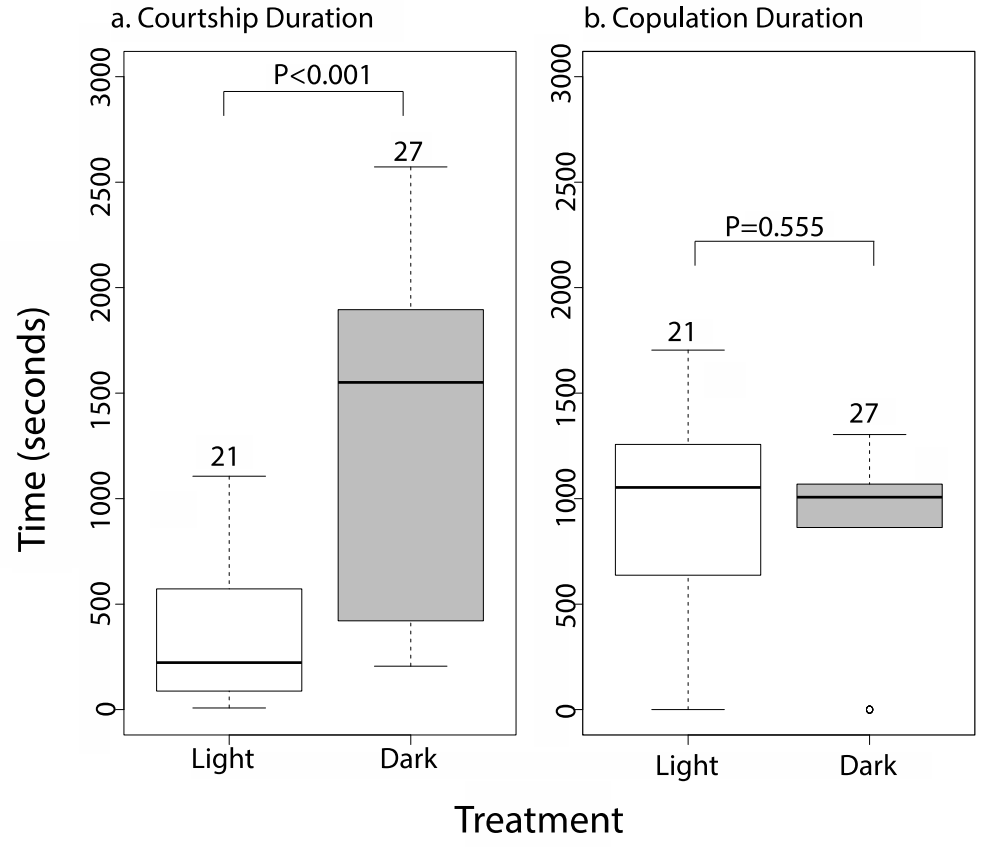


Figure 1.6. Effect of light on copulation parameters in *D. biarmipes*. Mating pairs were observed for 60 minutes in either light or dark. The a) courtship duration was significantly longer for males in the dark but b) copulation duration did not differ between treatments. Upper and lower quartiles of 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.

Discussion

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 (Singh and Chatterjee 1987; Hegde et al. 2005; Parkash et al. 2013). 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 (True et al. 1999; Gompel et al. 2005; Prud'homme et al. 2006; Yeh et al. 2006; Werner et al. 2010; Arnoult et al. 2013; Yeh and True 2014; Koshikawa et al. 2015), 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 did not find evidence for choice by females of either type of male, 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* 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 hours (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 (Singh and Chatterjee 1987; Hegde et al. 2005; Parkash et al. 2013).

Females show no significant preference for 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. Robert et al. 1985; Wilkinson and Reillo 1994; Hill and McGraw 2004) 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 of 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 unlikely to play 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 most likely have spots further apart than small males due to differences in wing span. Differences in fighting ability should be tested, however, if one phenotype was more successful at fighting than the other then 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 (Singh and Chatterjee 1987; Hegde et al. 2005; Parkash et al. 2013) 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 may have failed to see an effect of phenotype on female choice as a result of low power due to sample sizes. With 95% confidence intervals placed on the proportion of spot chosen, the potential for a significant choice of spots (or no spots) still could be possible for all species. To detect a true lack of preference we would need sample sizes of approximately 200 or higher to more confidently reject the alternative hypothesis. Fuyama (1979) ran 200 choice trials and did not see a significant preference for spots when females were kept in standard light-dark conditions, so failure to see an effect is not unreasonable (at least for *D. suzukii*).

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.

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 as compared to in white light. 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), thus 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 (Spieth and Hsu 1950; Grossfield 1971). 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 males in 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 facilitates rapid acceptance in *D. biarmipes*.

The role of the wing display 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 & True, 2002; personal observation, Chapter 2). Through the dark experiments, we can determine the necessity of visual cues. The fact that the elimination of spot does not significantly affect female mating proportions (within the scope of this study), 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.

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 may not directly 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. Wing movements could be the signal and the spot could enhance wing detection in more complex environments.

Chapter 2: Whole genome phylogeny and examination of the evolution of sexually dimorphic traits in spotted winged *Drosophila*

Abstract

Some organisms have elaborate and conspicuous traits that evolve through sexual selection. The mode by which sexual dimorphisms are gained or lost can be identified in the patterns of trait transition across related species and species groups, but for such analysis a reliable phylogeny is required. Wing spots are a conspicuous sexual dimorphism observed in some species within the *Drosophila melanogas*ter group, commonly referred to as "spotted winged *Drosophila*". Sexual selection has been implicated in driving the evolution of wing spots, particularly because the morphology is always paired with a frontal courtship display (wing display), but phenotyping of the morphology and the behavior in the spotted winged *Drosophila* subgroups needs confirmation and the origin and transitions of the wing spot remains unclear. More modern techniques such as maximum likelihood and binary trait signal analysis are available to identify the origin, transition, and phylogenetic signature of morphological traits. In this study, we constructed a phylogeny of the spotted winged *Drosophila* subgroups using whole genome sequence data. We produced a phylogeny with high branch support for the spotted winged subgroups that resolved some species relationships and confirmed others. We then examined patterns of wing spot trait evolution using ancestral character reconstruction and found that we are unable to determine if wing spot was the ancestral condition to the group or if it was repeatedly gained because of the rapid evolutionary rate of the trait. The wing spot character did not have a phylogenetic signal, thus wing spot is not a conserved trait. We confirm that wing spot is tightly correlated with wing displays, thus the two traits are likely coevolving. Our findings in total suggest rapid fluctuations of sexual selection across the spotted winged *Drosophila* subgroups causing the observed patterns of male sexual dimorphisms.

Introduction

Most sexually reproducing species (excluding hermaphrodites) are inherently sexually dimorphic (e.g. gametes), but some organisms evolve elaborate and conspicuous sex-limited secondary characters such as extravagant plumage in birds of paradise (Irestedt et al. 2009), or colorful dewlaps in anoles (Nicholson et al. 2007). The evolution of elaborate male dimorphisms (secondary sexual characteristics) by sexual selection was first proposed by Darwin (1871) and female choice driving the evolution of conspicuous male dimorphisms has substantial support from the literature (see Lande 1980; Andersson 1994; Andersson et al. 2002; Andersson and Simmons 2006). Because dimorphisms can evolve as a result of female preferences (see Fisher 1915; Lande 1980; Kirkpatrick and Ryan 1991; Andersson 1994), we expect that changes in female preferences can also contribute to losses of dimorphic traits (e.g. due to changes in signal efficacy in a particular environment, physiological changes that affect the sensory system and reception of a particular signal, or natural variation in female choosiness, see Jennions and Petrie 1997; Bradbury and Vehrencamp 1998; Cotton et al. 2006; Stevens 2013). We therefore expect sexual selection to influence transitions to and from conspicuous dimorphisms over evolutionary time.

Loss of dimorphic male traits, even those presumably maintained by female choice, occur relatively frequently (Wiens 2001), suggesting that evolutionary forces opposing sexual selection can act on dimorphic traits (e.g. natural selection due to increased conspicuousness to predators). Identifying independent gains/losses of a trait (convergence) versus presence of trait due to common ancestry (conservation) across related species can uncover the mode by which sexual dimorphisms are gained or lost. With phylogenies we can identify relevant patterns of trait transition at the species level and test hypotheses of the role of selection in driving traits of

interest (Ord and Martins 2010), including dimorphisms. For example, mapping male anole display traits onto a phylogeny allowed researchers to implicate both habitat use and sexual selection in the diversification of sexual dimorphisms (Ord and Martins 2006). The identification of where gains and/or losses of characters have occurred, particularly within clades and between sister species, can aid us in deducing how, and potentially why, traits are different among related species (Wiens 2001).

In six subgroups of the *Drosophila melanogaster* group, a striking sexual dimorphism is observed in ten species. In these species, males have a "wing spot" and perform a conspicuous, species-specific frontal courtship display (Kopp and True 2002b; Prud'homme et al. 2006). Wing spot morphology is hypothesized to be a component of courtship signaling and to be sexually selected, but the function of the wing spot in female choice sexual selection has conflicting evidence; some studies support female choice (Singh and Chatterjee 1987; Hegde et al. 2005; Parkash et al. 2013) while others refute female preference (Fuyama 1979; Roy and Gleason 2019). These studies are primarily on *Drosophila biarmipes* (though three spotted species in the *suzukii* subgroup were examined in Roy & Gleason, 2019).

Phylogenetic analysis of both male wing spot and wing display traits may allow us to identify the model of evolution that best describes the observed trait distributions across the spotted winged *Drosophila*. Across the spotted winged *Drosophila* subgroups, the presence of wing spot and wing display appear more dispersed than one might expect, suggestive of fluctuations in selection. If traits are not correlated, as previously reported, there may be evidence for genetic drift operating on the evolution of traits as opposed to fluctuating sexual selection. Wing display is reported for all species with wing dimorphism (Kopp and True 2002b). Even *D. eugracilis*, a species with dilute pigmentation along the wings instead of

conspicuous spot, performs wing displays (Kopp and True 2002b). One issue with current phenotyping of wing spots and wing displays is that the description of one species, *D. lutescens*, differs from our observations: we find that it has pigmentation and behavior similar to *D. eugracilis*, a species that has dilute pigmentation along the wing and performs wing display (Kopp and True 2002b) and it is reported as having clear wings and not performing wing displays (see Figure I.1). Correlation of wing spots with wing display behavior needs confirmation.

Currently, the origin and transitions of wing spots are inconclusive regarding the ancestral state of wing spots and if apparent trait convergence is a result of gains or losses. Kopp and True (2002b) used parsimony to propose the potential origin of the wing spot in the *melanogaster* subgroups (see Figure I.1). The random distribution of the trait makes proposals for both gains and losses of wing spot possible (Kopp and True 2002b). Prud'homme et al. (2006) used Bayesian ancestral character reconstruction of wing spots. Their analysis suggests that the trait is ancestral to the *melanogaster* subgroups, arising as a single gain and then lost independently six times (Prud'homme et al. 2006). There is discrepancy in phenotyping of wing spots between the Kopp and True (200b) and Prud'homme et al. (2006) studies. In Prud'homme et. al (2006) the wing spot trait was assigned to *Drosophila takahashii*; however, we observe *D. takahshii* to have clear wings and it was described originally as having clear wings (Sturtevant 1927). The ancestral phenotype and nature of transitions, therefore, cannot be confirmed from either study.

The distribution of wing spot presence along the phylogeny as measured by phylogenetic signal can give insight to the mechanisms behind the transitions to/from spotted states. Phylogenetic signal is defined as the statistical nonindependence among species trait values due

to their phylogenetic relatedness (Revell et al. 2008), or in other words, the tendency for close relatives to resemble each other. The extent to which a trait found in existing species varies due to underlying shared ancestry is reflected in the phylogenetic signal produced by the trait (Ord and Martins 2010). From the presence of phylogenetic signal we can infer conservation of a trait, while from the absence of phylogenetic signal we can infer the lability of a trait (Blomberg et al. 2003). Conservation of traits could reflect phylogenetic inertia due to either low mutation rate or genetic, developmental, or physiological constraint (Ord and Martins 2010). Conservation could also reflect stabilizing selection (Hall 2013). Convergence suggests a change in selective pressures, typically due to a change in environment (Ord et al. 2015) or stochastic changes in traits. In spotted winged *Drosophila*, wing spot phenotypes appear to be randomly distributed within and across the clades that contain dimorphic species (Kopp and True 2002b; Prud'homme et al. 2006) so fluctuation of selective pressures is probable, most likely because of sexual selection due to the dimorphic nature of the wing spot trait and potential correlation with other dimorphic courtship traits (wing displays). Genetic drift could produce a comparable pattern in morphology along the phylogeny and therefore cannot be ruled out. Identification of phylogenetic signal of wings spots will guide future research on the possible mechanisms underlying trait changes within the spotted winged *Drosophila* subgroups.

Identifying the origin, transition, and phylogenetic signal of the wing spot requires an accurate tree. Multiple phylogenies have been proposed for the *takahashii, suzukii, elegans* and *rhopoloa* subgroups (Schawroch 2000; Kopp and True 2002a; Lewis et al. 2005; Prud'homme et al. 2006; Da Lage et al. 2007; van der Linde and Houle 2008), though some species placements are unresolved. Within the *Drosophila takahashii* subgroup, species relationships are represented by either a ladder phylogeny or as unresolved (Kopp and True 2002a; Prud'homme et al. 2006;

van der Linde and Houle 2008). An additional problem at present is that all of the current phylogenies are missing one or more species (Supplementary Table S2.2) that are helpful for the comparative behavioral analysis (Chapter 3). A species available to us in the laboratory, *D. subpulchrella*, has never been included in a phylogeny. Changes in the species included in the phylogeny could potentially alter the inferred topology of the species tree for these subgroups. Even small changes in tree topology could affect the inference of character states (Ryan and Rand 1993b; Martins 1996).

In this study, we sequenced the genomes of eight species of *Drosophila* covering the *takahashii*, *suzukii*, *elegans*, and *rhopaloa* subgroups that are the subjects in our study of courtship behavior. We used our sequence data in conjunction with genomic sequencing from thirteen additional species to build a maximum likelihood phylogeny and reconstruct the ancestral history of the wing spot characters. We then tested for phylogenetic signal and dispersal pattern of the wing spot to identify potential mechanisms for the observed pattern of spot morphology across the spotted winged *Drosophila*.

Materials and Methods

Strains and cultures

For new genome sequences, we used eight species from the National *Drosophila* Species Stock Center (Table 2.1). Cultures of 20-50 adults were established in 25 mm x 95 mm vials containing Bloomington standard formula (corn syrup, cornmeal, soy flour, yeast, and agar) *Drosophila* food, and maintained at 23-25°C with 12:12 hour light:dark cycle. For species with sequenced genomes, we used raw genomic data from the National Center for Biotechnology Information Sequencing Read Archive (Table 2.1). The *D. suzukii* reference genome and annotation were provided by Dr. Nicolas Gompel. The *D. melanogaster* BDGP6.95 reference genome was downloaded from Ensembl (Zerbino et al. 2017).

Library Preparation and Genome Sequencing

Females were collected from vials and starved by placing them onto damp cotton two hours prior to preservation. Specimens were preserved in 70% ethanol at -20 ˚C for seven days prior to DNA extraction. We extracted total genomic data from 10 female individuals from each of eight species (Table 2.1) following a DNA isolation protocol (Puregene Cell and Tissue Kit, 158388; Qiagen) in the lab of Rob Unckless at the University of Kansas, yielding sufficient DNA for library preparation and sequencing (~20-61 ng/ μL). From the extracted DNA, sequencing libraries were prepared with the Quick Nextera sequencing protocol (Nextera DNA Flex Library Prep Kit, 20018705; Illumina) following the manufacturer's protocol. Adapter barcoded libraries (Illumina Incorporated) were pooled into a single sample in equimolar concentrations as verified by qPCR and Tape Station at the University of Kansas Genome Sequencing Core. The pooled

Table 2.1 Stocks used for whole genome sequencing and the source of their phenotype characterization

Species Subgroup	Species[1]	Genome Origin	Original Phenotype Characterization[5]
ananassae	*D. ananassae*	SRR3946371	Bock and Wheeler (1972)
elegans	***D. elegans***	SRR345540	Bock and Wheeler (1972)**
	D. gunungcola	Massey & Wittkopp[2]	Sultana et al. (1999)**
eugracilis	***D. eugracilis***	SRR345543	Bock and Wheeler (1972)
ficusphila	*D. ficusphila*	SRR345541	Kikkawa and Peng (1938)
melanogaster	*D. melanogaster*	SRR8439107	Meigen (1830)
	D. simulans	SRR869579	Sturtevant (1919)
	D. yakuba	SRR2318687	Burla (1954)
montium	*D. auraria*	SRR6655883	Peng (1937)
rhopaloa	***D. fuyamai***	SAMN11310175*[3]	Burla (1954)**
	D. rhopaloa	SRR345538	Setoguchi et al. (2014)
suzukii	***D. biarmipes***	SRR345536	Malloch (1924)**
	D. lucipennis	SAMN11310176*	Bock and Wheeler (1972)**
	D. mimetica	SAMN11310177*	Bock and Wheeler (1972)**
	D. subpulchrella[4]	SAMN11310178*	Takamori (2006)**
	D. suzukii	SRR942805	Matsumura (1931)**
takahashii	***D. lutescens***	SAMN11310179*	Okada (1975)**
	D. paralutea	SAMN11310180*	Bock and Wheeler (1972)**
	D. prostipennis	SAMN11310181*	Bock and Wheeler (1972)**
	D. pseudotakahashii	SAMN11310182*	Mather (1957)**
	D. takahashii	SRR345539	Sturtevant(1927)**

[1]Spotted species in bold type
[2]Genome sequences in FASTQ format were kindly provided by J. Massey and P. Wittkopp prior to publication. The strain DgunSK was collected in Sukarami, West Sumatra, Indonesia (1999).
[3]Genomes sequenced in this study denoted with an * are each part of Bioproject PRJNA530273, this study.
[4]Strain was kindly provided by M Turelli, having originated with the Chin Lab in 2014.
[5]All descriptions marked with an ** were also phenotyped in this study using the stocks in our laboratory

sample was paired-end sequenced on an Illumina HiSeq 2500 at the Oklahoma Medical Research Foundation, and data in the form of FASTQ files were received electronically.

In addition to the eight species that we sequenced, we used raw reads from *D. gunungcola* provided by Jon Massey (lab of Patricia Wittkopp) and 12 additional species through SRA (Table 2.1) in raw FASTQ. We included species from the *D. melanogaster* subgroup, as well as the outgroup species *D. ananassae* and *D. auraria* to include all of the subgroups and outgroups represented in Prud'homme et al. (2006).

Alignment, Variant Calling, and Phylogenetic Construction

Illumina sequence data were analyzed using FastP (v.0.19.6; Chen et al. 2018) to trim adapters and quality check the raw sequences. Trimmed sequences were aligned to the *D. suzukii* and *D .melanogaster* reference genomes in separate pipelines using BWA (v0.7.17; Li and Durbin 2009) and converted to BAM format using SAMTools (v.1.4; Li et al. 2009). From the aligned sequences, variants were called using SAMTools bcf tools -mpileup (v.1.9; Li 2011). The vcf files were filtered with bcftools -filter to remove indels and the surrounding 20 base-pairs, heterozygotes, variant sites with a depth of over 10, and sequences with a call rate of 13 or more to eliminate enrichment bias and low-quality markers that may confound downstream phylogenetic analysis. A concatenated vcf file containing all filtered variants from each species was then annotated and further filtered to only include coding regions and exclude non-informative characters and singletons.

The annotated variants from each reference alignment were used to generate Maximum Likelihood trees in RAxML (v.8.2.12; Stamatakis 2014) using the GTR GAMMA model. Both alignments (to *D. suzukii* and to *D. melanogaster*) were examined. Branch support was computed by estimating 100 non-parametic bootstrap replicate trees. Datasets for both alignments will be made available on Dryad.

Ancestral Character State Reconstruction and Phylogenetic signal

Tip states were determined by the presence of melanization on the wings (wing spots). If male wings had any pigmentation, they were assigned the spotted phenotype. If male wings were hyaline, or devoid of pigmentation, they were assigned the spotless phenotype. For species that we did not have in the lab, we used the literature to determine the phenotype (Table 2.1). While most pigmentation is manifested as a conspicuous patch at the tips of the wings, two species, *D. lutescens* and *D. eugracilis*, have less obvious pigmentation. *Drosophila lutescens*, a species that we had in the lab, was characterized as spotless in Kopp and True (2002) but we observed pigmentation similar to that described in *D. eugracilis*, which was characterized as spotted in Kopp and True (2002a) so we designated it as spotted (Table 2). *Drosophila rhopaloa* was reported as pigmented (Setoguchi et al. 2014) and we characterized it as spotted for this study. For every spotted species included in this study, we observed frontal wing display during courtship and we determined that from other studies that both *D. eugracilis* (Kopp and True 2002b) and *D. rhopaloa* (Setoguchi et al. 2014) also perform displays. Species that do not have spots do not perform wing displays during courtship (personal observation, Kopp and True 2002b). Thus, all species with pigmentation perform wing displays and none of the species without pigmentation perform wing displays; this correlation allows us to interpret our results for wing spots as applying to wing display.

The RAxML "best tree" from the *D. suzukii* alignment filtered dataset was imported into R Studio v.1.1.463 (R Core Team). Ancestral character state reconstruction of wing spot was performed with an equal rates (ER) and variable rates (ARD) models for discrete characters with the ace function in the package APE (v.5.2; Paradis and Schliep 2018), to estimate ancestral

character states and the associated uncertainty using a maximum likelihood model (Pagel 1994) and joint estimation procedure similar to that described in Pupko et al. (2000).

We performed a "total garbage test" (Harmon 2018) to determine if the evolution of the wing spot is too rapid to infer evolutionary history. We compared the log likelihoods of the total garbage and ER models used in APE for ancestral character reconstruction. Likelihoods values not significantly different between the two models (determined using a Chi square test) indicate that evolution is too rapid for reconstruction to infer the history of the character. All potential trait value estimates for the condition of a phenotypic trait at a given node, if greater than a certain value, will have the same likelihood.

We tested the phylogenetic signal (whether wing spot is phylogenetically clustered or is distributed randomly) of the wing spot phenotype using the D statistic for binary traits from Fritz and Purvis (2010) implemented with the caper package in R (Orme et al. 2013). The D statistic is calculated by scaling the observed sum of sister-clade differences in a given phylogeny with the mean values of the two expected character distributions from the given model (random and Brownian). If D is equal to zero, the observed trait pattern is indistinguishable from Brownian motion. If the D is equal to one, binary traits have a phylogenetically random distribution across the tips of the phylogeny. Numbers significantly less than zero indicate a clumped pattern (conservatism), and numbers significantly greater than one indicate overdispersal of the traits (non-conservatism). The significance of the observed D value is determined from the distribution of scaled D from the simulated data (Fritz and Purvis 2010).

Though D is the recommended statistic to assess the phylogenetic signal of binary traits, the most appropriate sample sizes for included taxa for the analysis are 25 or above (though authors note that error rates are generally acceptable and D's power to detect signal was only

reduced by very small phylogenies, Fritz and Purvis 2010). Because we have 21taxa, we also tested for phylogenetic signal using Pagel's lambda using the geiger package (Harmon et al. 2014). A lambda value of zero indicates no phylogenetic signal, and a lambda value of one indicates strong phylogenetic signal.

Results

Phylogeny

The whole-genome libraries averaged 26.9 M raw read pairs (range of 4.5 M to 55.0 M, Supplementary Table 2.1). We aligned to both *D. suzukii* and *D. melanogaster* to check for reference bias in phylogenetic analysis (for all statistics on reads, see Supplementary Tables S2.3-S2.7 and Supplementary Figures S2.1-S2.3). We observed a relationship between reads mapped and phylogenetic distance (measured as distance from the reference species using the distTips function in the R package adephylo, Jombart et al. 2010) when we aligned to *D. suzukii* (Pearson's Correlation = 0.77, $P < 0.001$, Supplementary Figure S2.2a) that we did not see when we aligned to *D. melanogaster* (Pearson's Correlation = 0.39, $P = 0.09$, Supplementary Figure 2.1a). In both reference alignments, mean depth of coverage increased with an increased number of reads (Supplementary Figures S2.1b and S2.2b). The number of divergent nucleotides increased as phylogenetic distance from the reference increased (Supplementary Figures S2.1c and S2.2c) but was only significantly correlated in the *D. melanogaster* reference alignment (Pearson's Correlation = 0.48, $P = 0.03$, Supplementary Figure S2.1c). Depth of coverage was not affected by distance to the reference in either alignments (Supplementary Figures S2.1d and S2.2d). The *D. melanogaster* genome is well annotated allowing us to assess chromosomal and sex-autosome biases, which we did not see (Supplementary Table S2.3). We could not determine sex-autosome bias in the *D. suzukii* alignment because sex chromosomes are not defined in the

annotation (though we do not see any obvious bias towards any particular scaffold in the annotation, Supplementary Table S2.4).

Alignment to *Drosophila melanogaster*, filtering, and annotation yielded 789 K sites for phylogenetic analysis whereas alignment to *D. suzukii* yielded 515K sites. The number of variants called from the *D. melanogaster* reference alignment for the RAxML input ranged from 10,358 (*D. gunungcola*) to 773,515 (*D. melanogaster*, Supplementary Table S2.7). The number of variants called from the *D. suzukii* reference alignment ranged from 12,937 (*D. gunungcola*) to 503,555 (*D. suzukii*, Supplementary Table S2.7). The number of variants called for each species was correlated between the two reference alignments (Pearson's Correlation = 0.66, P = 0.001). The number of variants called in each species was not a function of phylogenetic distance (*D. melanogaster*: Pearson's Correlation = -0.17, P= 0.47; *D. suzukii*: Pearson's Correlation=-0.32, P = 0.16; Supplementary Figure S2.3).

The total number of unique alignment patterns (the set of molecular characters that exist at a given sequence in the taxa, which excludes singletons, repeats, and non-informative characters) used by RAxML to build the phylogenies was 454,675 (*D. melanogaster* reference alignment) and 372,685 (*D. suzukii* reference alignment). The RAxML analysis produced a well-supported hypothesis of the relationships of the spotted winged *Drosophila* subgroups for both reference alignments (Figures 2.1 and 2.2). All nodes for the *D. melanogaster* reference alignment tree had 100% bootstrap support with two exceptions: one node in the *takahashii* subgroup (97%) and one node determining the placement of *Drosophila ficusphila*, (69%, Figure 2.1). All nodes for the *D. suzukii* reference alignment tree had 100% bootstrap support with the exception of the node determining the placement of *Drosophila ficusphila* (54%, Figure 2.2)

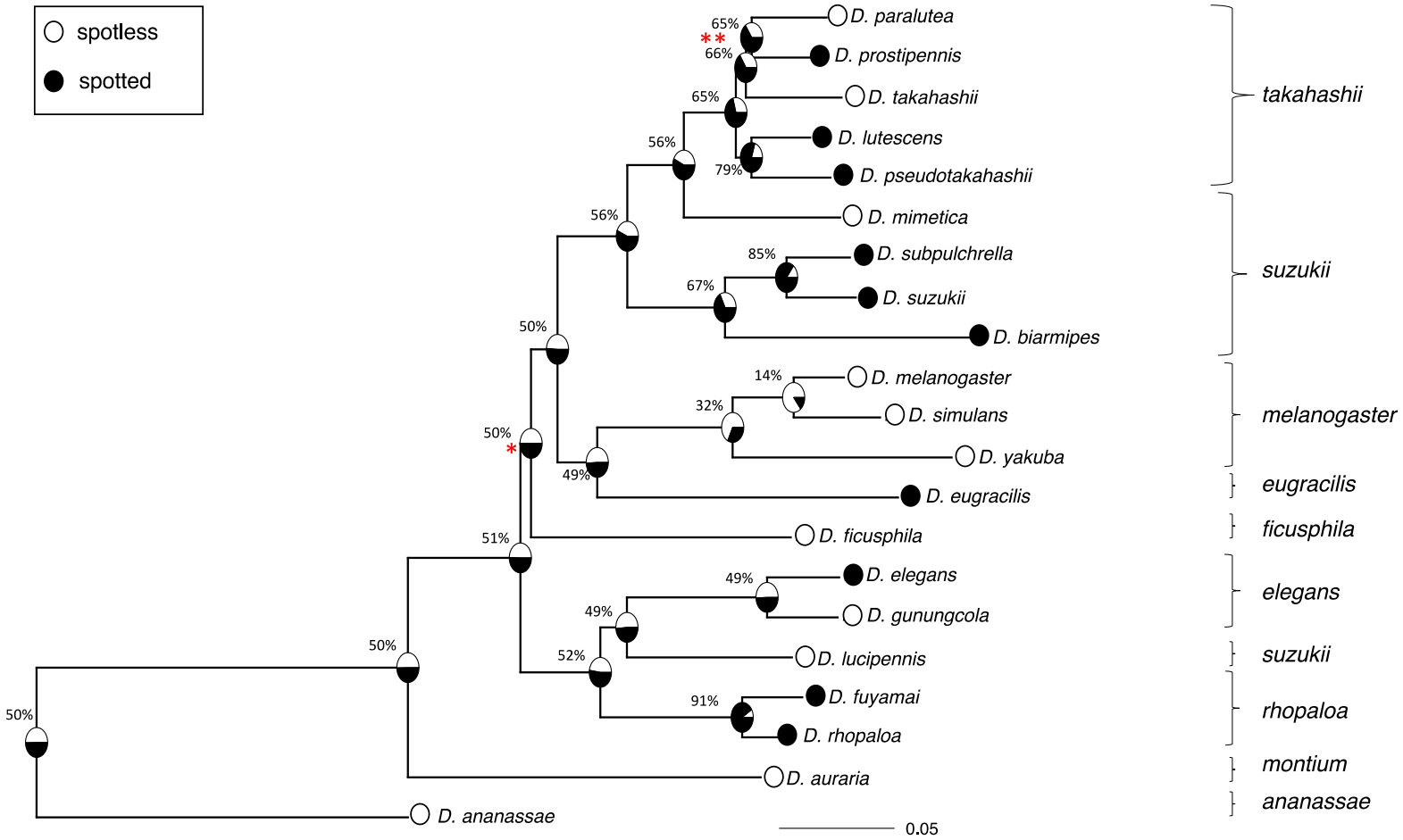


Figure 2.1 Ancestral character reconstruction of wing spots on the *Drosophila melanogaster* reference best fit tree. Generated from 454,675 unique alignment patterns with 100 bootstrap replicate trees under the GTRGAMMA model in RAxML. Ancestral state was modeled using maximum likelihood assuming equal rates. Estimates at each node were generated with joint estimation and scaled to represent proportions. Percentages at each node represent the scaled likelihoods for the spotted trait. Ancestral state of the spot from the root are uncertain due to the rapid evolution of the character. Asterisks designate a bootstrap support value below 100 percent (*=69% and **=97%). Scale bar corresponds to branch length, representing the mean number of nucleotide substitutions per site as estimated by RAxML.

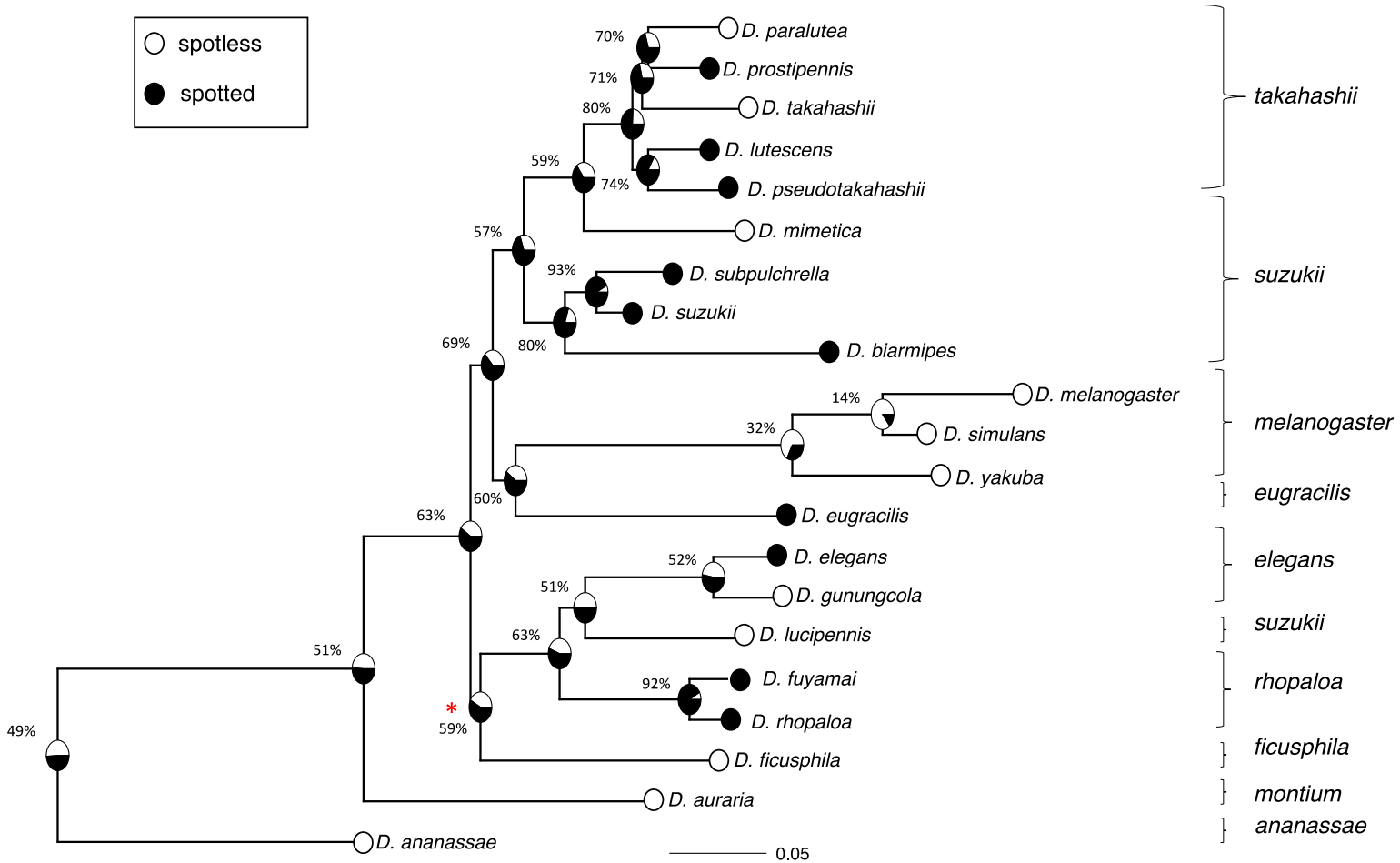


Figure 2.2. Ancestral character reconstruction of wing spots on the *Drosophila suzukii* reference best fit tree. Generated with 372,685 unique alignment patterns with 100 bootstrap replicate trees under the GTRGAMMA model in RAxML. Ancestral state was modeled using maximum likelihood assuming equal rates. Estimates at each node were generated with joint estimation and scaled to represent proportions. Percentages at each node represent the scaled likelihoods for the spotted trait. Ancestral state of the wing spot from the root are uncertain due to the rapid evolution of the character. Asterisks designate a bootstrap support value below 100 percent (*=54%). Scale bar corresponds to branch length, representing the mean number of nucleotide substitutions per site as estimated by RAxML.

The two phylogenies were almost entirely concordant, with only *Drosophila ficusphila* placed differently between the two phylogenies (Figures 2.1 and 2.2). In the *D. melanogaster* reference tree, *D. ficusphila* is sister to the clade encompassing the *takahashii, suzukii, melanogaster*, and *eugracilis* subgroups (Figure 2.1). In the *D. suzukii* reference tree, *D. ficusphila* is sister to the clade encompassing the *elegans* and *rhopoloa* subgroups (Figure 2.2).

The subgroups are monophyletic, with the exception of the *suzukii* subgroup. In this subgroup, *D. mimetica* is sister to the *takahashii* subgroup and *D. lucipennis* is sister to the *elegans* subgroup (Figures 2.1 and 2.2). For the relationships among the subgroups, our trees confirm that the *takahashii* (with *D. mimetica*) and *suzukii* subgroups are sister subgroups, and form a clade with the *melanogaster* and *eugracilis* subgroups (Schawaroch 2002; Kopp and True 2002a; Prud'homme et al. 2006; van der Linde and Houle 2008). Previous research has also placed the *elegans* and *rhopaloa* subgroups as sister subgroups (Kopp and True 2002a; Prud'homme et al. 2006; van der Linde and Houle 2008).

Relationships within subgroups are well resolved. For the *takahashii* subgroup, only van der Linde and Houle (2008) and Prud'homme et al. (2006) had resolution of the *takahashii* subgroup species, but the species were arranged in a ladder (Figure 2.3). Both of our phylogenies (Figure 2.1 and 2.2) have sister groupings for *D. paralutea* with *D. prostipennis* and *D. lutescens* with *D. pseudotakahashii,* though branch lengths uniting species in this subgroup are very short, indicating rapid diversification of the subgroup. For the *suzukii* subgroup, *D. subpulchrella* was not included in other phylogenetic reconstructions, but here occupies the place of *D. pulchrella*. *Drosophila subpulchrella* was originally described as a morph of *D. pulchrella,* but was assigned to its own species when differences in melanization patterns were described and the two species

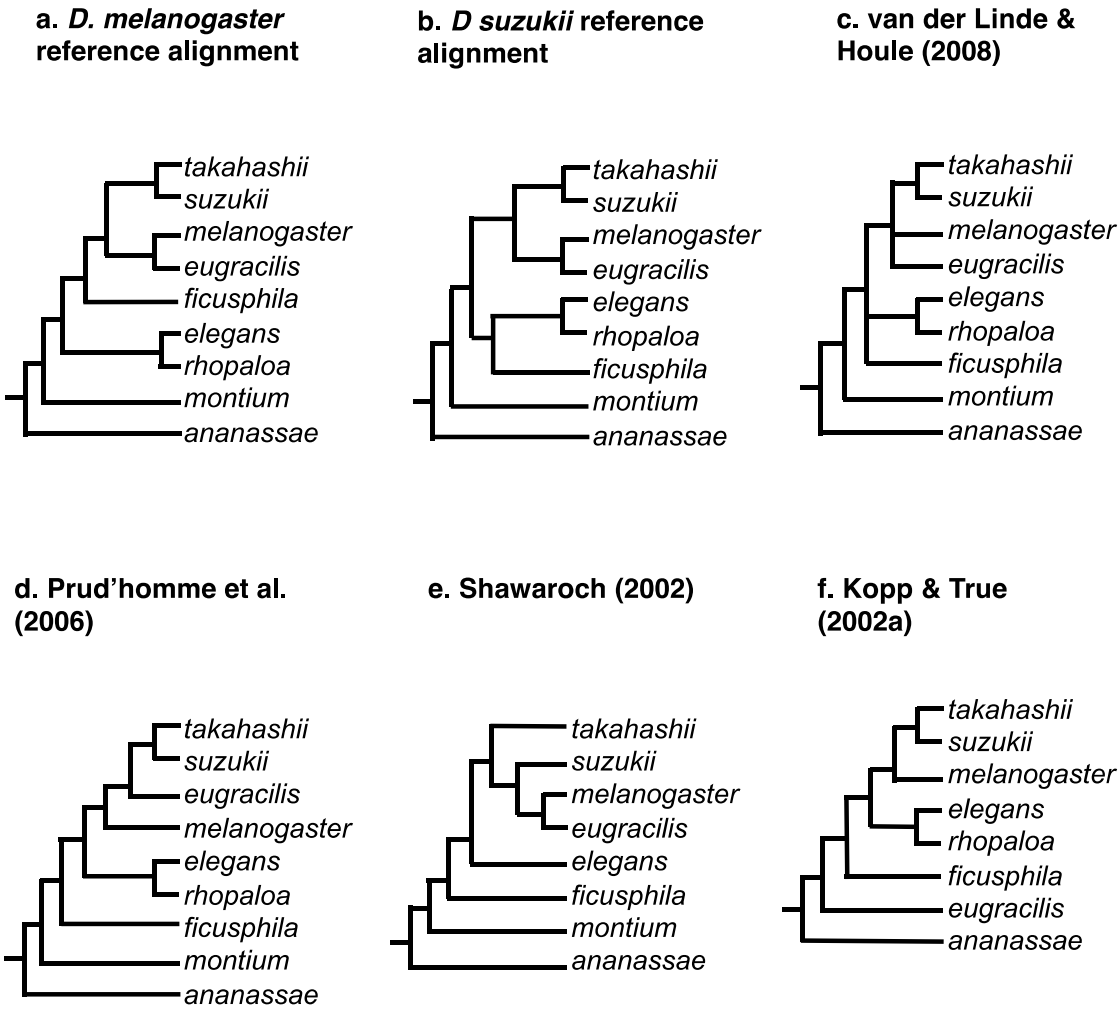


Figure 2.3 Tree topologies for the *melanogaster* group species subgroups. Proposed topologies for the *takahashii*, *suzukii*, *melanogaster*, *eugracilis*, *elegans*, *rhopoloa*, and *ficusphila* subgroups for a) the *D. melanogaster* reference alignment; b) the *D. suzukii* reference alignment; c) van der Linde and Houle (2008); d) Prud'homme et al. (2006); e) Schawaroch (2002); and f) Kopp & True (2002a) . The *montium* and *ananassae* groups are outgroups for all trees except for f) because *montium* was not included in the study. The *rhopaloa* subgroup was not included in e). The *suzukii* subgroup is paraphyletic, with *D. mimetica* falling in the *takahashii* clade and *D. lucipennis* falling in the *elegans* clade. The placement of both *D. mimetica* and *D. lucipennis* is consistent between all of the phylogenies represented.

were found to not interbreed (Takamori et al. 2006). *Drosophila suzukii* and *D. subpulchrella* are more closely related to each other than either is to *D. biarmipes* (Figures 2.1 and 2.2).

Ancestral Character Reconstruction and Phylogenetic Signal

We performed all subsequent analyses with both the *D. melanogaster*- and *D. suzukii*-referenced phylogenies. We performed ancestral character reconstruction for the wing spot trait with two underlying models: the equal rates (ER) model and all rates different models (ARD). The two models produced similar log likelihoods for both reference alignments (Likelihood Ratio Test, $P = 0.53$, Table 2.2). We present the ER model (Figures 2.1 and 2.2) because it is the simpler model.

Table 2.2. Summary statistics for ancestral character reconstruction and phylogenetic signal testing with the *D. melanogaster* and *D. suzukii* reference alignments

Analysis			Alignment	
			D. melanogaster	*D. suzukii*
Equal Rates (ER) Model		log likelihood[1]	-13.64	-13.62
		rate estimate.	12.73 ± 8.91[4]	15.39 ± 10.90[4]
All Rates Different (ARD) Model		log likelihood[1]	-13.54	-13.43
	Rate Estimate	spotted to spotless	11.34 ± 6.38[4]	14.98 ± 9.30[4]
		spotless to spotted	7.89 ± 6.62[4]	11.81 ± 9.01[4]
Total Garbage Test Log Likelihood			-7.21	-7.21
Fritz and Purvis' D Statistic[2]			0.48	0.58
Pagel's Lambda Statistic[3]			0.00	0.00

[1]Statistics generated as R outputs with the ace function in the APE package (Paradis and Schliep 2018)
[2]Statistics generated as R outputs with the phylo.d function in the caper package (Orme et al. 2013)
[3]Statistics generated as R outputs with the geiger package(Harmon et al. 2014)
[4]Numbers after ± represent standard error

At the basal nodes, ancestral character reconstructions indicate that spotted and spotless phenotypes were almost equally likely at the nodes ancestral to all species groups and subgroups, (Figures 2.1 and 2.2). The likelihoods favoring one character state (spotted or spotless) over another increased at nodes closer to the tips of the trees, and were slightly different between the two trees (Figures 2.2 and 2.2). To determine if the differences in tree topology affected the ancestral character reconstruction results, we measured the correlation between scaled likelihood values at the nodes encompassing the same species (18 nodes were included, and two were

eliminated). We found that the ancestral character scaled likelihood values between the comparable nodes were correlated (Pearson's Correlation = 0.94, $P = 0.002$, Supplementary Figure S2.4) indicating that the two phylogenies yielded similar results.

Because of the relatively high rate estimates and large standard errors on the rate estimates for both the ER and ARD models (Table 2.2), the likelihood surface for our ancestral character states are likely flat, and therefore the uncertainty of character states (most notably at the deeper nodes, see Figures 2.1 and 2.1) is largely due to the rapid rate of evolution of the trait (Harmon 2018). To confirm this, we performed a total garbage model test (Harmon 2018) and compared its likelihood to the ER model test to determine if evolution is too rapid to determine ancestry. The garbage dump model was as likely as the ER model given the data for both reference alignments (Likelihood Ratio Test, $P = 1$), meaning evolution of wing spot in these subgroups is too rapid to determine the ancestral state (Table 2.2).

The wing spot trait does not have a phylogenetic signal. The Fritz and Purvis' D statistic is 0.48 and 0.58 for the *D. melanogaster* and *D. suzukii* alignment trees respectively (Table 2.2); neither values are statistically different between Brownian motion (*D. melanogaster:* permutation test, $P_{D=0} = 0.15$; *D. suzukii*: permutation test $P_{D=0} = 0.25$) or random model distributions (*D. melanogaster:* permutation test, $P_{D=1} = 0.26$; *D. suzukii*: permutation test $P_{D=1} = 0.23$; Supplementary Figure S2.5). Pagel's Lambda statistic for both alignments equals zero (Table 2.2), confirming that the state of the wing spots is not conserved.

Discussion

Whole Genome Phylogeny Resolves melanogaster Subgroup Relationships

Our phylogenic analysis using whole genome sequence data provides a solid hypothesis for the evolutionary relationships of the *melanogaster* subgroups. Four previous phylogenies

assess the oriental lineages using nuclear gene sequence data (Schawaroch 2002; Kopp and True 2002a; Prud'homme et al. 2006; van der Linde and Houle 2008) and have the most species overlap with our analysis. Because gene trees and species trees are often in discordance (Maddison 1995) we use whole genome data instead of select genes (e.g. four nuclear and two mitochondrial, Kopp and True 2002a; two nuclear and one mitochondrial, Schawaroch 2002; six nuclear and five mitochondrial, Prud'homme et al. 2006). Whole genome datasets are preferable to select genes because they provide more input for phylogenetic analysis and can help resolve both long and short branches (Girault et al. 2014). The large amounts of data produced by whole genomes, however, can make data processing and filtering relatively complicated (Stephens et al. 2015) and can potentially lead to ascertainment bias due to filtering pipelines.

Clearly the reference genome used for the alignment influences tree topology, revealing that our alignment method using BWA to align to our ingroup reference genomes results in reference bias. We aligned to an outgroup species, *D. ananassae*, to eliminate such bias but did not get good enough alignment to yield sufficient data for phylogenetic analysis. We recognize that a more robust consensus for *D. ficusphila* may be achieved by aligning to the outgroup reference with an assembler designed for more divergent reference genomes (e.g. Stampy, Lunter and Goodson 2011), or by assembling with a method that does not require a reference genome (e.g. target restricted assembly, Allen et al. 2017). We note that the differences in the trees did not significantly affect our downstream analysis because we have concordance of node estimates for our ancestral character state reconstruction even though the estimates between the two trees are not identical.

With these caveats in mind, our analysis nonetheless provided two well-supported hypotheses of the relationships of the species of interest for our future comparative analyses. The

only low bootstrap support for both of our trees is for the node determining the placement of *D. ficusphila*, a species in its own subgroup and not well resolved in previous studies. A supertree analysis places *D. ficusphila* in a polytomy with a clade containing the *takahashii, suzukii, melanogaster, eugracilis* subgroups and a clade containing the *elegans* and *rhopaloa* subgroups (Figure 2.3a, (van der Linde and Houle 2008), which is consistent with both of our phylogenies. The most resolved tree (Figure 2.3c, Prud'homme et al. 2006) places *D. ficusphila* sister to all of the spotted winged subgroups, while our analysis places *D. ficusphila* as sister to the clade encompassing the *takahashii, suzukii,* and *melanogaster* subgroups (Figure 2.3a) or sister to the *elegans* subgroup (Figure 2.3b).

Both of our trees place *D. eugracilis*, a species also in its own subgroup, sister to the *melanogaster* subgroup. We resolve the polytomy in the supertree analysis (van der Linde and Houle, 2008, Figure 2.3a) and find it more closely related to the *melanogaster* subgroup than in other analyses (Prud'homme et al., 2006, Figure 2.3c; Schawaroch 2002, Figure 2.3d) in which it is placed sister to the clade encompassing the *takahashii, suzukii,* and *melanogaster* subgroups.

Relationships Within Subgroups

At the species level both phylogenies resolved relationships within species groups in the same manner. Our analysis supports the paraphyly of the *suzukii* subgroup. Two species, *D. lucipennis* and *D. mimetica* were classified in the *suzukii* subgroup according to genital morphology (Bock 1980). In all studies to date that have included these species *D. mimetica* is sister to the *takahashii* subgroup (Schawaroch 2002; Kopp and True 2002a; Prud'homme et al. 2006; van der Linde and Houle 2008). The same is true for *D. lucipennis*, which is always sister to the *elegans* subgroup (Schawaroch 2002; Kopp and True 2002a; Prud'homme et al. 2006; van der Linde and Houle 2008). Broader sampling within the sister and other closely related subgroups of both of these species, as well as analysis of morphology in addition to genitalia, is needed to determine if *D. lucipennis* or *D. mimetica* are in their own subgroups or are members of the established subgroups; neither belongs with the *suzukii* subgroup. With new species of *Drosophila* yet to be discovered (a new species closely related to *D. rhopoloa* was described less than a year ago, Gompel and Kopp 2018), close relatives of both *D. mimetica* and *D. lucipennis* may exist.

Ancestral Character State of Wing Spots are Uncertain

We are unable to determine the ancestral state of the wing spot with our analysis due to the rapid evolution of the character. We saw a small difference between the two trees in the ancestral estimation for the state of wing spot at the node ancestral to all subgroups, as well as differences at the nodes at the base of each subgroup (see Figures 2.1 and 2.1), but differences did not change estimations at the deeper nodes on the trees and all nodes encompassing the same species were highly correlated. The change in topology did not change the conclusions of the ancestral character states (because no models were preferred over another for either tree and

suggest evolution too rapid to determine character state) or our phylogenetic signal analyses (because no signal suggesting conservation was detected in any of our analyses).

Previous reconstruction suggests that wing spots were ancestral and have been lost in extant lineages as opposed to gained (Prud'homme et al. 2006). One major difference between our analysis and that of Prud'homme et al. (2006) is the placement of *D. ficusphila* (as discussed above). In both of our trees, *D. ficusphila* is placed within the clade encompassing all spotted winged subgroups, while in Prud'homme (2006) *D. ficusphila* is placed outside of all subgroups. This particular topology could change ancestral character estimations. We know that the changes in the placement of *D. ficusphila* do not affect our analysis because estimations were similar at nodes encompassing the same species subgroups, but node estimation may change if *D. ficusphila* is placed outside of all subgroups. We would need to run our analyses on the data of Prud'homme (2006) to identify how the placement of *D. ficusphila* affects ancestral reconstruction outcomes.

Relative branch length affects ancestral character estimations because both Bayesian and Maximum Likelihood methods incorporate branch lengths to make inferences about the rate change for the trait of interest. Our reconstructions may differ from that of Prud'homme et al. (2006) because of differences in relative branch length estimations between the studies. We cannot know for sure if this is the case because branch lengths are not reported in Prud'homme et al. (2006), so we were unable to compare their relative branch lengths to ours. Their study includes the *obscura* group, a more distant group with several spotted, but mostly spotless species. Including the *obscura* group in the analysis and inferring ancestral states using the same model applied over the entire tree most likely lowers rate estimates and raises the confidence of

ancestral character estimates at the nodes ancestral to the *melanogaster* group species because the two groups differ in phenotypic representation.

The differences in phenotyping between our study and that of Prud'homme et al. (2006) may have contributed to the differences in ancestral character estimate. *Drosophila takahashii* is incorrectly phenotyped as spotted by Prud'homme et al. (2006). According to our personal observations and the original species description (Sturtevant 1927) *D. takhahashii* has clear wings and does not perform frontal courtship displays. Prud'homme also phenotypes *D. trilutea* as spotted, but its phenotype cannot be confirmed: Kopp and True (2002b) describe it as spotless, but it is described by Bock and Wheeler (1972, 1980) as having a dimorphic, "dusky" wing. The courtship behavior of *D. trilutea* (*D. takahashii* subgroup) has not been described so we do not know if the species performs frontal wing displays. If one or both of these species is incorrectly phenotyped, the analysis by Prud'homme et al. (2006) could be flawed.

Though we cannot determine if the ancestor to the spotted winged *Drosophila* groups was spotted or spotless, or if spots were subsequently gained or lost, losses of complex traits are generally more likely than gains (reviewed in Wiens et al. 2011) and multiple losses of a complex trait are more plausible than multiple gains because losses can potentially occur in a single step, and gains require multiple coordinated gene products (Prud'homme and Gompel 2007), particularly when paired with simultaneous gain of a behavior pattern (wing display). The genetics of the wing spot are complex, with multiple genes regulating spot production, intensity, and pattern independently (Wittkopp et al. 2002; Gompel et al. 2005; Yeh and True 2014). Although cis-regulatory modifications at *yellow* have produced large changes in Yellow protein expression in the wings of several species, *yellow* alone is not sufficient to create a wing spot (Gompel et al. 2005). Loss of the wing spot occurs with the deletion of ten nucleotides in the

regulatory region of *yellow* in *D. elegans* (Prud'homme et al. 2006), indicating that disruption of gene expression is easier than the gain of the regulatory element. Wing display is also complex: multiple genes regulate the production of wing display, and different genomic regions regulate different elements of the display (Yeh and True 2014). The precise genes regulating wing display have yet to be identified, though *yellow* contributes to wing extension in *D. elegans* (Yeh and True 2014), and therefore wing display could also potentially be lost gene expression disruption. Characterization of the genetic elements implicated in the production of wing spot and wing display, and which elements are ancestral to subgroups of spotted winged *Drosophila*, will elucidate how the wing spot and wing display traits have transitioned over evolutionary time.

Pattern of Wing Spot and Wing Display Implicate Rapid Fluctuations in Sexual Selection

Changes in complex traits, such as courtship displays observed in the spotted winged *Drosophila*, can be a result of direct female preferences (Basolo 1990; Cummings et al. 2007) and may evolve rapidly, leading to a disrupted pattern of evolution and decreased phylogenetic signal (Rendall and Di Fiore 2007). Whether not ancestrally derived and gained, or ancestrally derived and lost, multiple convergences of the wing spot transition have occurred rapidly within groups of closely related species because pairs of closely related species exist in which one has the spot and another does not (e.g. *D. gunguncola* and *D. elegans*: *D. paralutea* and *D. prostipennis*) .

Signatures of Brownian motion will not necessarily inform the relative importance of selection versus drift. Brownian motion is often equated to genetic drift, but traits evolving under multiple selective models can have a signature of Brownian motion (Harmon, 2018). We can only infer from our data what best explains the observed pattern of wing spot in extant species in the context of conservatism vs. convergence, because our results were not significantly different

than expected under Brownian motion. Wing spots are sexually dimorphic characters directly correlated with courtship display behaviors (personal observation, Kopp and True 2002b; Setoguchi et al. 2014) and thus wing display behaviors are likely also rapidly coevolving with morphology between species in the spotted winged subgroups. The pattern of wing spots we observe across the phylogeny is likely the result of rapid fluctuation in sexual selection because drift is unlikely to result in the evolution of two characters involved in courtship displays identically in all extant species.

Low phylogenetic signal and rapid male trait evolution likely indicate rapid shifts in selective regime (Ord and Martins 2010) either from a change in direct female preference for courtship displays or a change in environment affecting courtship signal efficacy. Though evidence for direct female choice sexual selection on wing spots has mixed support (Fuyama 1979; Singh and Chatterjee 1987; Hegde et al. 2005; Roy and Gleason 2019), sexually dimorphic morphologies commonly accompany specific courtship behaviors to enhance courtship displays (e.g. wolf spider leg ornamentation and leg waving, Hebets and Uetz 2000; bird of paradise plumage and courtship dances, Scholes 2008; *Drosophila prolongata* enlarged, pigmented forelegs and leg shaking, Setoguchi et al. 2014). How wing spots potentially enhance wing movements is an avenue of research that has yet to be explored, but the correlation of wing spots and wing movements suggest that they could both function in courtship. Further exploration of differences in female preferences and ecological impact on mating success could elucidate potential causes of shifts in phenotypes between closely related species differing in wing spot phenotype (e.g. *D. elegans* and *D. gunungcola*, or *D. prostipennis* and *D. paralutea*).

We used a simple presence-absence characterization of wing spots (and wing display, because it is always performed in spotted species) for our analysis, but pigmentation does differ

in both intensity and distribution among those with spots. Wing display is also different in each species. Each species moves its wings in different patterns and holds its wings at different angles. In some species, males display at a 45-degree angle, while in others males stand directly in front of females (Yeh 2009; Revadi et al. 2015). Some species also shake their bodies simultaneously as they display (Mazzoni et al. 2013; Yeh and True 2014). To more precisely map both wing spot and wing display, and determine how each element may have evolved, we need to quantify pigmentation in intensity and/or area covered on the wing. Each element of wing display for each species should also be dissected and mapped to identify shared and derived elements of displays to better understand how wing display and wing pigment are coevolving.

Sexual selection theory predicts a coevolution between male sexual ornamentation and female preferences (Fisher 1915; Lande 1980; Andersson 1994). In some spotted species vision is either required for or facilitates mating success (Chapter 1; Roy and Gleason, 2019), but the contribution of vision to mating success has yet to be explored in the non-spotted species of the spotted winged *Drosophila* subgroups. Associations between visual dependency on mating with spotted phenotypes (Chapter 3) may provide support for sexual selection influencing the evolution of sexually dimorphic traits across the spotted winged *Drosophila* clades. The courtship behavior in the species from this study should be examined comparatively to understand the evolutionary mechanisms driving the gains or losses of sexual dimorphisms between and across these species.

Conclusions

We constructed a phylogeny of the spotted winged *Drosophila* subgroups of the *D. melanogaster* group from whole genome sequences, sequencing eight new species, and explored the potential evolutionary avenues of the wing spot character through ancestral character state

and phylogenetic signal analyses. We find evidence that the evolution of the wing spot is very rapid and does not have phylogenetic signal, suggesting that spots are independently gained or lost in extant species. Wing spot and wing display are correlated and support rapid fluctuations in sexual selection in spotted winged *Drosophila* subgroups.

Chapter 3: Comparative assessment of visual dependence and mating outcomes across species varying in dimorphic courtship displays

Abstract

Courtship communication enables information exchange for the identification of suitable mates and contributes to biological fitness and species distinction. Many *Drosophila* species, such as spotted winged *Drosophila*, exhibit novel courtship behaviors, through novel sexual dimorphisms of correlated visual display traits (wing spot and wing display). These species are predicted to be reliant on vision for successful copulation, but little experimental evidence exists. In this study, we tested light dependency across four species subgroups of *Drosophila* that vary in wing spot and wing display by pairing males and females in darkness and observing their behaviors at short (60 minutes) and long (ten days) time points. We uncovered a trend of high dependency of vision in sexually dimorphic species with less dependency in sexually monomorphic species. We also mapped the effect of vision on the phylogeny of the spotted winged subgroups and tested for phylogenetic signal. We found little phylogenetic conservation in courtship behaviors and found male courtship and long-term mating have lower phylogenetic signal than expected under a Brownian motion model. We also tested for associations between the effect of darkness and phenotype and found that dimorphic species and long-term mating suppression were significantly associated. Our results suggest that sexual selection is operating on wing spots and/or wing displays, and that the presence of male-limited dimorphisms in spotted winged *Drosophila* are predictive of reliance on visual courtship signals.

Introduction

Courtship communication behaviors are critical components in prezygotic isolation and species delimitation (Ritchie 2007) because individuals must be able to distinguish between appropriate and inappropriate potential mates to maximize fitness (Mendelson and Shaw 2012). Courtship communication involves highly ritualized and stereotyped behaviors that are often innate and have associated morphologies to make them reliable and conspicuous (Hebets and Papaj 2004; Brown 2014), because signals that allow animals to locate, attract, or appropriately identify mates experience a selective advantage (Zahavi 1987; Endler 1992; Stevens 2013). In many instances, conspicuousness is male-limited, and females choose mates based on courtship displays (e.g. elaborate coloration and calls in birds of paradise, bright coloration and leg movements in peacock spiders, anole head bob and dewlap displays, túngara frog mating calls).

Courtship communication systems are complex, and evolution can determine their composition in many different ways. Sexual selection can drive signal divergence via intersexual preferences (Lande 1980; Kirkpatrick and Ryan 1991), intrasexual competition (Darwin 1871; West-Eberhard 1983), or character displacement (Brown and Wilson 1956; Alexander et al. 1997). The ecological environment of the species may also change the dynamics of signal efficacy, imposing or relaxing natural selection on traits that may change signal efficacy and/or the dynamics of signal preferences (Endler 1992; Ryan and Rand 1993a; Cummings et al. 2007; Ryan and Cummings 2013). Alternatively, diversification could result from stochastic forces such as genetic drift (Boake et al. 2003) or genetic correlations between different elements of a phenotype (Ord and Martins 2010). Furthermore, multiple evolutionary forces could shape signals in parallel (e.g. habitat structure and species recognition on anole dewlap displays, Ord and Martins 2006) or selective forces may oppose one-another (e.g. predation and mate choice

on tail size in guppies. (e.g. predation and mate choice on tail size in guppies, Endler 1984). We can disentangle such complexities by identifying the morphological and behavioral factors that vary among species and employing phylogenetic comparative methods (ancestral character reconstruction, phylogenetic signal estimation, and correlated trait evolution analysis) to infer the probable drivers of trait variation among species (Ord and Martins 2010; Harmon 2018).

Courtship signals, and the behaviors associated with them, may show homoplasy due to their rapid evolution, may not evolve in a stepwise fashion, and may show decreased phylogenetic signal as a consequence (Rendall and Di Fiore 2007). A lack of phylogenetic conservation, therefore, could be indicative of selection (Gleason and Ritchie 1998) but could also result from genetic drift (Harmon 2018). Alternatively, congruence of behavior with molecular phylogenies may be suggestive of either rapid phenotypic drift directing the evolution of lineages along increasingly divergent paths as time increases (Ord et al. 2015), or of stabilizing selection (Rendall and Di Fiore 2007). Selection may be distinguished from drift if convergent phenotypes are associated with fitness components (e.g. predation evasion or mating success). The correlation or disassociation of signaling traits with mating success can allude to the potential role of sexual selection in shaping signal design (Martins 1996; Ryan 1996; Ord and Martins 2010).

Drosophila species are excellent to study sexual selection and mate choice evolution because many species groups exhibit novel courtship behaviors. The spotted winged *Drosophila* species have conspicuous sexual dimorphism, a common correlate of strong sexual selection (Andersson and Simmons 2006). Spotted winged *Drosophila* species are found in several subgroups of the *melanogaster* group, and are noticeably different from other species in the group because males of some of the species have a novel, conspicuous patch of melanization at

the tips of their wings that is paired with a frontal wing display behavior: males orient themselves in front of the female and extend their wings out to the side, waving or flicking them in a species-specific manner (Spieth 1974; Yeh 2009; Mazzoni et al. 2013; Revadi et al. 2015). Males of species lacking wing spots do not perform frontal displays (Chapter 2; Kopp and True 2002b; Prud'homme et al. 2006). Thus, novel wing morphology and their associated behaviors may change the dynamics of courtship communication in these lineages compared to those previously studied.

Species using conspicuous courtship displays, as observed in spotted winged *Drosophila*, are hypothesized to be dependent on vision to mate (Ewing 1983). Vision dependency in *Drosophila* has been determined using a classification system by Grossfield (1971). The system uses insemination rates of pairs left in continuous darkness compared to pairs left on a normal 12:12 light cycle. Class I species are light-independent and do not require light to mate. Class II species are light-facilitated and mate at a higher rate in the light compared to the dark, and Class III species are light-dependent and will not mate in darkness. Two spotted species in the *D. suzukii* subgroup of spotted winged *Drosophila* are light-dependent (Class III, *D. suzukii* and *D. subpulchrella*), and one species is light facilitated (Class II, *D. biarmipes*; Chapter 1, Roy and Gleason 2019). Wing spot presence (and presumably the wing display) is evolving very rapidly, so much so that we cannot identify the ancestral state of wing spots (Chapter 2), thus we do not know if spots have been gained or lost in extant lineages. Multiple independent transitions in spot phenotypes are apparent (Chapter 2). The dimorphic nature of the spots and correlation with stereotyped courtship behaviors (wing displays) suggests that rapid fluctuations in sexual selection are occurring in extant species.

Recent work suggests that the wing spot may not influence female choice as strongly as previously thought, at least in laboratory conditions (Chapter 1, Roy and Gleason 2019), but visual signals could still be mediating courtship behavior and mating success. If visually mediated mating behaviors produce a phylogenetic signal indicative of selection, and if behavioral traits correlate with the wing spot phenotype, we can form better inferences of how visually mediated courtship behaviors contribute to evolution of conspicuous courtship traits across taxa or vice versa. We may also better understand if dimorphic traits may or may not play a role in sexual selection. The spotted winged *Drosophila* species provide a unique opportunity to explore the evolution of visual signaling behavior and sexual dimorphisms using a comparative framework.

We examined visual courtship signaling across four species subgroups of spotted winged *Drosophila* (*takahashii*, *suzukii*, *elegans*, and *rhopoloa*) using detailed observations of both male and female behaviors to elucidate how visually mediated courtship behavior evolves in relation to wing spot morphology. We first described the mating behaviors of all species in the context of visual dependence when paired in the dark and classified them according to Grossfield (1971) to discretely categorize and describe their behavior. We also quantified the effect of darkness on mating behavior according to courtship and copulation outcomes (defined as treatment effect). We used continuous treatment effect values to test for phylogenetic signal of male courtship behavior and female courtship acceptance behavior. We tested for associations between the morphological wing spot character and behavior both along the phylogeny and as independent groups to understand how courtship signals and their associated behaviors might be coevolving. Our results allowed us to identify the potential evolutionary processes driving visually mediated courtship behaviors.

Materials and Methods

Strains and Maintenance

We obtained 11 species from the National *Drosophila* Species Stock Center (Supplementary Table S3.1). *Drosophila suzukii* and *D. subpulchrella* were provided by Dr. Michael Turelli, and *D. gunungcola* was provided by Dr. Patricia Wittkopp. Cultures of 20-50 adults were established in 25 mm x 95 mm vials containing Bloomington standard formula Drosophila food (corn syrup, cornmeal, soy flour, yeast, and agar), and maintained at 23-25°C with a 12:12 hour light:dark cycle. The light-dark assays for *D. subpulchrella*, *D. suzukii*, and *D. biarmipes* are published in Roy and Gleason (2019). All data for other species are new to this study.

Virgin flies were collected under mild CO_2 anesthesia within four hours of eclosion. Females were housed in groups of five and males were housed individually in small food vials (16.5 mm x 95mm) plugged with cotton. All individuals were checked after 48 h and only males without wing defects and, for spotted specimens, with two well-formed spots, were used in trials. Flies were aged 3–7 days before all trials, and all mating trials took place within four hours of lights on.

Light-Dark Assays

To determine the effect of vision on mating success, we followed the procedure of Roy and Gleason (2019). Briefly, we placed a single virgin male and virgin female of a species together in small food vials in either the light or the dark. For 60 minutes after introduction, pairs were observed either in white light or in the dark under red light (because they cannot see in red light (Hanai et al. 2008)). For each pair, copulation success, courtship latency (the time from the introduction of male and female until the initiation of male courtship), courtship duration (the time from male courtship initiation to copulation acceptance) and copulation duration (the time

from copulation acceptance until the end of copulation) were measured. After the 60-minute observation period, flies in the dark condition were kept in continuous darkness for ten days (even if observed to mate in the first 60 minutes). Pairs from the light condition were kept in a normal 12:12 LD cycle if they had not yet mated. The pairs that mated in the light were split randomly: half were placed in the dark to test if darkness inhibits egg laying behavior. The other half were left in a normal 12:12 LD cycle. 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 mating had occurred.

Data Analysis for Behavioral Observations

For the initial 60-minute assay, we measured courtship frequency, courtship latency, copulation frequency, courtship latency, and copulation duration. We calculated long-term mating success as the proportion of total vials containing larvae after ten days (ten-day copulation frequency). Vials of pairs who had mated in the light during the 60-minute observation but were moved to the dark to lay eggs were included in the light dataset (for sample numbers, see Supplementary Table S3.4-S3.6).

All data analysis was performed in R Studio v.3.5.3 (R Core Team; http://r-project.org/). The number of individuals courting or pairs copulating for the two treatments (light and dark) were compared for significant differences using a Fisher's exact test. Timing data were compared between the two treatments using a Student's t-test.

Phenotyping

To determine the level of dependence on light for mating in a way comparable to those previously studied, we binned our results discretely using the classification system from Grossfield (1971) and used the three class types as the score for the species. We modified the system to include all of our data to 1) determine if males vs females were mediating any

Table 3.1 Measurements used for classification of visual reliance

	Male	Female	Joint	Grossfield (1971)[1]
Class I	No significant difference in both courtship frequency and courtship latency between treatments	No significant difference in copulation latency and/or courtship duration between treatments	Neither sex had lower copulation success in the dark.	No significant difference in larval presence after 10 days
Class II	Significantly lower courtship frequency or longer courtship latency in dark treatment	Significantly lower copulation frequency after 10 days, or longer courtship duration in dark treatment	Significant decrease in at least one sex, but no elimination of copulation in either sex.	Significantly fewer vials with larvae after 10 days in dark treatment
Class III	Males do not court females in the dark	No copulation after 10 days	Complete elimination of mating in copulation frequency at 60 minutes and 10 days	No larvae in any vials after 10 days in dark treatment

[1]Grossfield (1971) used insemination rates by dissecting females. Our methods were modified to check for larvae.

repression of behavior (e.g. lack of courtship would infer male mediated mating outcomes) and 2) consider timing data in the initial observation period (summarized in Supplementary Table S3.2). When mating was eliminated in the dark, the species was scored Class III (light dependent). When mating was only partially eliminated, but courtship frequency, courtship latency copulation frequency, or courtship duration were significantly decreased in the dark, the species was scored Class II (light facultative). When mating was unchanged in darkness, the species was scored Class I (light independent). The classification system used in this study is summarized in Table 3.1.

Character Traits and Mapping

The frequency data (courtship, copulation after 60 minutes, and copulation after 10 days) were used to estimate the effect of darkness on courtship and copulation (treatment effect). Not all pairs mated in the control treatment, so the treatment effect is expressed as:

$$Treatment\ effect = \ log_2 \frac{proportion\ of\ successful\ outcomes\ in\ treatment}{proportion\ of\ successful\ outcomes\ in\ control}$$

The log of zero is undefined, so in instances when the frequency of the behavior was zero, we replaced it with 0.01. By taking a $\log_2$ of the ratio of the proportions, the treatment effect is normalized to represent the change between the number of pairs that performed in the light and the number of pairs that performed in the dark. A value of zero means that pairs in the light and dark performed the behavior at the same frequency. A negative value means that darkness hindered courtship or copulation frequency, and a positive value means courtship or copulation frequency increased in darkness. Treatment effect values were designated as tip states onto a maximum likelihood consensus phylogeny constructed from whole genome short reads aligned to *Drosophila suzukii* (Chapter 2). The tree was pruned to include only the species for which behavioral data was collected. The tree file (Nexus and Newick) will be deposited in Dryad.

Phylogenetic Signal Analysis

We used Blomberg's K to determine if behavioral outcomes produce phylogenetic signal against the stochastic Brownian motion (BM) model, enabling us to detect if behavioral outcomes are likely a result of phylogenetic conservatism or not (Blomberg et al. 2003). To infer conservatism *vs*. lability, we implemented a model simulation to compare likelihood values between the observed data and 1,000 simulations of the data under the BM assumption (Revell et al. 2008). A K value of 1 indicates that our data are consistent with Brownian motion. K values significantly lower than 1 indicate low phylogenetic signal, and K values significantly higher than 1 indicate strong phylogenetic signal.

Analysis of Phenotype Associations

To determine if suppression of behavior in darkness differed by morphological type, we split our species into two groups, spotted and spotless. Closely related species are more likely to share traits, so we considered the association of wing spot and treatment effect considering

evolutionary history. We used phylogenetic ANOVA and using the phylANOVA function in phytools (Revell 2012) and phylogenetic logistic regression (LR) using the phylolm function (Ho et al. 2018) to assess the phylogenetic independence of trait association. The phylogenetic ANOVA analysis assumes Brownian motion only, but the LR analysis allows for multiple models including Brownian motion (assumes that traits evolve stochastically), the Ornstein-Ulenbeck Process (assumes that traits evolve towards an optimum), and Pagel's Lambda (assumes that traits evolve towards an optimum given the data). We ran all data with each model and assessed model fit using Akaike Information Criterion (AIC) values, because they indicate the relative quality of statistical models given our data. The AIC values were similar between models, and the results from each model followed the same pattern given each data type (Supplementary Table S3.3), so we present our data modeled under Brownian motion to maintain consistency of our analysis (because Blomberg's K uses Brownian motion as the model and spot signal was previously assessed against Brownian motion).

Results

Light-Dark Assays and Phenotypic Classifications

We described the behaviors and mating outcomes in darkness as compared to light in each species to identify major differences in visual reliance on mating success between spotted and spotless species. The classification system, modified from Grossfield (1971), allows us to identify gross patterns in visual reliance in our results using information about female-mediated behaviors (measured by copulation frequency, courtship latency, and ten-day copulation frequency), male-mediated behaviors (measured by courtship frequency and latency), and males and females together (considering all behaviors, Table 3.1).

There are eight mismatches between male and female class types; in six the female behavior is more light-dependent than is male behavior. By our joint classification method, six of the 13 species are classified as light facilitated rather than as light independent if they would be classified with the Grossfield (1971) method based on larval production after ten days (Table 3.2). Five of these species exhibit reduction in mating success mediated by female behavior. The exception is *D. mimetica* (a spotless species), for which males are more affected than females by darkness, courting significantly less often in the dark compared to the light (decreased from 100% in the light to 65% in the dark, Supplementary Table 3.4). When male *D. mimetica* court, females accept them at the same frequency in darkness as in light (Table 3.2, Supplementary Table S3.4).

All spotted species experience a reduction in mating success in the dark, with all but one species (*D. biarmipes*) experiencing reduced copulation frequency in either the first 60 minutes or after ten days in the dark. In three of the eight spotted species, copulation is eliminated completely in the dark. All spotless species can copulate in the dark in the first 60 minutes, though some do so at a reduced frequency. Darkness does not significantly reduce copulation frequency after ten days in any spotless species (Table 3.2).

Effect of Darkness on Behavioral Outcomes

We can use the proportion of successful behavioral outcomes in the treatment and control groups to estimate the effect of darkness. The effect of treatment on the suppression of behavioral traits is variable (Table 3.3). The treatment effect on courtship frequency ranges from the most suppression (-1.97) in *D. prostipennis* to a small increase (0.01) in *D. suzukii*. The range of treatment effects on copulation frequency during the initial 60-minute observation period was wider, from -6.64 in *D. paralutea* to 0.03 in *D. mimetica* (Table 3.3). Copulation frequency after

10 days has a similar range, but the extremes of the observations were *D. suzukii* (-6.58) and *D. paralutea* (0.11). All the positive treatment effects were very small, with non-significant differences in behavior between the light and dark treatments (Table 3.3).

Table 3.2 Significance of tests comparing pairs in light and dark[1]

	Frequency of occurrence[2]		Timing data[3]			Copulation Frequency after 10 Days[4]	Darkness effect class[5]			
Species	Courtship	Copulation	Courtship latency	Courtship duration	Copulation duration[6]	Larvae Presence	Female	Male	Joint	Grossfield Method
Spotted species										
D. biarmipes	NS	NS	Longer**	Longer***	NS	NS	II	II	II	I
D. elegans	Less**	Less***	Longer***	-	-	Less***	II	II	II	II
D. fuyamai	NS	Less*	NS	-	-	NS	II	I	II	I
D. lutescens	Less*	Less***	Longer*	-	-	Less***	II	II	II	II
D. prostipennis	Less***	None***	-	-	-	No***	III	II	III	III
D. pseudotakahashii	NS	NS	NS	-	-	Less***	II	I	II	II
D. subpulchrella	NS	None***	Longer***	-	-	No***	III	II	III	III
D. suzukii	NS	None***	NS	-	-	No***	III	I	III	III
Spotless species										
D. gunungcola	NS	Less**	NS	-		NS	II	I	II	I
D. lucipennis	NS	None***	Longer***	-	-	NS	II	II	II	I
D. mimetica	Less*	NS	Longer***	NS	NS	NS	I	II	II	I
D. paralutea	Less*	None*	NS	-	-	NS	II	II	II	I
D. takahashii	NS	NS	NS	NS	Shorter*	NS	I	I	I	I

[1]Summary statistics for each treatment, including sample sizes, are given in Supplementary Table 3.4-3.6

[2]Significance of Fisher's exact tests comparing the frequency of the event in the dark with that in the light within 60 minutes, thus "less" indicates that the number of pairs performing in the dark is less than the number performing in the light. A dash indicates that there was not enough data for a comparison because control pairs, treatment pairs, or both control and treatment pairs did not copulate at high enough frequency (fewer than ten pairs).

*P < 0.05; ** P < 0.01; *** P < 0.001

[3]Significance of Student's t-test comparing timing data between pairs in the dark and pairs in the light, thus "longer" indicates that the time for the trait was longer in the dark than in the light, and "shorter" means that the duration was shorter in the dark than in the light. Dashes and asterisks as above indicate fewer than ten pairs performing the behavior in either the control group, treatment group, or both groups to perform statistical analysis.

[4]Significance of Fisher's exact tests comparing the frequency of larval presence between dark and light:dark incubated pairs for 10 days.

[5]Species were classified based on Grossfield (1971) I, light independent; II, light facilitated; III, light dependent. Classification criteria are explained in the Materials and Methods and Supplementary Table 3.2. Classification was also determined based on the original method (Grossfield 1971) using only insemination rates (copulation frequency after 10 days). Shaded cells indicate where the Grossfield classification differs from the joint classification.

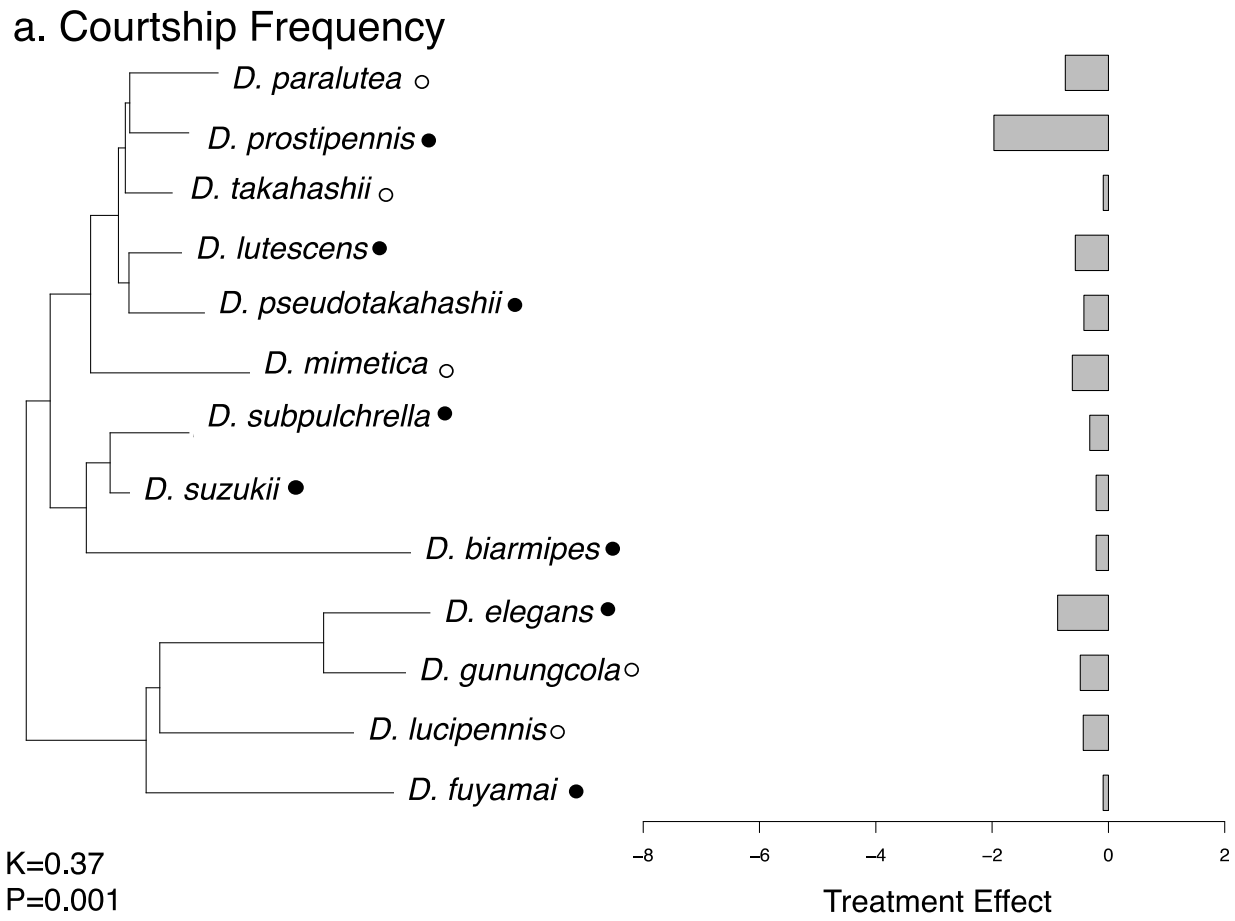
Table 3.3 Treatment effect on behavioral outcomes

Species	Treatment Effect: Courtship Frequency[1]	Treatment Effect: Copulation Frequency	Treatment Effect: Copulation Frequency (10 Days)
Spotted Species			
D. biarmipes	-0.2123036	-0.19264	-0.043943
D. elegans	-0.8744691	-3.70044	-4.392317
D. fuyamai	-0.0901978	-1.62803	-0.796466
D. lutescens	-0.569855	-2.42884	-2.584963
D. prostipennis	-1.968291	NA[2]	-6.491853
D. pseudotakahashii	-0.4218267	0.00000[3]	-1.981853
D. subpulchrella	-0.3219281	-6.24792	-5.044394
D. suzukii	0.017277	-6.18982	-6.584963
Spotless Species			
D. gunungcola	-0.4854268	-2.18220	-0.344648
D. lucipennis	-0.4329594	-6.08746	-0.070389
D. mimetica	-0.6214884	0.03136	-0.185866
D. paralutea	-0.742503	-6.64385	0.112474
D. takahashii	-0.089267	-0.18442	-0.095157

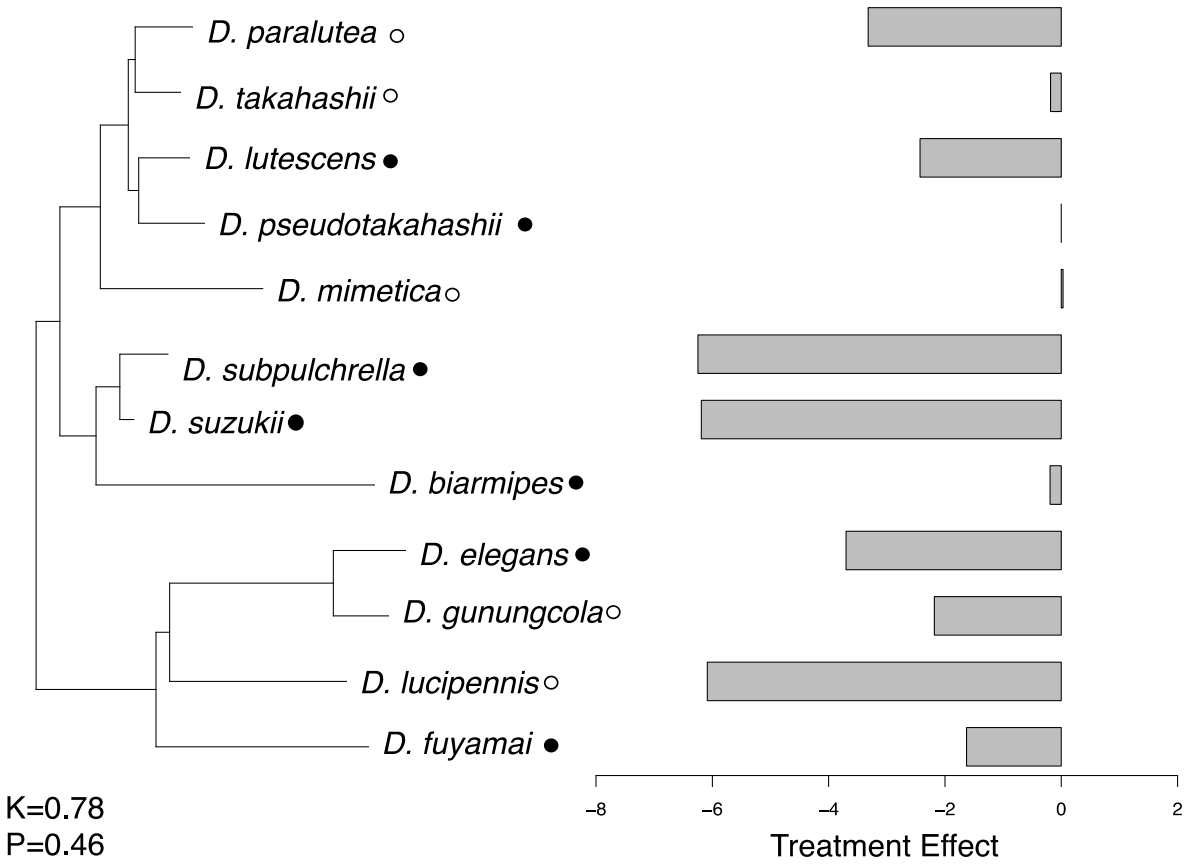
[1] Treatment effect was measured by dividing the proportion of successful outcomes in the treatment divided by the successful outcomes in the control $\log_2$ transformed for normalization.
[2] *D. prostipennis* could not be assessed because no pairs in control or treatment groups mated in the first 60 minutes
[3] Zero effect for *D. pseudotakahashii* was the result of the proportion of pairs in control and treatment groups mating at equal frequencies in the first 60 minutes.

Phylogenetic Signal of Behavior and Association of Morphology and Behavior Phenotypes

The effect of darkness on courtship frequencies and copulation frequencies at both 60-minutes and ten days were mapped on the species tree (Chapter 2) and tested against a stochastic Brownian motion null model to understand the evolutionary processes that have shaped the observed behaviors. Copulation frequency in the first 60 minutes does not have phylogenetic signal statistically different from stochastic change (K=0.78, *P*= 0.45, Figure 3.1b). Courtship frequency


a. Courtship Frequency
D. paralutea ○
D. prostipennis ●
D. takahashii ○
D. lutescens ●
D. pseudotakahashii ●
D. mimetica ○
D. subpulchrella ●
D. suzukii ●
D. biarmipes ●
D. elegans ●
D. gunungcola ○
D. lucipennis ○
D. fuyamai ●
K=0.37
P=0.001
−8
−6
−4
−2
0
2
Treatment Effect

b. Copulation Frequency (60 Minutes)



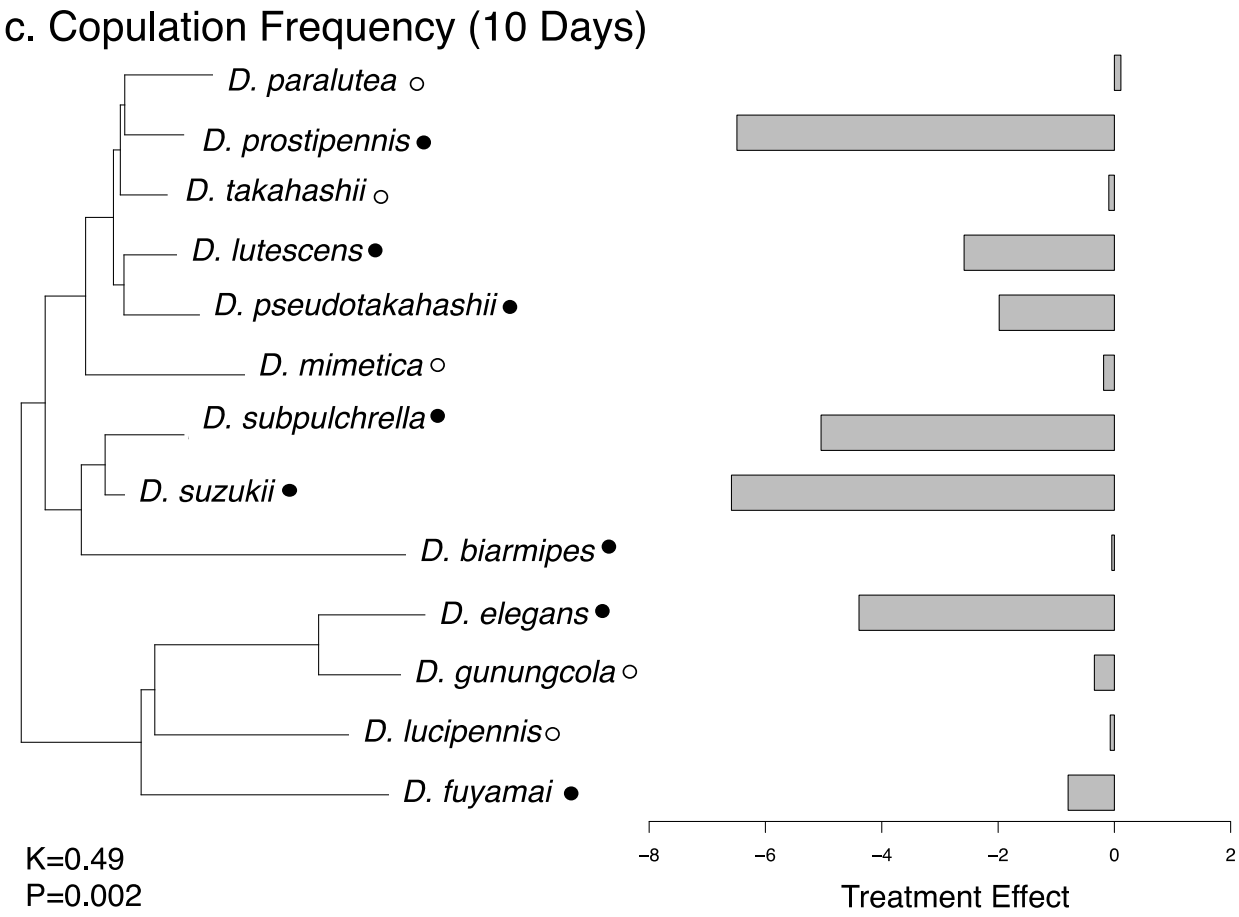


Figure 3.1 Treatment effect plotted with evolutionary history. The phylogenetic signals of treatment effect for a) courtship frequency; b) copulation frequency after 60 minutes; and c) copulation frequency after ten days, were determined using Blomberg's K in phytools (based on 1000 simulations under a Brownian motion model) and then plotted next to the best fit RAxMLphylogeny (Chapter 2) to visualize patterns of treatment effect on each courtship parameter. The tree was derived from whole genome sequence data aligned to *D. suzukii* and pruned to include only species from which behavioral data was taken. In panel b) *D. prostipennis* was removed because the controls did not mate and thus there were no data. A treatment effect of zero indicates no difference between the light and dark treatments. The more negative a value is, the larger the suppressive effect of darkness on the mating parameter. Open and closed circles next to species names represent spotless and spotted species, respectively. Blomberg's K values and the associated P value representing difference of observed values from the model are in the upper left corner of each bar plot. Courtship frequency signal is significantly lower than signal from a stochastic process of evolution. Copulation frequency in the first 60 minutes is not significantly different from a stochastic process of evolution. Copulation frequency after ten days is significantly lower than signal from a stochastic process of evolution.

and copulation frequency after ten days have significantly lower signal than expected from a Brownian motion model (K=0.37, *P*= 0.001 and K=0.49, *P*= 0.002, respectively, Figure 3.1a and 3.1c). ANOVA and LR analyses reveal a significant association between suppression of mating after ten days in darkness for those with spots and low suppression of mating after ten days in darkness for spotless species (Table 3.4). No associations between spot type and suppression of courtship or copulation acceptance in the first 60 minutes were found.

Table 3.4: Association between morphology and behavior adjusted for evolutionary history

Mating Parameter	phlANOVA[1]	LR[2]
Courtship Frequency	0.698	0.119
Copulation Frequency (60 min)	0.697	0.454
Copulation Frequency (10 days)	**0.006**	**0.002**

[1]*P* values from the phylogenetic ANOVA analysis for each behavior parameter. Bolded numbers are statistically significant.
[2]*P* values from the phylogenetic logistic regression under a Brownian motion model. Bolded numbers are statistically significant.

Discussion

Long Term Mating Success Data Infers Selection

Suppression of long-term mating success (reflected by the ten-day copulation frequency data) is associated with dimorphic species. While correlation does not always imply causation, our results in combination with phylogenetic signal analysis suggest that the associations are not arbitrary. Long-term mating success also has significantly lower phylogenetic signal than expected under a stochastic model of evolution, which can be attributed to rapid changes in within-clade variation (explained by the small Blomberg's K value). Convergent trait evolution is often indicative of a deterministic process as opposed to drift (Losos 2010). The phylogenetic signal analysis of long-term mating success is consistent with that of the dimorphic male phenotypes (wing spot and wing display, Chapter 2), thus consistent with the hypothesis that changes in sexual dimorphisms along the spotted winged *Drosophila* evolutionary history are likely a result of rapid fluctuations in sexual selection.

The phylogenetic correlation of traits can be indicative of their function (Ord and Martins 2010). In almost all spotted species, darkness completely eliminates mating success, suggesting that visual cues play a major role in courtship and mating success in species with conspicuous display traits. Mating behaviors are complex because they can be condition dependent, multimodal, and can vary in attractiveness due to receiver preferences (Andersson 1994; Stevens 2013). Considering these complexities, we still find a significant association between mating success in the context of visual reliance and conspicuous sexual dimorphisms. The significant association between spot phenotype and the suppression of long-term mating success in darkness suggests female choice on either the wing spot, the frontal wing display, or both. Because wing spots, wing displays, and mating success are all correlated along the phylogeny, it is highly unlikely that drift is the reason for the observed patterns of sexual dimorphisms across extant species.

If females have a strong preference for particular visual signals, only males who produce the correct visual displays during courtship will mate successfully. Assuming that both the production of visual signals and the preference for visual signals are genetically determined, they will become genetically linked over successive generations and will be strongly associated with one another throughout the population in a "runaway" process (Fisher 1930). A visible wing spot linked to visual courtship displays and mating success suggests that male traits and female preference for those traits are coevolving. The genetic basis of the wing spot is known for several species within the spotted winged *Drosophila* (Gompel et al. 2005; Prud'homme et al. 2006; Yeh et al. 2006; Yeh 2009; Yeh and True 2014), and regulation of wing spot and wing display have some shared genetic underpinnings in *D. elegans* (Yeh et al. 2006; Yeh and True 2014) but additional independent loci are associated with either wing spot or wing display (Yeh and True

2014). The elements of visual courtship displays that females find particularly attractive and the genetic basis of those preferences are not known for any spotted winged *Drosophila*. Further work exploring which genotypes are preferred by females, and if alteration of those genotypes in females affects their preferences could test the hypothesis that traits and preferences are coevolving via runaway sexual selection.

Visual courtship elements may also result from direct selection if elements of visual displays are direct indicators of fitness (Andersson 1994). Although we characterize all pigmented flies as spotted or spotless because we are interested in a sexual dimorphic wing phenotype vs. a sexually monomorphic wing phenotype, we recognize that pigment intensity and distribution varies among and within species (Wittkopp et al. 2003). Further examination of courtship signaling among those species with male-limited dimorphisms (particularly the level of pigmentation and the differences in wing display elements) should explore a quantitative difference between wing spots of species (area covered, intensity, or both). By quantifying wing traits we may identify variation in wing spots and wing displays and then comparatively assess their associations with fitness outcomes (e.g. longevity, viability, desiccation resistance, thermal tolerance) and test hypotheses that particular elements of visual displays are honest signals reflecting male fitness.

Variation in Copulation Success in the First 60 Minutes

We found no association between visual reliance on mating and copulation acceptance within the first 60 minutes of courtship, potentially because the analysis was only based on females who were actively courted by males in the first 60-minutes. Some males did not initiate courtship until after the initial observation period in some species because more females produced larvae after 10 days than were initially courted. The elimination of any single sense

may disorient the normal mating process for a period of time before the receiver detects a "backup" or redundant signal (Hebets and Papaj 2004). Because vision is one of the first modalities employed during *Drosophila* courtship (Greenspan and Ferveur 2000), the courtship sequence may be interrupted even in species that do not require vision to successfully mate. One way to resolve the issue of adjustment periods to the dark environment would be to watch the mating pairs for a longer period of time, or place individuals in the dark for a longer period of time before trials.

Additionally, some mating success in the initial observation period may not be attributed to female acceptance. Males sometimes achieve mating success despite female resistance though aggression and coercion. We have no way to differentiate or measure vigorous courtship followed by female acceptance versus male coercion/forced copulations because female acceptance behaviors are not well studied and are often difficult to observe with the naked eye (e.g. spreading of anal plates, Greenspan and Ferveur 2000). We observed that males of some species were more aggressive in pursuing females than others, and that some females appeared unable to forcefully repel males. For example, female *D. suzukii* actively rejected male copulation attempts by kicking at them if they attempted to mount. Female *D. lutescens*, however, appeared unsuccessful at dislodging males a few times because they would shake and kick at mounted males, but were unable to dismount them, which is likely why two males mated successfully in the dark.

Vision and Male Courtship Initiation

The phylogenetic signal of male courtship frequency is significantly lower than predicted by Brownian motion, but the results need to be interpreted differently than long-term mating success. We did not expect darkness to suppress male courtship, because no suppression was

observed in earlier experiments on three spotted species (Chapter 1, Roy and Gleason 2019). The largest effect of treatment is relatively small, meaning the overall effect of darkness on courtship initiation is relatively small. The initiation of male courtship behaviors as facilitated by different signal modalities in spotted winged *Drosophila* have not been studied, but males likely receive the necessary signals to initiate mating in darkness, implying that those signals are not visual. We observed that when males did not court, both the male and the female were mostly motionless for the entire duration of the trial. We did not quantify overall movement of individuals in this study, but low general activity of certain species and high general activity noted in others could explain differentiation in courtship frequencies between species.

Consideration of Behavior to Classify Light Dependency

We are the first to examine *Drosophila* light-dependence with direct behavioral observations. Until now, light-dependence was described using insemination frequency, a system developed by Grossfield (1971). Studies comparing light dependency in *Drosophila* (Markow and O'Grady 2005b; Jezovit et al. 2017) use the Grossfield (1971) system of classification and do not account for courtship behavior. Comparative methods can find trends in behavioral data, but depending on the model, the inferences of evolutionary process are stronger if backed up by details of how the organisms are behaving. Signatures of Brownian motion are often equated to random genetic drift, but similar patterns can arise by selection (Harmon 2018). Differences in male and female behaviors in response to the elimination of vision within a species may change their classification and also allow us to hypothesize sources of behavioral isolation by identifying which sex mediates behavioral outcomes. Without direct observation of both male and female behaviors in courtship signal studies, the identification of how courtship or copulation behavior is breaking down, or which sex requires a particular signal for normal mating, is impossible. By

classifying each species separately by sex and then jointly, we are able to understand the potential dynamics that contribute to overall courtship outcomes mapped to the phylogeny for comparative analyses.

One discrepancy between our classification system and that of Grossfield (1971) highlights the importance of direct behavioral observations. We describe *Drosophila mimetica,* a spotless species, as Class II by our system where as it would be Class I by the Grossfield (1971) classification. In general, female behaviors were more suppressed in darkness than male behaviors, except for those of *D. mimetia*. Courtship frequency was significantly reduced during the initial observation period, but almost all females who were courted in darkness during that time accepted mating. After ten days, most pairs of *D. mimetica* had mated in the dark, indicating that males may have used other sensory signals to locate and successfully court females and that females receive enough information without vision to accept mating. In many *Drosophila* species, males receive visual signals from females required to initiate courtship, and some males will not attempt to court or mount unless they receive specific signals from a female (Markow and O'Grady 2005b). *Drosophila nebulosa* (*willistoni* group) males perform a wing waving behavior (thought to waft pheromones) and experience a complete elimination of mating in the dark. Manual blinding of each sex reveal that males depend on vision to initiate courtship while females mate normally without vision (Gleason et al. 2012). Males therefore may be light dependent in this species.

Drosophila prostipennis is described as a Class III by our system, which would be in agreement with the Grossfield (1971) system. Both male and female mediated behaviors contribute to the lack of mating, because only 23% of males courted in the first 60 minutes, and no mating was observed in darkness after ten days. Because *D. prostipennis* is a spotted species,

orientation requiring vision may be a critical step in courtship initiation; lack of proper visual cues for orientation to perform displays could explain the dramatic decrease in courtship frequency in the dark. Females were unresponsive to courtship and did not accept mating because they too require visual stimulation to accept mating, a result consistent with most spotted species.

Only two species from this study, *D. takahashii* and *D. suzukii*, were described in the original Grossfield (1971) study. Grossfield (1971) characterizes *D. takahashii* as light-facilitated (Class II) based on the observations of Spieth and Hsu (1950). The original study by Spieth and Hsu (1950) showed that *D. takahashii* mated in the dark (at the same rate as in the light) when females checked for insemination were alive at the time of data collection (light independent, Class I), but Grossfield (1971) based their characterization on the total data reported, which did not account for female mortality. Our classification of *D. takahashii* as a Class I species supports the original interpretation of Spieth and Hsu (1950). Our classification of *D. suzukii* as Class III is consistent with the classification of Grossfield (1971), originally described by Manning (1965).

Drosophila biarmipes as Insight for Potential Modality Shifts

We expect species with novel wing spots and frontal courtship display behavior will show primacy in visual signaling for courtship communication. However, *Drosophila* courtship communication is multi-modal and female preferences for one modality over another may change. *Drosophila biarmipes*, a spotted species, mates in the dark at frequencies no different from the light, though males have to court females significantly longer in the dark than in the light to achieve mating success. Visual cues may stimulate *D. biarmipes* females, but other signals may be used to make mating decisions in the absence of vision. Unlike *D. suzukii*, *D.*

biarmipes produces a multi-component song during courtship (Yeh 2009; Mazzoni et al. 2013). Auditory communication via male singing is species-specific and necessary for mating acceptance in some species of *Drosophila,* but variation in modality use exists between related species (Markow 1987; Ritchie et al. 1999; Klappert et al. 2007; Gleason et al. 2012; Veltsos et al. 2012; Dyer et al. 2014; Colyott et al. 2016). If a shift in female preference for male signals in *D. biarmipes* exists, the change in signal preference could explain why visual signals contribute less to mating success in *D. biarmipes* as compared to the other spotted species. In the future, *D. biarmipes* males may lose traits associated with visual signaling if they are costly. The interplay of modalities in all of the spotted winged *Drosophila* should be explored to better understand the role of vision in courtship communication and the potential direction of trait transition (gain vs. loss) and shifts in modality use.

Geography and Interacting Selective Forces

Geographical ranges and light dependent mating have an apparent association with in *Drosophila* (Jezovit et al. 2017). Most cosmopolitan species are Class I (light-independent), while most tropical species are Class II (light facilitated) or Class III (light-dependent, (Jezovit et al. 2017). Relaxed abiotic selective pressures and an increase in relative density in tropical regions are hypothesized to increase sexual selection (primarily due to increased population densities), resulting in more elaborate and conspicuous visual displays (Jezovit et al. 2017). Some spotted winged *Drosophila* distributions are known, placing them primarily in tropical and subtropical regions with a wide range of available breeding sights that might relax natural selection (Markow and O'Grady 2005a; Markow and O'Grady 2008). However, we observe variation in behaviors related to visual signaling within groups sharing ecotypes and covering similar ranges. If species ranges are mostly allopatric but have sympatric zones, and the species

have different wing phenotypes as reported for some spotted winged *Drosophila* (e.g. *D. lutescens* and *D. takahashii*, Fukatami 1984), transitions in phenotypes could be a result of character displacement. Complex species interaction effects beyond range-based geography need to be considered (e.g. allopatry and sympatry, altitudinal differences, diurnal activity, and micro-habitat preferences), and more detailed studies on the ecology of *Drosophila* are needed.

Conclusions

We present the first study of responses to visual courtship signals of the spotted winged *Drosophila* in a phylogenetic, comparative framework. Our results identify a general association between long-term mating success and sexual dimorphisms (wing spots and wing displays). The suppression of mating in the dark (long-term) also behaves evolutionarily similar to male dimorphisms in that it is not conserved. The association between mating success and male dimorphisms is strong indication that changes in phenotypes among the spotted winged *Drosophila* are a result of sexual selection. We observe one spotted species, *D. biarmipes*, to be an exception because it mates in the dark, thus the effect of other signaling modalities on mating success should be tested. Reliance on visual signals for mating success is associated with the presence of wing spots and wing displays, but recent work suggests that the wing spot may not significantly influence female choice. Quantification of wing displays, wing spots, and an examination of the ecological aspects of visual efficacy have not yet been explored. For these reasons, the signals that influence female mating decisions within and between species remains unclear. A dissection of the function of the wing display elements in female mate choice will clarify the role of wing display in courtship communication. Additionally, further exploration of *Drosophila* ecology and additional sensory systems that influence mating will enrich this data set

to paint a picture of the complexity of visual signaling and its influence on sexual isolation and species delimitation.

Chapter 4: Connecting the spots: alternative considerations for the presence of conspicuous dimorphisms

Introduction

The remarkable diversity of morphological traits in nature has long inspired questions about how and why traits emerge and differentiate along particular lineages, and what underlying mechanisms operate to build complex phenotypes (reviewed in Pigliucci and Preston 2004). Many complex traits involve both morphological and behavioral components (West-Eberhard 2003), each responding to various selective pressures. My doctoral work explores visual courtship signals in both functional and phylogenetic contexts to address how visually mediated courtship behaviors and female choice sexual selection influence morphological and behavioral phenotypes and vice versa.

We cannot determine if wing spot morphology or wing display behavior traits have been gained or lost over time, or if shifts in courtship behaviors precede morphological transitions along the evolutionary history due to the rapid and labile nature of trait evolution. The lability and rapid evolution of courtship characters in spotted winged *Drosophila* are not surprising considering that courtship signaling is complex, involves the sharing of information among multiple individuals across changing environments, and can involve multiple modalities (de Queiroz and Wimberger 1993; Martins et al. 2004).

The most intriguing aspect of my work is that visually mediated courtship behavior in species with wing spots supports sexual selection as a mechanism for the evolution of male sexual dimorphisms (wing spot and wing display), but the wing spot itself may not be preferred by females. All spotted species need preference tests to confirm our findings to generalize to all spotted winged *Drosophila*; however, the data from the *D. suzukii* group provides support against direct female preference on wing spots. The question then becomes, ‘what function does

the spot have, if any?' In this chapter, I detail considerations for the function and maintenance of wing spots alternative to direct female preference.

Stuck on You: Wing Spot and Courtship Display Are Linked Genetically

The wing spot trait may be maintained because it shares a genetic basis with the wing display. The genetic mechanisms responsible for differentiation of wing pattern in some spotted species are well known (Wittkopp et al. 2002; Gompel et al. 2005; Prud'homme et al. 2006; Arnoult et al. 2013). Wing spots are a result of the modification of cis-regulatory and trans-acting elements co-opted from highly conserved genes that regulate wing shape and venation pattern (Gompel et al. 2005). The gene *yellow*, known for its role in body pigmentation regulation, is globally expressed in all *D. melanogaster* and *D. biarmipes* females (Wittkopp et al. 2002). Changes in the regulatory elements of *yellow* and *ebony* produce discrete, tissue specific expression in male *Drosophila biarmipes* wings (Wittkopp et al. 2002; Gompel et al. 2005). Species-specific melanization patterns are a result of differential modification of multiple sites in the wing spot regulatory elements in conjunction with the co-option of cis-regulatory elements in *yellow* (Wittkopp et al. 2003; Gompel et al. 2005; Prud'homme et al. 2006; Simpson 2007). The identified contributors to pigment patterns (both body and wing) in addition to *yellow* are *ebony tan* (reviewed in Wittkopp et al. 2003; Simpson 2007; Wittkopp and Beldade 2009; Massey and Wittkopp 2016) but in *D. elegans ebony* and *tan* could not be implicated in spot regulation (Yeh and True 2014). There are multiple genes acting to regulate wing spot, and they may be different depending on the species.

Less is understood about the genetics of wing display behavior, but backcross studies reveal that wing spot and frontal courtship displays are both polygenic and linked because hybrids of *D. elegans* (spotted and performs courtship displays) and *D. gunungcola* (spotless and

does not perform displays) do not fully extend their wings in displays, only extend one wing at a time, and perform fewer displays (Yeh et al. 2006). Quantitative trait loci (QTL) research implicates *yellow* as a component for male courtship display differences between *D. elegans* and *D. gunungcola*, confirming that frontal courtship behaviors and wing spot share genetic architecture, but the genetics of display behaviors is complex (Yeh and True 2014). Seven QTLs are associated with courtship displays, and different elements of frontal display behavior (circling, body shaking, and wing extension) have different genetic architectures (Yeh and True 2014). The components of courtship display on which females base mating decisions could be those that share genetic architecture with wing spots, and even if wing spots are not being chosen by females, they could be maintained because of the selection on the display trait being controlled by the same genes. Identifying loci that contribute to specific visual elements of courtship traits preferred by females allows us to identify any genetic overlap between preferred traits with wing spots and could explain how wing spots could be maintained as a result of female preferences for linked courtship display behaviors.

Shifting Spots: Signaling in a Complicated World

Spot contribution to signaling may be context dependent. In a noisy environment, wing spots may be more important than in the laboratory environment. Signals have two components: strategy and efficacy (Stevens 2013). The strategic elements are those that convey the actual content or message to be extracted by the receiver (e.g. “I am toxic”, “I want to mate with you”), while efficacy refers to how the signal is constructed over evolution to influence the response of the receiver most effectively (e.g. “I will kill you if you eat me” versus “I WILL KILL YOU IF YOU EAT ME”). Light-dark experiments (Chapter 3) suggest that visual signals from males convey a message that they are a ready and/or suitable mate for the female. The wing spot,

however, may not be a part of strategic elements of the visual signal because females do not choose mates based on the presence or absence of the spot in the three species I observed. Conspicuous traits sometimes act as amplifiers of other signals to increase the efficacy of a signal without themselves being used directly for mate choice (Hebets and Uetz 2000), and could explain the function of the wing spot in courtship display.

One aspect of signal efficacy is how easily a signal can be distinguished from the background. In general, signals with higher intensity and contrast compared to the background environment should elicit responses most effectively (reviewed in Stevens 2013). Efficacy can be achieved by redundancy; multiple signals communicated in the same channel increase the intensity of the signal or provide additional contrast in a given environment. Wing spots could be redundant to wing display, either increasing the intensity of the visual signal, providing contrast in a given environment, or both. We saw no difference between spotted and spotless males in the length of time they had to court females before successfully copulating in *D. biarmipes*, *D. suzukii*, or *D. subpulchrella* (Roy and Gleason 2019), but this could be because the laboratory environment that we tested them in was devoid of background noise under bright lighting. The laboratory conditions could make it easier for females to see the necessary contrasts of the wing display without spot, making the spot less relevant for mate choice.

In certain environments, wing spots may increase signal efficacy so that males do not have to exert as much energy and can more effectively secure mates. No studies have measured differences in courtship vigor (e.g. the number of wing displays or amount of time spent actively courting between courtship initiation and copulation) between spotted and spotless males. Investigating different aspects of the environment that make visual signals difficult to receive (e.g. background noise, low light levels), and the effort that males with spots have to put into

courtship compared to those without spots in a given environment, could highlight the role of wing spots in increasing the efficacy of visual courtship signaling.

Keeping Spots: Natural Selection and Function in Thermoregulation

Wing spots may be a by-product of differential thermoregulation between males and females and selective advantages for males with a wider range of thermoregulation capabilities. Differential fitness between pigmented males independent of female choice could lead to higher mating success in melanized males due to a higher tolerance to a wider range of temperatures (particularly cooler) and earlier emergences during breeding seasons (reviewed in True 2003). Darker colors absorb more light, leading to greater heat absorption for a given level of solar radiation (May 1979; Burtt 1981). Darker individuals, therefore, could benefit at lower temperatures by raising the individual's body temperature closer to its optimum faster in order to acquire mates (Huey and Kingsolver 1989). At warmer temperatures, the body temperature of darker individuals may be increased past an optimum for general activity (particularly wing movement, Church 1960) that would put them at a selective disadvantage (Huey and Kingsolver 1989) and thus constrain the evolution of dimorphic pigmentation by geographic region (Watt 1969; May 1979; Moore et al. 2019). We would expect to see populations of *Drosophila* occupying temperate regions expressing more pigment than in the tropics.

Melanization in *Drosophila* (as demonstrated in *D. melanogaster*) is plastic (Pool and Aquadro 2007) and geographically variable (David et al. 1985; Telonis-Scott et al. 2011), with darker fly morphs likely adapted for colder conditions (David et al. 1998) and lighter morphs for warmer climates (Gibert et al. 1998). Natural selection could be affecting male and female pigmentation differently, considering shown demonstration of differences in thermal effects between sexes on other morphological traits in *D. melanogaster* (e.g. body size, Crill et al. 1996;

recovery from chilling, David et al. 1998). In *Drosophila suzukii*, darker pigmentation (including the darker wing spots in males) is correlated with cold conditions (Hamby et al. 2016; Shearer et al. 2016).

Drosophila lutescens and *D. takahashii* are closely related species that occupy Northern and Southern regions of Japan, respectively. At the borders of their regions overlap of species has been observed (Fukatami 1984). *Drosophila lutescens* has pigmented wings. In *D. lustescens*, male pigmentation could be present because of the shared genetic basis with wing display (which could be under character displacement to avoid mating mistakes in sympatric zones) but is maintained because males with more pigment have broader thermal tolerances and therefore better access to mates than those with lower thermal tolerances. If other pairs of spotted and spotless relatives have similar geographic range patterns, they are not reported. A trend in spotted species occupying colder climates, a closely related relative occupying a warmer climate, and areas of species overlap, could implicate natural and sexual selection working in parallel to maintain both wing spot and wing display traits.

Know Your Enemies: Intrasexual Selection

Though less likely, considering that there was no observed significant difference in mating success between spotted and spotless males in choice test (Chapter 1, Roy & Gleason 2019), males may use wing spots to assess other males and establish hierarchies prior to intersexual interactions. Male competition is observed in Hawaiian *Drosophila*, a system in which mating success is less dependent on female choice and more dependent on position in a mating territory (Droney 1992). Males may possibly compete for "territories" at which they have best access to females. We observe *D. biarmipes* males pushing one another to access females during competition experiments, but we have not yet examined territory establishment prior to

exposure to females. Further exploration of male-male interaction and competition could shed light on the potential role of wing spots on intrasexual selection.

Final Words

Many questions remain as to what role morphology and specific associated behaviors have in contributing to visual courtship communication, but the foundation of work to date highlights the utility of spotted winged *Drosophila* as a model for the study of the function of sexual dimorphisms and courtship communication evolution. Future studies examining the functional courtship elements of the wing display and the accompanying genetic architecture, as well as directing research to encompass sensory ecology, will provide valuable contributions to many fields of biology, particularly evolutionary ethology.

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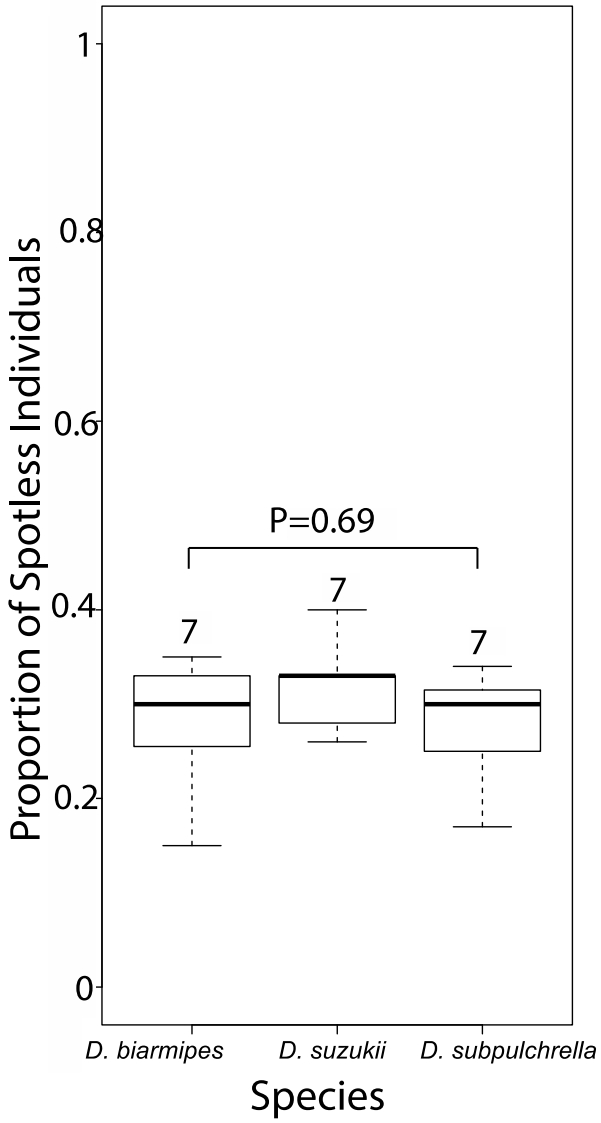
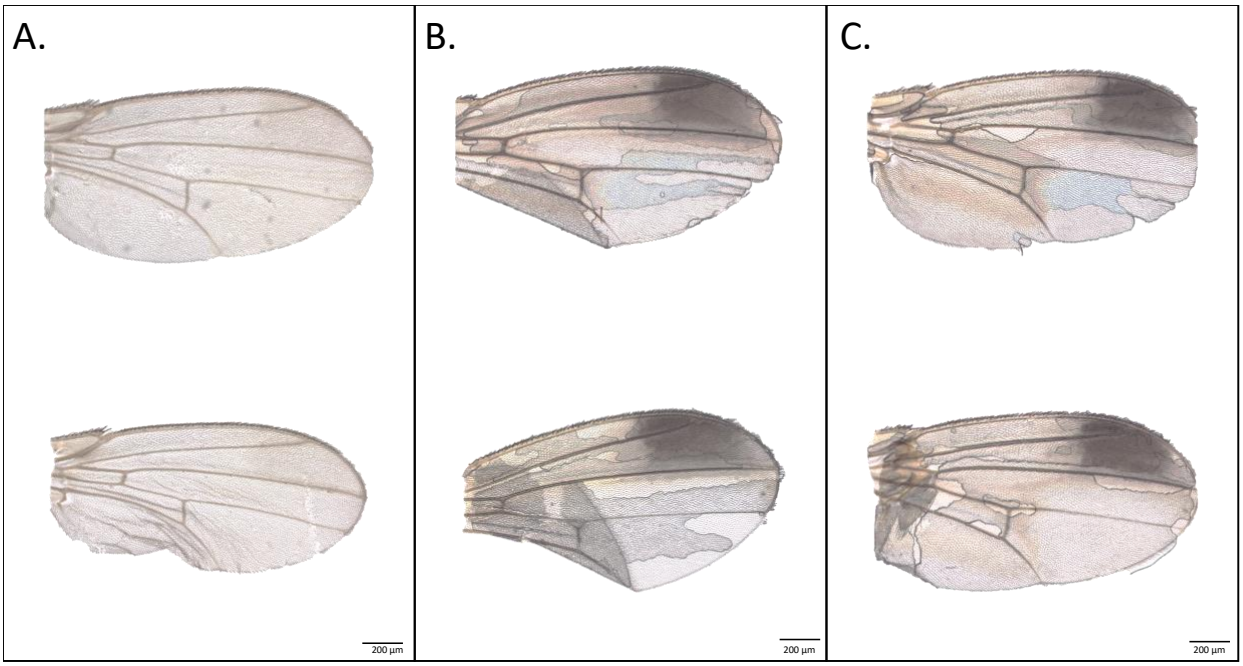
Appendix 1

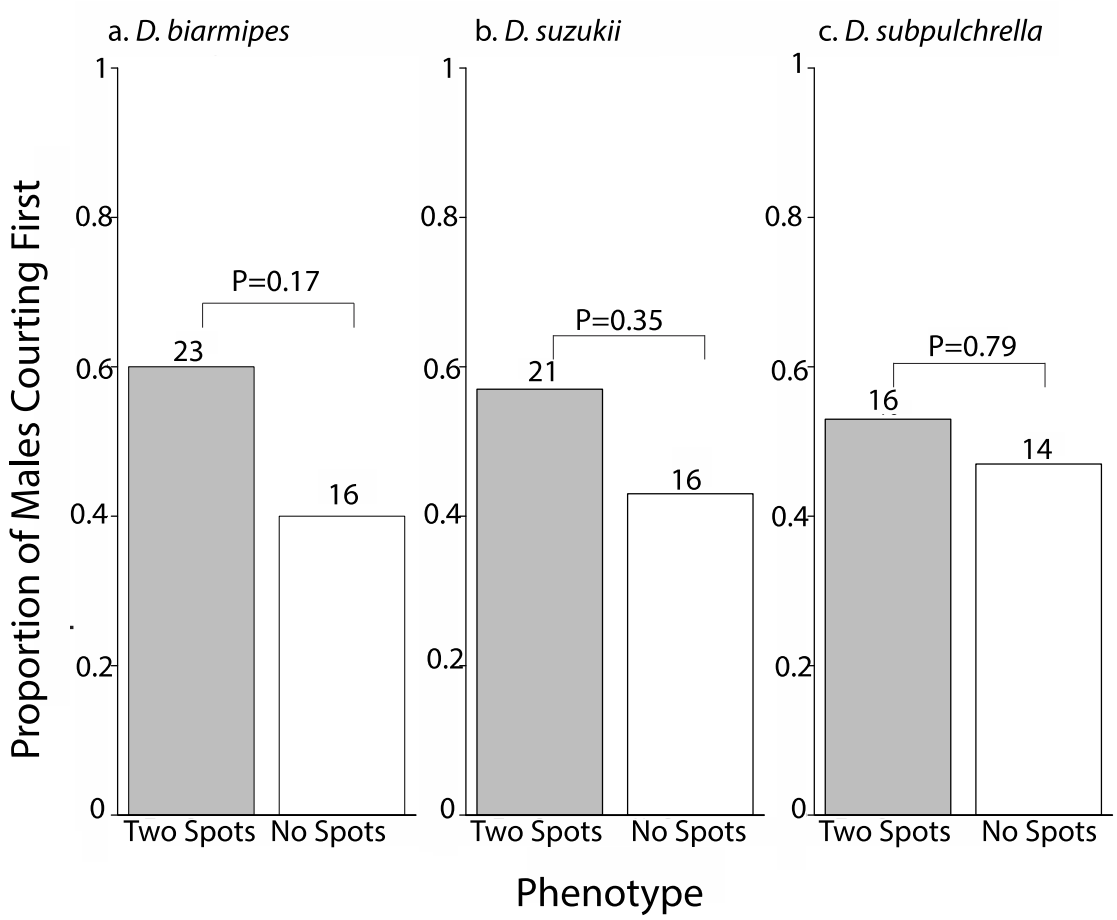
Appendix 1.1: Supplementary Figures for Chapter 1



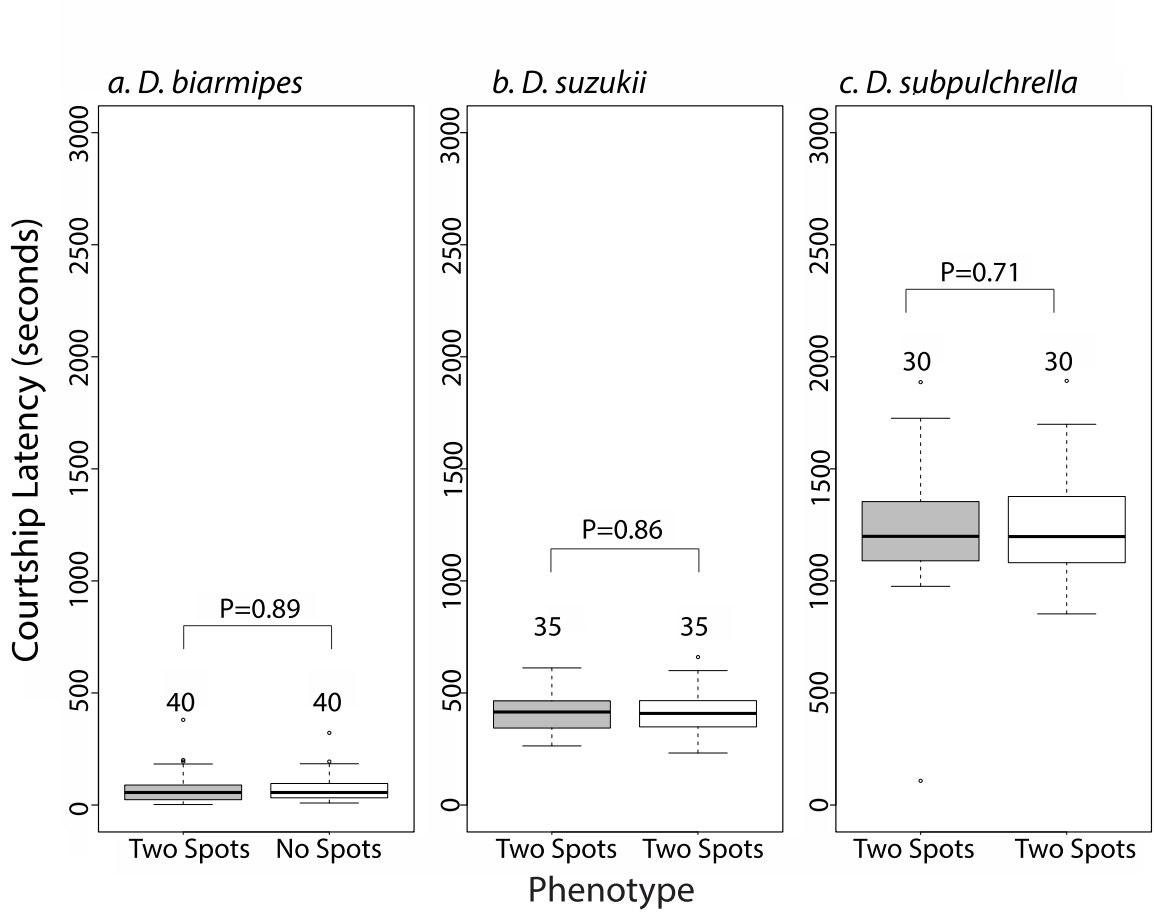
Supplementary Figure S1.1. Proportion of spotless individuals treated with CO_2. Newly eclosed flies were anesthetized with carbon dioxide for three minutes, before placing males in vials to recover. Wing phenotype was scored after 48 hours. For each species, the average proportion of spotless individuals over all sets collected was calculated and then compared using a one-way ANOVA. The proportion of individuals lacking spots across collection sets was not significantly different between species (P = 0.686). Numbers on top of each column are the number of collection sets.



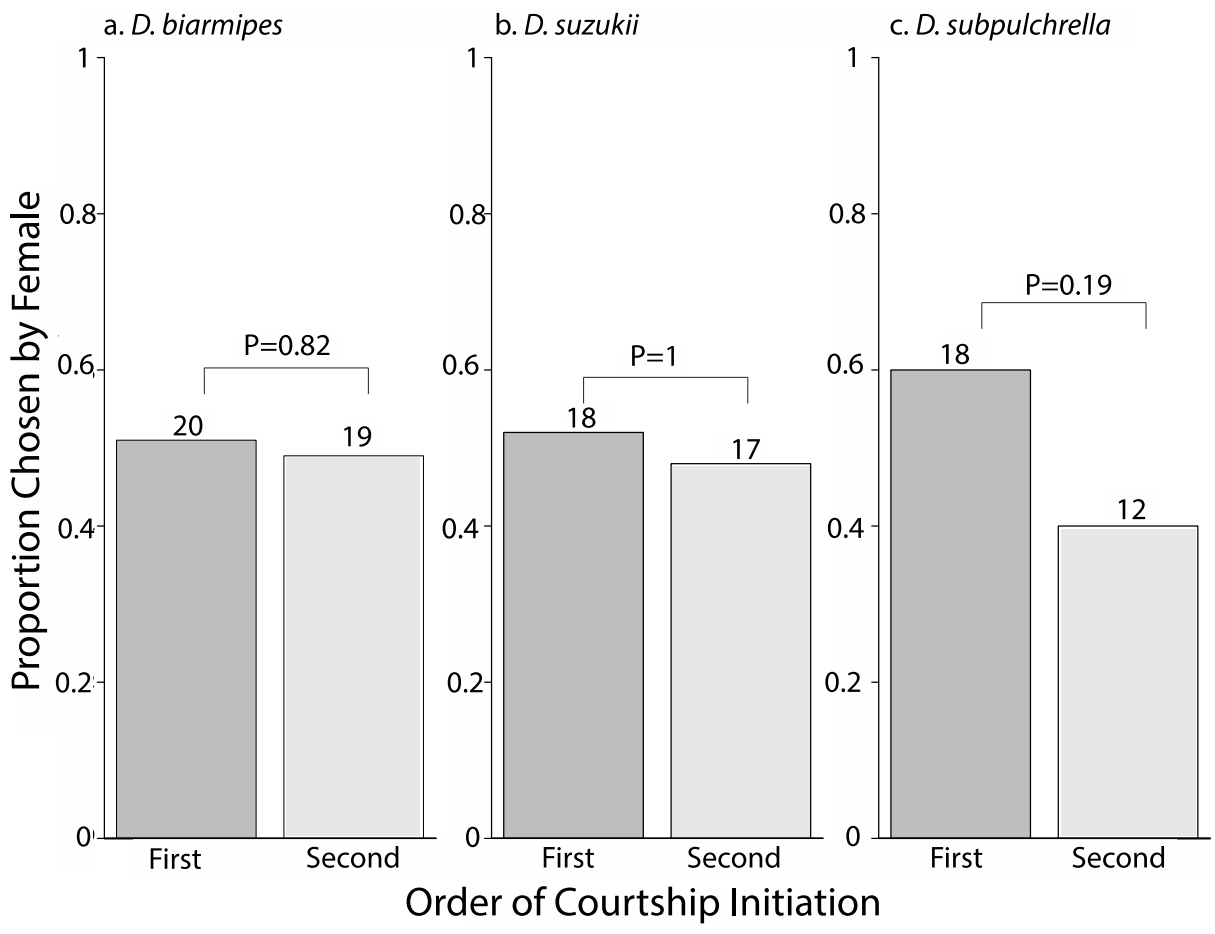
Supplementary Figure S1.2. Wing phenotypes of *D. biarmipe*s treated with CO_2 or aspirated. Newly eclosed flies were anesthetized with carbon dioxide for three minutes, before placing males in vials to recover. Wing phenotype was scored after 48 hours. Shown are examples of A. males treated with CO_2 that lost wing spots; B. males treated with CO2 that retained wing spots and C. males that were aspirated with the natural phenotype. All aspirated males had wing spots.



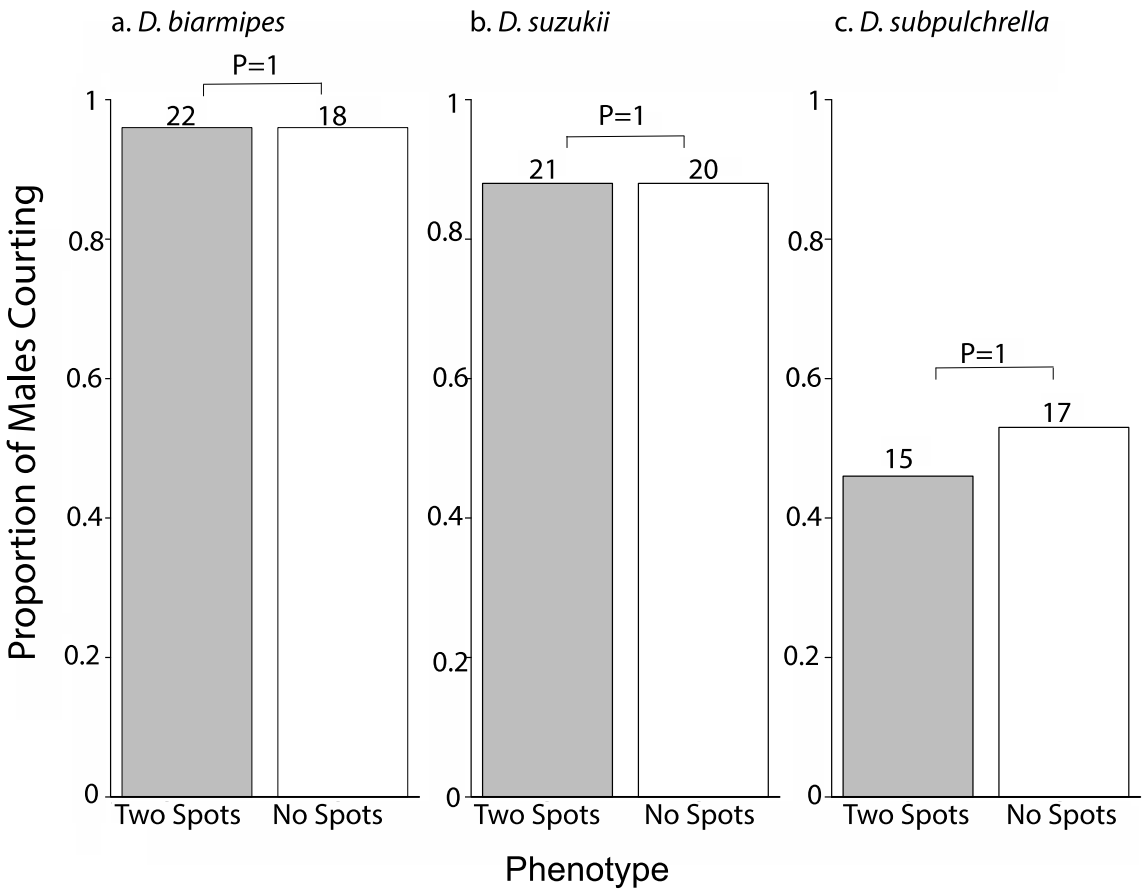
Supplementary Figure S1.3. **Proportion of males courting first in competition experiments**. Mating pairs were observed for 60 minutes and time to courtship initiation was recorded. Spotted and spotless phenotypes were compared using a Student's T-Test. The frequency at which males courted females first was not different between males with two spots and males with no spots (*P* = 0.17, *P* = 0.35, *P* = 0.79, *D. biamipes*, *D. suzukii* and *D. subpulchrella*, respectively). Numbers on top of each column are sample sizes.



Supplementary Figure S1.4. **Courtship latency of males of different phenotypes in competition.** Effect of phenotype (spotted and spotless) courtship latency. Mating pairs were observed for 60 minutes and courtship order was recorded. Phenotypes were compared using a Student's T Test. The courtship latency times were not different between males with two spots and males with no spots (P = 0.89, P = 0.86, P = 0.71, *D. biamipes, D. suzukii* and *D. subpulchrella*, respectively). Numbers on top of each column are sample sizes.

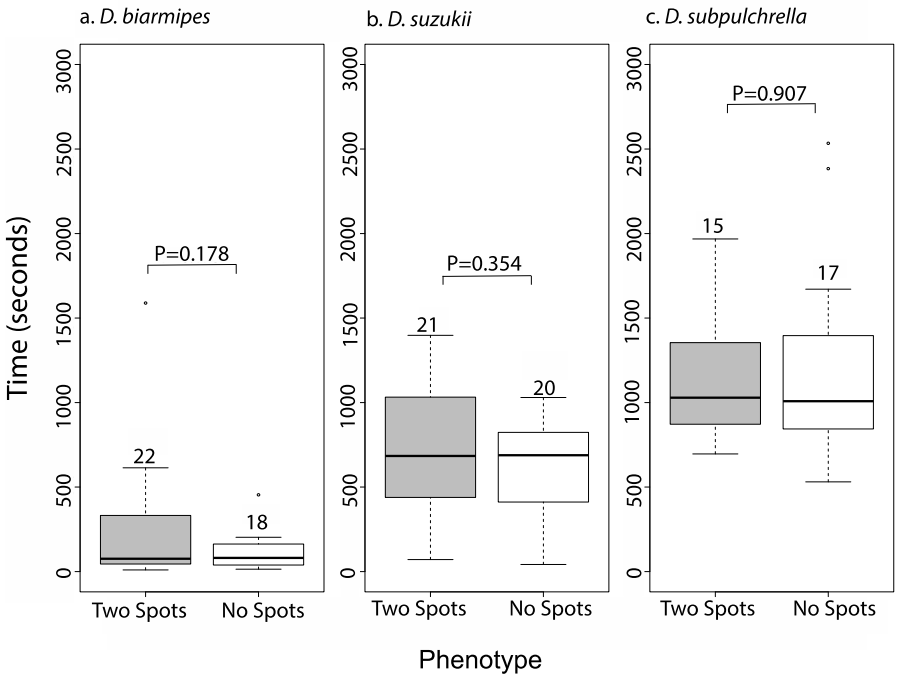


Supplementary Figure S1.5. **Mating success of males by order of courtship**. Mating pairs were observed for 60 minutes and time to courtship initiation and mating success was recorded. The male who courted first was compared to the male that courted second in the context of being the successful mater. The order in which the males began courtship did not significantly affect their mating success ($P = 0.82$, $P = 1$, $P = 0.19$, *D. biamipes*, *D. suzukii* and *D. subpulchrella*, respectively). Numbers on top of each column are sample sizes.

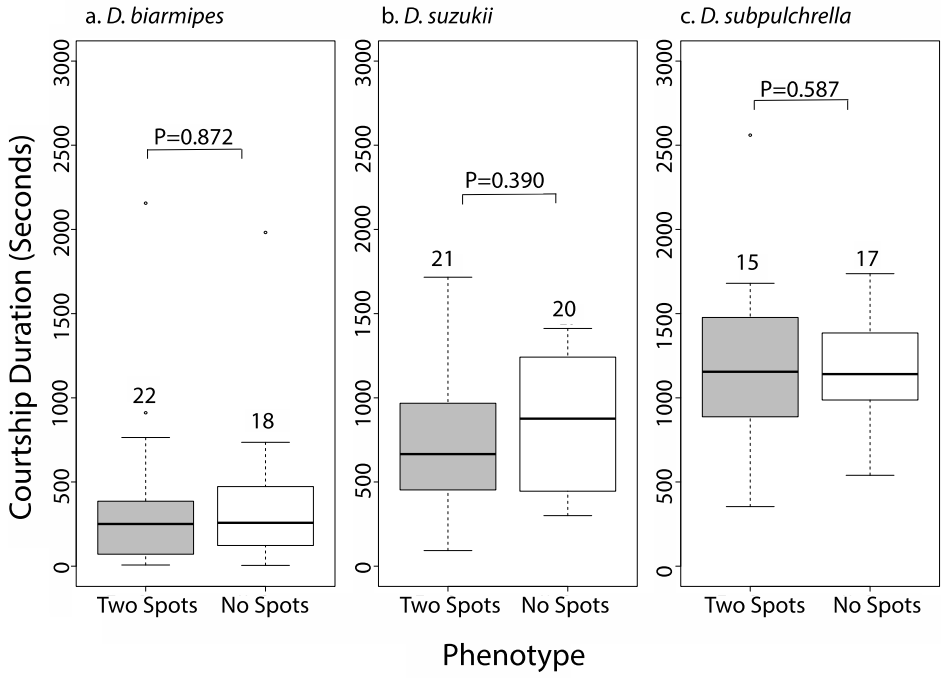


Supplementary Figure S1.6. Effect of phenotype on courtship frequency. Mating pairs were observed for 60 minutes and observed for frequency of courtship. Phenotypes were compared using a Chi-squared test. The frequency at which males courted females was not different between males with two spots and males with no spots ($P = 1$, $P = 1$, $P = 0.651$, *D. biamipes, D. suzukii* and *D. subpulchrella*, respectively). Numbers on top of each column are sample sizes.

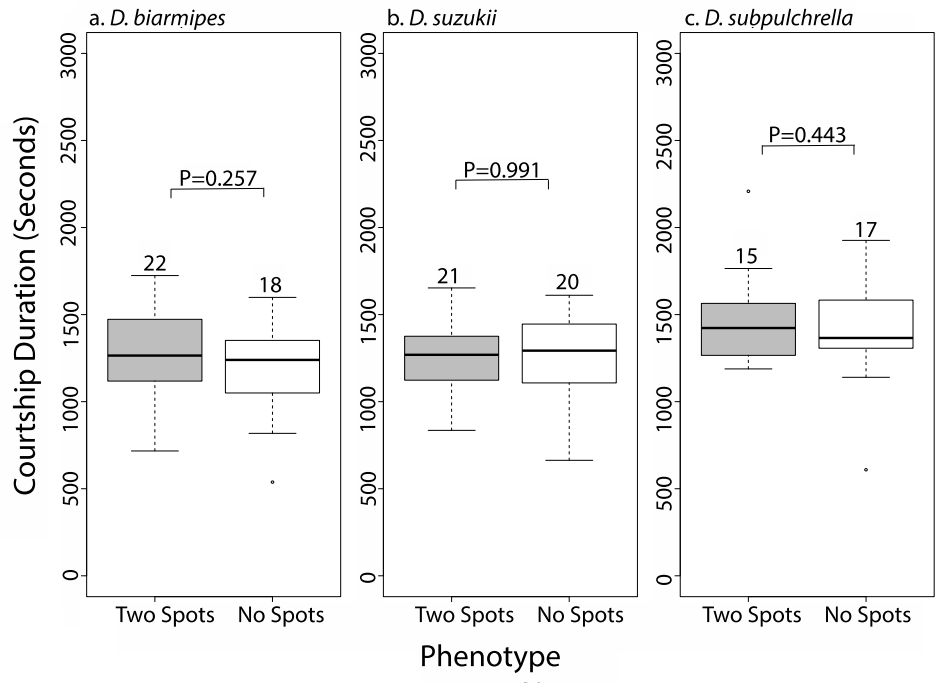
Supplementary Figure S1.7A Courtship latency



Supplementary Figure S1.7B Courtship duration



Supplementary Figure S1.7C Copulation duration



Supplementary Figure S1.7. Effect of phenotype on courtship timing parameters in no-choice assays. a) Courtship latency b) Courtship duration. c) Copulation duration. Mating pairs were observed for 60 minutes and observed for courtship latency, courtship duration, copulation duration. Males with two spots were compared to males with no spots for all parameters using a Student's T-Test. 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. No treatments were found to be significantly different for any of the parameters measured. Numbers on top of each column are sample sizes.

Appendix 2

Appendix 2.1 Supplementary Tables and Figures for Chapter 2

Supplementary Table S2.1. Species of spotted winged *Drosophila* included in the phylogenies of this study and four additional phylogenies most-similar to this study

Species Subgroup	Species	This Study	van der Linde and Houle (2008)	Prud'homme et al. (2006)	Schawarock (2002)	Kopp and True (2002a)
takahashii	*D. lutescens*	x[1]	x	x	x	x
	D. paralutea	x	x	x	x	
	D. prostipennis	x	x	x	x	x
	D. pseudotakahashii	x	x	x		x
	D. takahashii	x	x	x	x	x
	D. trilutea		x	x		x
suzukii	*D. biarmipes*	x	x	x	x	x
	D. lucipennis	x	x	x	x	x
	D. mimetica	x	x	x	x	x
	D. pulchrella		x	x		
	D. subpulchrella	x				
	D. suzukii	x	x	x		x
elegans	*D. elegans*	x	x	x	x	x
	D. gunungcola	x	x	x		x
rhopoloa	*D. fuyamai*	x	x	x		x
	D. rhopoloa	x				

[1]An x esignates its inclusion in the tree

Supplementary Table S2.2. Phenotype assignments from this study and previous studies

Species Subgroup	Species	This Study	Prud'homme et al. (2006)	Kopp and True (2002b)
takahashii	*D. lutescens**[1]	spotted	spotted	spotless
	D. paralutea	spotless	spotless	N/A
	D. prostipennis	spotted	spotted	spotted
	D. pseudotakahashii	spotted	spotted	spotted
	*D. takahashii**	spotless	spotted	spotless
	*D. trilutea**	N/A	spotted	spotless
suzukii	*D. biarmipes*	spotted	spotted	spotted
	D. lucipennis	spotless	spotless	spotless
	D. mimetica	spotless	spotless	spotless
	D. pulchrella	N/A	spotted	N/A
	D. subpulchrella	spotted	N/A	N/A
	D. suzukii	spotted	spotted	spotted
elegans	*D. elegans*	spotted	spotted	spotted
	D. gunungcola	spotless	spotless	spotless
rhopoloa	*D. fuyamai*	spotted	spotted	spotted
	D. rhopoloa	spotted	N/A	N/A

[1]An * indicates disagreement in phenotyping between studies

Supplementary Table S2.3 Alignment and filtering data for genomes aligned to *D. melanogaster*

Species	Raw Read Pairs	Fastp Filtered Read Pairs	Percentage Reads Mapped	Mean Depth of Coverage	Median Depth of Coverage	Standard Deviation Depth	Percentage of Nucleotide Characters After Annotation				
							Informative				Non-Informative
							A	G	T	C	N
D. ananassae	28540678	28537702	24.96	5.60	3	6.58	4.98	9.19	9.18	5.03	71.61
D. auraria	34515189	3106264	20.15	4.51	3	4.89	2.94	6.91	6.84	2.93	80.37
D. biarmipes	31659664	31199002	44.04	16.20	16	7.85	15.02	26.44	26.32	15.25	16.97
D. elegans	35095287	33642560	31.51	7.85	9	8.00	12.32	12.51	12.45	12.41	50.30
D. eugracilis	34241670	32075257	36.35	8.52	9	5.97	10.92	12.63	12.51	10.95	52.98
D. ficusphila	31825159	29539191	31.99	7.37	7	5.63	7.62	11.73	11.71	7.65	61.29
D. fuyamai	13251295	12951995	51.38	5.17	4	3.93	3.44	3.82	3.66	3.70	85.39
D. gunungcola	4477455	4362812	54.36	2.35	2	2.25	0.38	0.29	0.29	0.41	98.63
D. lucipennis	19330136	18871396	50.32	6.38	6	4.35	5.00	7.24	7.15	5.36	75.25
D. lutescens	17324187	16883831	54.50	7.19	7	4.49	6.52	8.35	8.32	6.92	69.89
D. melanogaster	55011053	54365944	96.50	55.76	55	16.71	21.38	28.11	27.95	21.76	0.81
D. mimetica	19892168	19397851	51.73	7.17	7	4.49	6.19	9.11	9.04	6.60	69.06
D. paralutea	19209357	18714383	53.48	6.56	6	4.38	5.43	6.87	6.82	5.79	75.09
D. prostipennis	10971754	10631530	51.81	3.48	3	2.89	1.05	1.16	1.17	1.16	95.47
D. pseudotakahashii	19872587	19408505	55.76	6.76	6	4.42	5.60	7.74	7.64	5.94	73.07
D. rhopaloa	42338083	35951526	34.90	7.30	6	6.14	8.74	7.04	7.01	8.82	68.40
D. simulans	21450787	21153125	88.65	12.47	12	5.04	15.67	21.59	21.39	15.93	25.42
D. subpulchrella	25801350	24553938	51.71	6.02	5	4.20	4.45	6.20	6.11	4.69	78.54
D. suzukii	41111127	40410198	44.90	12.84	13	6.11	15.22	21.75	21.74	15.44	25.85
D. takahashii	19256479	18880788	44.24	9.89	10	6.05	11.78	15.05	14.99	11.98	46.20
D. yakuba	39632759	39035282	77.77	22.22	20	13.33	17.13	24.70	24.55	17.45	16.17

Supplementary Table S2.4 Alignment and filtering data for genomes aligned to *D. suzukii*

Species	Raw Read Pairs	Fastp Filtered Read Pairs	Percentage Reads Mapped	Mean Depth of Coverage	Median Depth of Coverage	Standard Deviation Depth	Percentage of Nucleotide Characters After Annotation				
							Informative				Non Informative
							A	G	T	C	N
D. ananassae	28540678	28537702	31.26	4.95	2	6.28	4.31	8.40	8.36	4.37	74.56
D. auraria	34515189	3106264	26.64	4.49	3	5.02	2.94	7.51	7.30	2.98	79.27
D. biarmipes	31659664	31199002	71.44	22.67	23	7.5	16.62	32.04	31.21	17.24	2.90
D. elegans	35095287	33642560	49.49	10.74	10	7.53	12.51	15.67	15.13	12.77	43.91
D. eugracilis	34241670	32075257	49.02	9.08	10	6.08	11.63	14.50	14.15	11.93	47.79
D. ficusphila	31825159	29539191	40.54	7.76	8	5.96	8.02	13.67	13.31	8.35	56.65
D. fuyamai	13251295	12951995	70.27	6.04	5	4.16	4.90	6.31	6.06	5.29	77.45
D. gunungcola	4477455	4362812	67.54	2.79	2	2.52	0.61	0.68	0.66	0.63	97.41
D. lucipennis	19330136	18871396	66.19	7.42	7	4.59	6.91	11.30	10.94	7.34	63.51
D. lutescens	17324187	16883831	76.83	9.78	10	4.52	11.12	16.85	16.40	11.68	43.95
D. melanogaster	55011053	54365944	60.14	23.28	23	14.47	17.07	23.81	23.15	17.61	18.35
D. mimetica	19892168	19397851	77.53	10.1	10	4.60	11.43	18.67	18.14	11.99	39.78
D. paralutea	19209357	18714383	76.96	9.58	10	4.59	10.82	16.32	15.84	11.37	45.65
D. prostipennis	10971754	10631530	72.51	5.13	4	3.40	2.77	3.85	3.77	2.92	86.69
D. pseudotakahashii	19872587	19408505	78.02	10.07	10	4.63	11.49	18.14	17.56	12.02	40.78
D. rhopaloa	42338083	35951526	51.01	8.26	8	5.92	10.22	10.61	10.34	10.40	58.44
D. simulans	21450787	21153125	43.80	4.91	4	4.70	3.77	6.74	6.62	3.95	78.91
D. subpulchrella	25801350	24553938	96.15	10.68	11	4.61	12.36	19.53	19.09	12.95	36.07
D. suzukii	41111127	40410198	98.81	22.17	21	7.99	18.80	30.03	29.33	19.58	2.25
D. takahashii	19256479	18880788	66.78	14.06	13	6.70	15.54	24.39	23.70	16.09	20.27
D. yakuba	39632759	39035282	55.72	12.29	11	10.3	10.40	18.12	17.76	10.84	42.87

Supplementary Table S2.5 Mean depth of coverage by chromosome for all species aligned and annotated with the *D. melanogaster* reference

Species	Chromosome Arm[1]						
	2L	2R	3L	3R	4	X	Y
D. ananassae	5.8	6.3	6.2	6.1	2.3	4.2	12.3
D. auraria	4.2	4.8	4.6	4.8	0.8	4.4	0.8
D. biarmipes	16.9	16.9	17.3	17.2	15.6	13.5	9.1
D. elegans	10.6	10.3	11.1	10.6	11.6	9.3	3.1
D. eugracilis	8.5	8.6	8.7	8.5	6.4	8.5	3.9
D. ficusphila	7.3	7.5	7.5	7.6	2.8	7.3	0.1
D. fuyamai	4.8	5.1	5.1	5.0	5.6	5.7	5.1
D. gunungcola	2.6	2.6	2.7	2.6	2.6	1.4	1.1
D. lucipennis	6.2	6.2	6.4	6.1	6.6	6.9	9.3
D. lutescens	7.3	7.3	6.9	7.3	9.7	7.0	6.0
D. melanogaster	61.8	61.6	60.7	60.7	62.5	38.5	41.6
D. mimetica	6.9	7.0	6.7	7.0	9.0	7.9	11.5
D. paralutea	6.3	6.2	6.0	6.3	8.9	7.5	12.5
D. prostipennis	3.3	3.2	3.1	3.3	4.7	4.2	5.3
D. pseudotakahashii	6.5	6.4	6.1	6.4	9.1	7.9	8.0
D. rhopaloa	7.2	7.0	7.6	7.1	8.2	7.5	6.5
D. simulans	12.1	12.0	11.8	11.8	12.5	14.2	9.5
D. subpulchrella	5.5	5.3	5.6	5.5	7.2	7.6	14.0
D. suzukii	12.5	12.4	12.7	12.6	14.1	13.6	16.5
D. takahashii	10.1	9.7	9.6	9.8	12.6	10.1	16.0
D. yakuba	19.4	20.5	19.4	19.7	5.3	31.1	9.2
Mean and standard deviation	10.8 ± 12.4	10.8 ± 12.4	10.8 ± 12.2	10.8 ± 12.2	10.4 ± 12.6	10.4 ± 8.8	9.6 ± 8.7
Mean and standard deviation without reference genome	8.2 ±4.3	8.3±4.4	8.3±4.3	8.3±4.3	7.8±4.1	9.0±6.1	8.0±4.9

[1]Indicates the chromosome arm of *D. melanogaster* (Ensembl reference BDGP6.95 from Ensembl (Zerbino et al. 2017))

Supplementary Table S2.6 Mean depth of coverage by scaffold for all species aligned and annotated with the *D. suzukii* reference

CH[1]	ana[2]	aur	bia	ele	eug	fic	fuy	gun	luc	lut	mel	mim	par	pro	pse	rho	sim	sub	suz	tak	yak
Andromeda	4.9[3]	4.1	23.7	11.1	8.9	7.5	5.9	3.2	7.2	10.2	23.9	10.1	9.7	5.1	10.1	8.4	4.2	10.6	22.9	14.5	10.2
Cepheus	5.4	4.5	23.8	11.4	9.0	7.9	6.0	3.2	7.4	9.8	24.6	9.9	9.2	4.9	9.7	8.5	4.6	10.3	22.0	14.0	11.1
Contig_10	5.8	5.2	23.5	10.8	9.1	8.4	5.9	3.2	7.4	10.0	24.5	10.1	9.5	4.9	9.9	8.1	4.4	10.2	21.2	14.4	10.8
Contig_101	0.0	0.6	17.4	24.1	10.9	3.6	3.4	10.0	7.0	9.2	30.1	5.8	5.6	3.0	9.6	27.4	3.2	8.4	15.8	13.4	3.3
Contig_102	0.0	3.4	23.8	5.5	0.8	1.8	11.3	0.8	0.0	13.4	15.1	11.0	21.3	11.0	7.8	10.2	0.0	4.8	28.8	43.8	0.5
Contig_105	3.1	3.2	26.3	17.7	5.1	2.9	5.1	3.1	7.2	13.4	19.4	13.2	9.4	5.7	10.2	6.5	4.7	17.7	20.1	18.6	1.3
Contig_106	4.1	4.6	20.2	17.0	7.5	2.9	7.0	3.1	6.1	10.8	24.9	8.3	10.2	4.5	9.6	10.4	5.1	12.5	19.0	16.4	2.5
Contig_108	0.0	0.0	13.7	18.3	2.7	0.7	10.7	3.0	6.0	5.7	14.0	3.3	14.7	5.7	15.0	24.0	1.3	11.0	28.7	25.0	3.3
Contig_109	2.3	0.6	21.0	3.8	2.9	0.5	2.0	1.8	7.6	19.4	28.1	11.9	9.1	4.9	12.0	4.4	0.4	17.9	29.1	11.1	19.1
Contig_11	4.7	4.8	17.6	9.2	8.7	7.5	6.7	1.5	8.2	9.1	20.1	10.4	9.5	5.8	10.7	8.3	6.5	11.3	20.9	13.3	18.1
Contig_110	0.0	3.8	19.9	10.2	7.5	12.6	3.3	1.6	6.0	6.7	19.2	15.0	9.7	10.3	7.7	4.6	3.1	12.7	20.5	15.3	24.0
Contig_114	2.7	2.1	26.2	11.4	5.7	4.9	4.2	3.9	9.8	8.9	10.2	16.1	10.1	7.3	12.0	8.5	0.3	11.9	28.3	21.4	1.6
Contig_12	1.5	0.7	23.2	13.8	5.1	2.0	6.0	2.3	6.8	11.4	13.2	12.9	13.5	5.4	11.8	8.8	2.1	17.7	23.8	18.9	5.6
Contig_120	8.6	9.7	14.3	1.4	2.3	8.8	7.1	3.7	5.7	8.2	28.0	10.2	10.2	5.2	11.3	2.5	11.0	8.3	30.7	17.2	11.0
Contig_13	5.1	4.8	23.8	11.0	8.0	7.8	5.8	2.9	7.4	10.4	24.6	10.3	9.5	5.2	9.8	8.2	4.7	10.3	22.2	14.1	11.7
Contig_130	0.0	0.2	19.0	8.7	4.3	0.2	7.9	0.4	2.7	12.6	9.0	11.4	14.3	10.2	15.9	5.1	1.1	16.0	29.5	20.4	1.5
Contig_133	4.2	0.8	18.5	15.1	3.8	1.5	14.3	0.9	13.7	8.7	5.9	14.9	8.3	9.3	13.1	22.3	0.4	10.1	17.0	3.5	1.1
Contig_135	0.0	0.9	20.7	12.4	5.7	6.9	15.9	5.2	5.3	10.8	29.7	9.4	9.3	5.0	7.7	9.5	4.7	14.3	22.6	16.6	3.4
Contig_137	8.1	6.9	23.2	18.8	13.1	4.9	5.1	2.1	2.1	10.8	14.3	10.1	9.5	4.8	8.9	16.4	2.5	9.3	23.0	17.9	0.9
Contig_138	5.0	0.0	14.5	28.5	10.0	4.0	3.5	3.0	1.0	6.0	57.5	11.0	2.0	5.5	10.0	11.0	10.0	7.5	16.0	19.0	1.0

Contig_14	4.3	2.8	22.9	13.9	7.3	5.5	8.1	2.6	12.5	11.8	25.1	9.4	10.3	4.0	11.7	7.1	3.9	12.1	19.2	10.6	2.1
Contig_15	2.9	3.0	19.4	6.3	7.7	5.7	7.0	1.0	7.1	11.2	19.5	10.9	11.2	6.5	11.4	5.9	5.7	12.5	21.7	16.2	16.1
Contig_155	9.3	0.0	23.9	12.3	2.4	0.0	8.8	2.4	6.8	5.4	27.2	8.1	7.2	8.6	10.1	18.3	5.3	9.6	25.4	12.6	1.3
Contig_157	5.9	5.8	23.0	9.6	8.1	8.4	6.1	3.2	7.2	9.0	29.5	10.5	8.7	4.4	9.6	7.3	5.7	9.2	21.3	13.4	12.4
Contig_16	5.8	5.3	23.7	11.2	9.4	8.5	6.0	3.1	7.3	9.2	25.6	9.8	9.1	4.8	9.8	8.3	4.8	10.5	22.1	12.8	11.7
Contig_161	0.1	2.9	20.8	19.2	1.9	1.6	3.4	2.3	6.3	13.6	17.1	19.1	8.4	5.1	10.8	10.4	2.3	12.8	33.2	21.6	1.1
Contig_166	8.5	1.5	29.7	8.3	9.5	8.0	3.3	0.7	9.3	4.2	19.8	7.8	7.0	2.8	10.2	5.7	11.3	8.8	35.2	19.2	18.0
Contig_17	1.6	0.8	25.1	14.3	7.5	3.8	6.8	3.0	7.7	12.1	18.8	11.8	11.4	6.4	11.4	10.4	2.8	11.5	23.0	18.1	1.8
Contig_172	0.0	0.0	12.3	15.8	0.0	0.0	2.5	2.7	5.0	11.7	25.5	15.5	13.7	11.5	10.7	0.0	0.0	14.7	16.5	13.8	0.0
Contig_174	0.0	0.0	20.0	0.0	0.0	0.0	2.0	6.0	6.0	10.0	55.0	14.0	10.0	5.0	15.0	0.0	6.0	10.0	34.0	10.0	12.0
Contig_176	4.5	3.9	21.7	7.0	7.5	7.5	4.9	1.9	6.6	9.3	25.2	11.7	9.9	6.2	11.7	7.3	4.1	10.5	15.4	16.3	11.8
Contig_18	5.3	5.4	24.6	11.2	9.2	7.2	5.5	3.1	7.1	9.0	25.3	9.8	9.3	4.9	9.8	8.0	4.6	10.4	23.0	13.8	11.9
Contig_19	3.9	4.8	19.4	8.8	7.2	8.9	7.0	1.5	7.6	8.9	22.6	9.6	9.7	5.9	10.9	7.5	7.2	10.6	21.4	11.8	21.0
Contig_191	0.0	0.9	16.7	15.5	7.5	2.2	4.0	2.7	14.2	12.6	2.2	13.9	16.5	7.8	17.6	9.8	0.5	12.2	15.2	18.2	1.7
Contig_194	8.5	4.2	26.7	0.5	3.2	11.0	3.7	0.3	1.2	13.2	19.8	8.5	10.5	2.3	7.2	6.7	1.8	15.0	37.0	11.2	17.2
Contig_20	0.5	1.0	15.6	2.4	12.6	10.3	2.2	2.5	9.1	6.9	46.6	15.5	8.9	3.4	12.0	1.9	1.5	10.0	30.5	15.7	9.6
Contig_200	0.3	1.5	18.0	6.6	11.8	8.7	1.6	1.9	4.9	11.3	25.1	9.8	12.1	5.3	12.0	4.6	8.7	14.2	15.5	20.1	11.6
Contig_204	7.7	6.3	16.9	14.8	5.3	6.0	6.7	0.7	9.3	8.0	33.1	12.6	6.8	3.1	10.3	7.3	3.2	13.8	14.6	16.8	6.1
Contig_21	4.1	2.3	22.4	15.0	6.2	3.4	9.3	3.8	7.5	11.1	17.8	10.8	11.0	5.2	11.1	12.0	2.8	10.0	23.9	16.6	2.1
Contig_22	6.0	5.1	23.0	10.6	9.1	7.9	6.0	2.9	7.3	9.7	26.3	9.9	9.6	5.0	9.8	7.6	5.1	10.5	22.0	12.7	12.2
Contig_226	2.0	3.0	27.5	11.0	14.0	8.0	0.0	1.0	14.5	18.0	24.0	17.0	13.5	5.0	11.0	0.0	5.0	2.5	13.0	18.5	5.0
Contig_23	9.7	4.8	15.3	7.8	6.7	16.3	2.9	3.5	5.6	5.9	37.9	11.9	7.3	4.0	9.9	7.9	10.3	3.3	18.7	11.5	14.1

Contig_239	2.9	6.4	19.8	22.5	7.2	6.0	5.1	2.2	4.8	14.1	22.8	10.2	11.0	7.6	11.1	8.4	2.6	10.7	20.0	16.8	1.7
Contig_25	3.1	2.4	19.4	7.2	9.9	6.2	5.4	2.0	6.7	10.7	30.9	9.3	11.4	4.7	11.3	6.5	4.9	11.9	21.8	14.0	14.3
Contig_251	0.5	1.4	26.1	4.7	10.6	4.6	1.2	2.7	8.2	7.8	39.4	14.6	11.8	6.6	12.0	2.6	12.4	6.1	12.7	14.5	17.1
Contig_26	0.5	3.2	22.9	12.2	7.4	5.5	5.2	2.5	7.1	10.0	30.2	9.8	10.6	7.3	9.7	8.9	3.6	12.3	21.6	16.6	6.7
Contig_261	0.3	1.5	16.7	0.0	1.5	0.0	0.4	0.1	0.0	15.1	35.8	14.5	12.2	6.6	13.7	0.1	1.0	13.7	13.9	14.9	41.0
Contig_27	3.6	3.0	26.2	7.8	5.7	5.2	5.8	2.8	7.0	11.5	20.6	11.0	10.7	5.7	11.4	8.1	3.3	12.0	26.8	17.1	7.7
Contig_285	4.5	2.1	18.4	15.3	3.5	4.0	9.2	4.1	6.6	10.2	24.9	8.6	11.1	5.9	12.2	11.7	4.5	8.0	14.6	17.0	11.5
Contig_289	0.7	0.8	3.8	12.2	12.0	12.2	1.7	0.3	5.0	10.2	59.0	4.5	7.8	0.8	5.5	2.7	11.5	10.7	13.3	23.3	24.0
Contig_29	10.1	5.4	17.6	8.9	6.8	9.4	7.3	3.3	7.9	9.0	40.5	8.9	8.5	3.1	9.9	4.3	4.9	10.9	23.4	10.5	10.2
Contig_33	4.8	4.3	23.7	11.5	9.4	7.9	5.9	3.4	7.0	10.3	22.7	10.1	9.9	5.1	10.0	8.4	4.1	10.5	21.6	14.4	9.8
Contig_34	4.8	4.4	20.6	10.1	10.2	8.3	6.5	1.9	7.9	8.6	20.5	9.8	10.8	5.1	10.9	7.9	6.4	10.9	21.3	11.4	18.0
Contig_35	3.6	0.4	27.5	8.3	4.5	7.5	4.3	2.4	8.7	13.5	16.5	12.0	10.7	6.5	9.6	2.6	3.4	14.7	33.6	14.9	3.1
Contig_36	3.8	2.8	23.0	10.3	12.0	8.5	5.6	2.7	6.4	10.9	29.6	12.3	8.7	4.7	8.7	10.6	3.9	15.3	17.7	16.9	3.7
Contig_37	7.4	5.0	23.4	11.6	8.1	8.5	5.8	3.1	7.7	9.5	22.5	9.1	8.7	4.7	9.6	8.2	4.8	10.7	22.9	13.4	11.9
Contig_38	0.2	0.0	25.5	8.1	1.3	1.0	5.1	8.9	8.3	13.9	5.8	13.1	12.3	6.4	13.9	8.4	0.4	14.6	27.6	21.8	6.0
Contig_39	0.9	0.0	22.9	3.7	4.9	2.3	7.0	3.1	7.4	13.9	18.1	10.1	11.8	4.7	10.2	4.9	4.9	12.6	31.9	14.2	20.0
Contig_40	0.4	0.0	23.2	12.3	6.2	3.0	6.6	2.5	9.3	6.4	27.6	11.7	10.2	4.9	15.7	2.7	5.2	9.8	25.2	16.8	12.8
Contig_42	0.0	8.2	13.6	12.9	1.0	0.7	10.4	5.5	7.6	11.4	17.3	12.3	5.5	8.8	9.5	10.7	3.0	12.3	28.9	15.5	5.3
Contig_43	0.0	0.0	21.0	3.0	0.0	0.0	2.0	4.0	0.0	10.0	20.0	10.0	6.0	10.0	15.0	16.0	11.0	38.0	45.0	5.0	0.0
Contig_432	1.2	8.8	10.2	11.0	8.6	1.0	8.6	2.0	9.4	7.2	40.4	11.0	11.4	5.8	17.0	5.6	7.6	17.6	15.0	12.8	11.2
Contig_437	4.8	2.6	21.9	14.4	1.4	3.4	7.1	3.6	9.8	11.2	19.8	11.7	8.1	6.9	13.4	12.5	5.1	9.1	14.8	21.9	6.4
Contig_44	6.2	3.5	24.0	10.4	8.9	9.6	5.7	3.1	7.6	10.2	24.3	9.9	8.7	5.2	9.7	7.9	4.9	10.7	21.3	14.3	11.3

Contig_45	0.0	0.0	20.4	6.6	3.7	1.9	10.5	1.0	4.4	15.8	6.9	10.4	20.1	10.4	28.1	12.4	2.4	9.4	14.5	13.3	0.0
Contig_47	3.7	1.0	19.3	7.4	7.2	5.8	3.8	2.4	6.2	9.7	24.6	11.7	12.7	5.2	10.6	4.6	3.6	11.2	26.1	15.8	19.8
Contig_49	4.5	4.6	23.8	10.8	8.7	7.8	5.9	3.2	7.2	10.2	23.3	10.1	9.6	4.9	10.3	8.1	4.8	10.2	22.4	14.6	11.7
Contig_494	0.0	0.5	30.1	6.7	3.5	8.2	3.6	1.8	3.1	12.3	26.9	13.6	4.2	2.0	14.8	11.3	2.4	14.7	16.1	10.4	11.5
Contig_495	0.3	2.7	20.3	5.0	11.2	5.8	3.7	0.8	1.1	9.7	29.5	14.3	10.8	3.2	12.6	8.1	9.9	7.6	21.0	18.0	9.4
Contig_5	5.3	4.7	24.0	10.3	8.9	7.9	6.0	2.9	7.2	10.3	23.9	10.3	9.5	5.2	10.0	8.0	4.5	10.4	22.9	14.1	10.8
Contig_50	3.6	5.9	23.4	16.1	8.5	7.3	6.7	4.4	8.0	10.9	25.0	9.6	10.2	5.3	10.1	5.3	5.4	10.7	18.6	17.5	3.8
Contig_51	4.0	1.7	21.7	8.2	8.3	2.8	7.7	3.2	5.8	5.7	22.3	9.8	12.6	4.4	10.9	8.3	1.7	13.5	21.9	19.3	20.0
Contig_52	1.6	0.0	8.3	11.1	0.0	3.8	12.9	3.8	0.4	2.9	29.1	15.2	13.3	2.0	6.5	12.3	0.9	19.3	26.0	13.0	12.6
Contig_54	3.1	6.3	23.7	17.3	5.3	3.7	5.5	3.4	9.9	12.1	19.9	13.1	9.9	6.1	11.8	10.5	1.9	11.0	21.3	17.5	0.9
Contig_547	0.0	0.0	19.4	13.0	0.3	3.4	7.6	2.0	11.9	3.9	32.4	13.1	8.3	3.8	10.6	7.4	9.5	13.3	19.8	16.9	2.9
Contig_56	5.2	4.9	23.1	10.2	8.9	8.4	5.9	3.3	7.6	10.0	25.7	10.0	9.1	4.9	9.5	7.3	5.6	10.3	22.1	13.8	13.0
Contig_569	0.0	0.0	25.3	10.7	4.2	11.3	4.5	0.5	7.2	11.6	41.6	9.1	10.7	7.1	21.0	10.4	4.0	1.9	11.4	7.3	17.5
Contig_58	3.2	5.6	21.9	15.1	17.5	3.7	11.0	3.7	10.8	13.2	21.8	6.9	7.8	4.6	8.0	11.4	4.3	11.0	17.1	20.1	1.9
Contig_6	4.8	4.5	23.6	10.4	8.6	7.6	5.8	3.2	7.3	10.1	24.4	10.2	9.6	5.0	10.1	7.9	4.6	10.4	22.2	14.7	11.6
Contig_607	2.0	2.9	20.0	13.7	4.4	8.6	4.8	2.3	6.6	13.3	25.0	10.1	9.5	5.4	12.7	10.5	4.8	7.7	14.2	20.9	6.4
Contig_61	1.4	0.4	13.1	9.4	6.9	0.3	7.5	0.4	8.6	5.9	16.3	19.0	10.9	6.9	12.3	10.4	2.1	15.5	26.6	12.9	4.0
Contig_62	0.1	0.0	22.4	2.2	0.4	3.7	10.3	3.4	2.2	7.2	28.2	10.6	11.7	7.1	13.7	3.6	0.2	13.7	35.7	11.5	16.8
Contig_63	2.5	2.3	19.3	14.1	5.5	1.9	6.8	3.5	7.0	10.6	23.4	11.3	11.0	6.6	9.8	15.3	2.6	13.1	18.9	17.2	1.5
Contig_64	9.3	1.9	22.8	10.3	0.7	3.1	12.2	3.6	7.1	7.3	12.5	19.2	9.2	2.8	15.2	14.6	1.8	10.7	17.5	16.6	4.1
Contig_66	8.4	8.4	18.1	12.5	6.1	6.5	6.6	2.2	6.6	13.1	23.1	7.7	15.7	5.6	9.0	11.1	1.5	11.2	12.7	21.0	6.6
Contig_67	3.6	8.0	21.7	15.1	5.7	1.9	5.6	12.6	6.0	10.8	25.1	10.0	8.8	4.9	9.7	9.3	2.1	17.9	19.4	15.7	2.3

Contig_69	0.0	0.0	42.2	4.0	0.0	0.0	10.8	0.6	13.0	10.2	29.8	11.8	0.0	2.8	11.6	5.2	2.2	9.6	26.0	17.8	7.2
Contig_7	4.8	4.6	17.9	10.0	9.7	8.7	6.7	1.5	8.2	8.8	20.7	9.7	9.5	5.2	10.2	8.5	7.0	11.1	20.2	12.8	18.6
Contig_75	1.8	2.5	22.8	13.3	7.2	5.5	7.5	3.6	5.3	9.2	21.5	11.2	10.9	5.3	8.2	12.5	3.5	9.3	13.6	18.8	11.6
Contig_76	0.0	0.2	12.6	0.0	12.4	6.3	4.2	0.1	9.6	11.9	15.3	13.5	15.5	2.2	13.7	2.8	2.5	12.5	35.4	27.5	1.0
Contig_8	5.1	4.8	23.8	10.3	9.8	8.1	5.9	2.9	6.8	9.9	23.2	10.3	9.5	5.0	10.1	8.0	4.7	10.3	22.7	15.4	9.4
Contig_80	0.8	1.2	22.2	16.8	7.5	1.9	1.2	4.2	6.6	10.5	18.2	9.9	10.2	6.2	7.5	10.3	0.8	34.1	21.9	23.5	2.0
Contig_82	6.6	7.5	23.4	2.0	2.5	8.9	6.8	0.4	0.6	4.5	39.5	12.0	9.5	5.9	13.8	1.3	5.0	12.0	25.3	13.6	17.4
Contig_85	3.4	3.8	16.2	10.0	8.5	9.4	6.6	1.6	8.3	9.5	19.3	10.6	9.9	5.8	10.7	9.1	6.4	11.9	20.1	13.4	16.0
Contig_87	7.4	0.8	27.0	9.7	8.2	7.6	2.5	2.5	6.1	10.2	17.4	11.2	7.4	2.9	10.7	6.1	2.6	10.2	35.3	14.4	12.4
Contig_88	0.8	0.1	19.2	4.1	0.0	1.0	4.7	1.3	3.8	9.6	29.0	18.0	9.6	7.1	14.0	11.8	0.7	14.1	27.4	17.8	1.0
Contig_89	0.0	0.0	19.1	16.6	10.0	0.6	0.9	1.0	7.0	14.8	23.7	11.5	10.0	0.1	14.7	7.5	6.1	15.2	25.9	15.3	0.0
Contig_90	4.8	4.7	23.3	10.6	9.0	7.3	5.5	3.1	7.2	9.9	25.1	10.2	9.6	5.1	10.0	7.7	4.5	10.8	22.6	13.9	11.9
Contig_90	4.7	3.3	19.7	2.5	9.1	1.2	1.7	2.9	12.6	11.3	5.8	12.0	11.9	7.0	20.5	1.3	0.1	19.6	27.8	14.1	1.3
Contig_93	0.0	0.0	21.5	0.0	0.0	5.5	10.5	0.0	0.0	16.0	29.0	5.5	10.0	5.5	13.5	2.0	10.5	13.0	37.5	10.5	0.0
Contig_94	9.5	6.2	18.1	9.0	6.0	11.3	4.1	3.8	6.7	7.2	26.0	14.0	10.3	3.5	10.0	5.3	0.9	10.8	28.6	14.3	5.6
Contig_96	0.0	0.2	25.5	7.3	0.0	0.5	3.0	2.6	12.7	12.1	0.0	16.2	11.2	13.1	14.6	5.9	0.0	16.4	29.7	12.7	0.0
Contig_97	0.0	0.0	24.0	14.0	0.0	0.0	3.0	0.0	10.0	10.0	11.0	6.0	10.0	5.0	7.0	21.0	0.0	12.0	25.0	25.0	0.0
Draco	5.0	4.6	23.9	10.8	8.9	7.5	5.9	3.1	7.2	10.2	23.3	10.2	9.6	5.1	10.2	8.2	4.3	10.5	23.2	14.3	11.1
Gemini	4.5	4.9	18.8	9.7	9.7	8.6	6.3	1.5	7.8	8.4	20.7	10.0	9.7	5.3	10.1	8.0	6.7	11.3	20.8	12.9	18.6
Short-Contig_418	0.0	1.0	21.0	10.0	1.0	6.0	1.5	4.5	12.0	10.0	55.0	5.0	4.0	4.0	10.5	10.0	1.0	13.0	19.0	3.0	26.0

[1]CH=Chromosome from *Drosophila suzukii* reference annotation provided by Dr. Nicolas Gompel

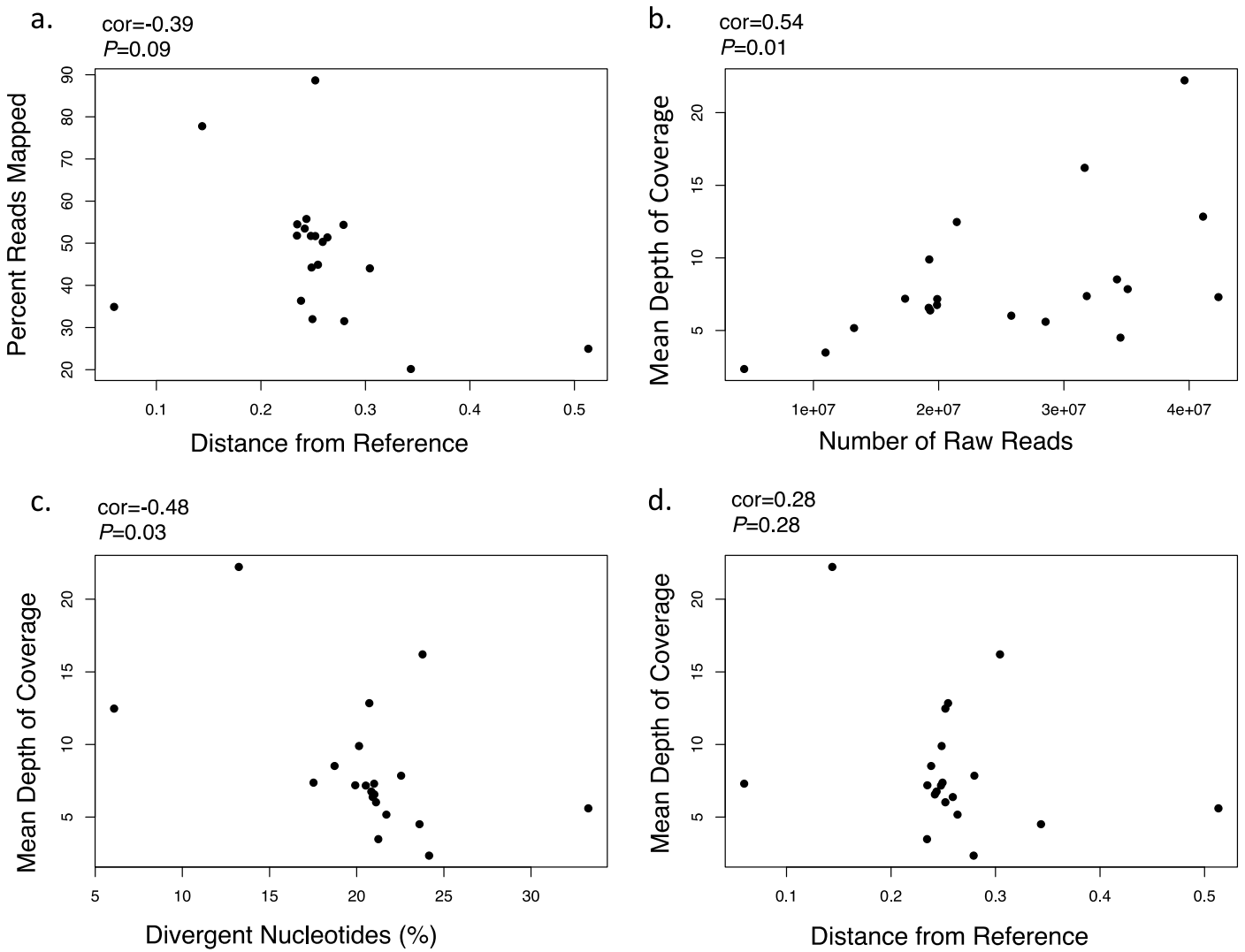
[2]First three letters of the species name.

[3]Values rounded to single decimal point for readability

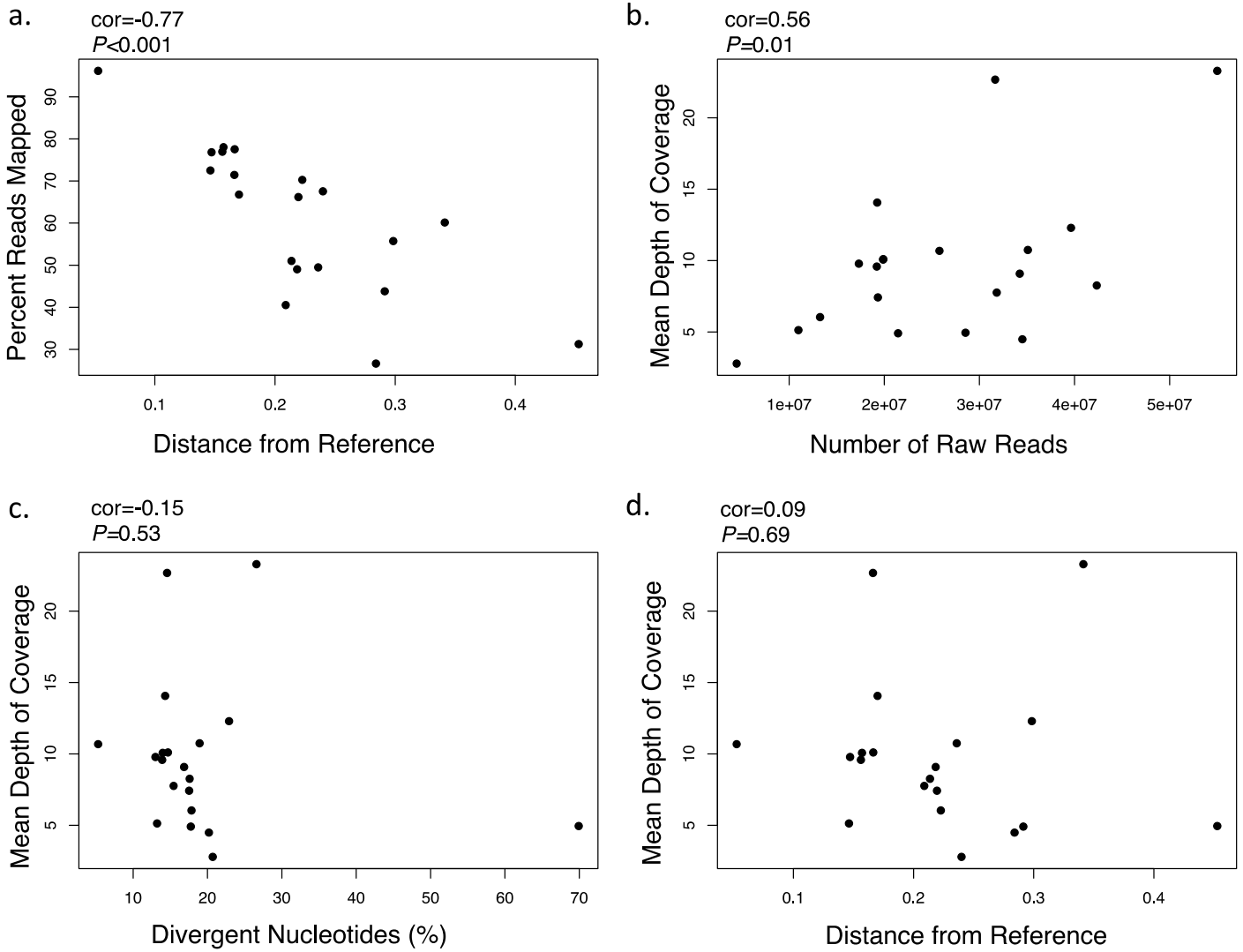
Table S2.7 Number of called variants by species

Species	*D. melanogaster* reference alignment	*D. suzukii* reference alignment
D. ananassae	188562[1]	114174[1]
D. auraria	141712	100127
D. biarmipes	611144	472593
D. elegans	363205	273898
D. eugracilis	344064	253076
D. ficusphila	286843	212602
D. fuyamai	109725	112552
D. gunungcola	10358	12937
D. lucipennis	184888	180802
D. lutescens	230761	285034
D. melanogaster	773515	389515
D. mimetica	233580	300784
D. paralutea	190105	274937
D. prostipennis	34849	67754
D. pseudotakahashii	205496	299671
D. rhopaloa	235677	206127
D. simulans	569823	107362
D. subpulchrella	164147	324035
D. suzukii	562555	503555
D. takahashii	407522	401576
D. yakuba	621800	282580

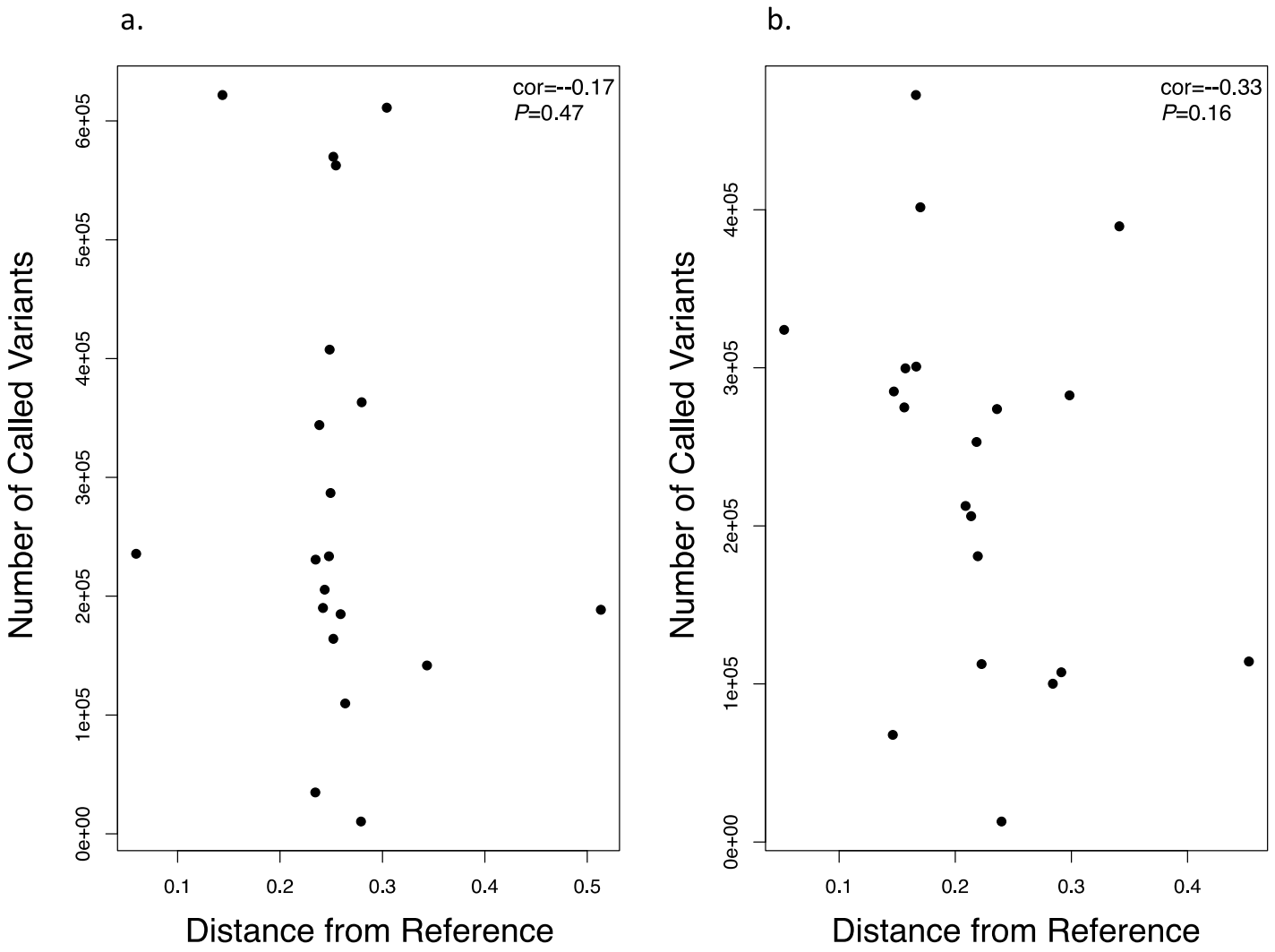
[1]Total number includes all informative characters (A,G,T,C) and does not include Ns or singletons



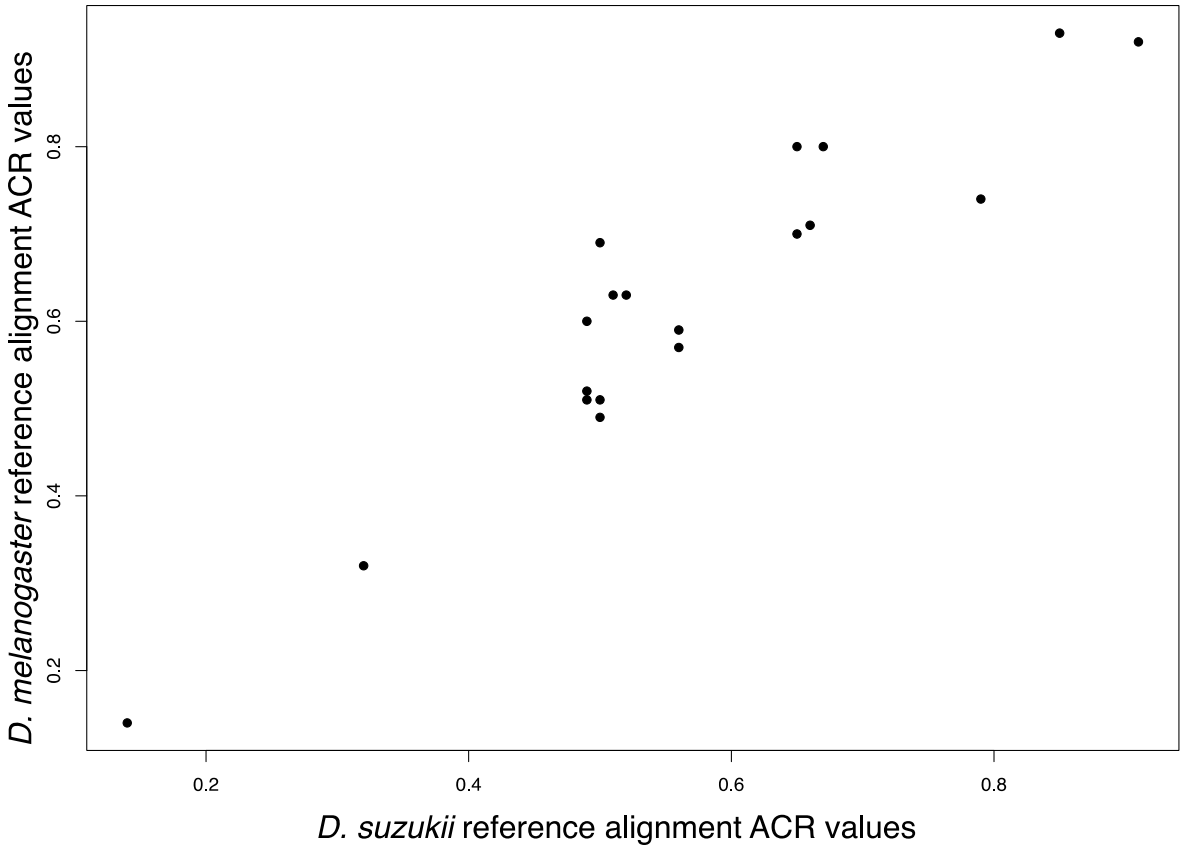
Supplementary Figure S2.1. Whole genome processing using the *D. melanogaster* reference genome. Showing: a) mapping percentage of each species compared to phylogenetic distance of each species to the reference calculated using the package adephylo package in R (Jombart et al. 2010), b)the mean depth of coverage for each species compared to the number of raw reads, c) the mean depth of coverage for each species compared to the divergence measured as the percent of divergent nucleotides in the annotated alignment and, and d) the mean depth of coverage for each species compared to the phylogenetic distance from the reference genome. Pearson's correlations and their corresponding *P* value are represented at the top left corner of each plot.



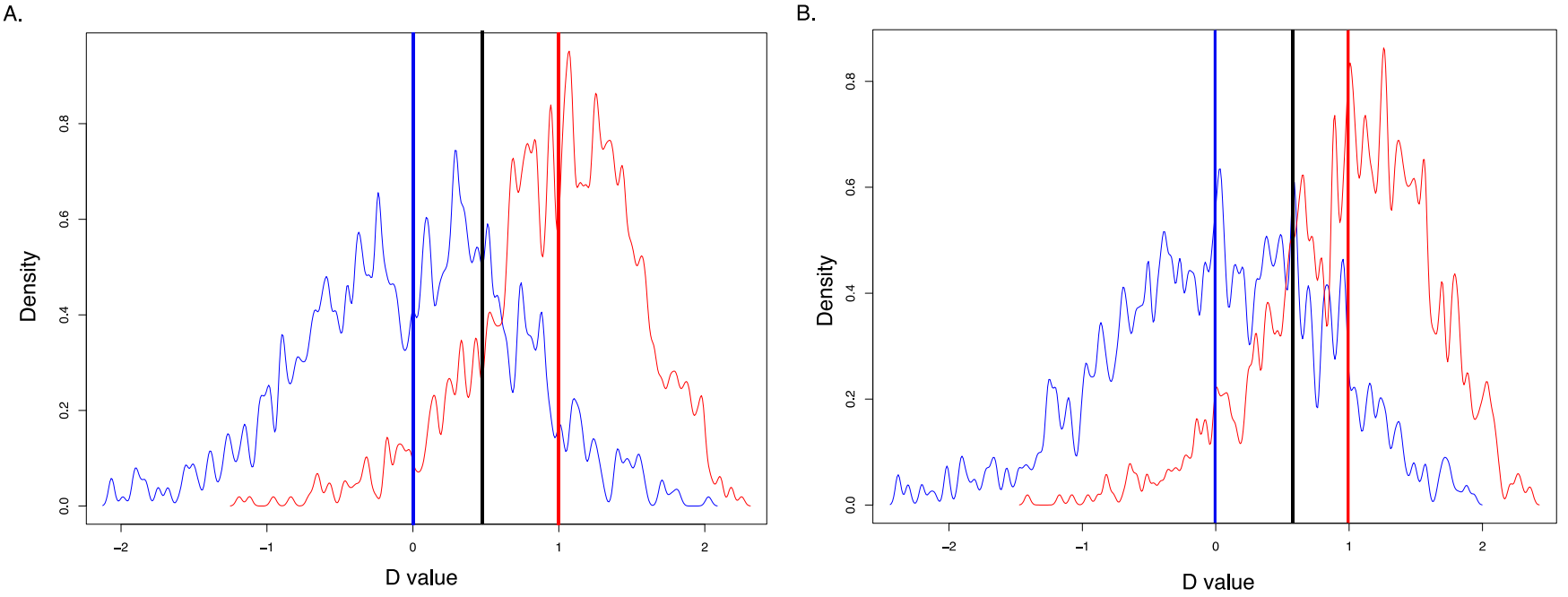
Supplementary Figure S2.2. Whole genome processing using the *D. suzukii* reference genome. Showing: a) mapping percentage of each species compared to phylogenetic distance of each species to the reference calculated using the package adephylo package in R (Jombart et al. 2010), b) the mean depth of coverage for each species compared to the number of raw reads, c) the mean depth of coverage for each species compared to the divergence measured as the percent of divergent nucleotides in the annotated alignment and, and d) the mean depth of coverage for each species compared to the phylogenetic distance from the reference genome. Pearson's correlations and their corresponding *P* value are represented at the top left corner of each plot.



Supplementary Figure S2.3. Number of Variants Called as a Function of Distance from the Reference Genome. The total number of variants (excluding non-informative characters and singletons) that were called for the phylogenetic analysis for each species aligned to a) the *D. melanogaster* reference genome and b) the *D. suzukii* reference genome. There was no relationship between the number of characters for a species due to its phylogenetic distance from the reference genome species. Pearson's Correlation and corresponding *P* values are found in the upper right-hand corner of each plot.



Supplementary Figure S2.4. Ancestral character estimation (ACR) values of the *D. melanogaster* reference alignment phylogeny as a function of ACR values of the *D. suzukii* reference alignment phylogeny. The nodes that encompassed the same species for each tree were compared to determine concordance of ancestral character estimation between the two tree topologies. Ancestral character estimations were comparable between the two reference alignment phylogenies (Pearson's Correlation=0.94, *P*=0.002).



Supplementary Figure S2.5 Phylogenetic signal of the wing spot. Distribution of 1000 simulations from the wing spot character distribution data of the A. *D. melanogaster* reference phylogeny and B. *D. suzukii* reference phylogeny under a Brownian motion model (in blue) and a Random Model (in red) used to estimate the phylogenetic signal (D) of the binary trait (spotted and spotless). Vertical lines represent the expected values of D under the model. The black vertical line represents the estimated value of D given the real data, which is not statistically significantly different from either model in either test for the *D. melanogaster* alignment phylogeny (D=0.48, $P_{D=0}$ = 0.15, $P_{D=1}$ = 0.26) or the *D. suzukii* alignment phylogeny (D=0.58, $P_{D=0}$ = 0.25, $P_{D=1}$ = 0.23)

Appendix 2.2 Command Line Code for RAxML Phylogenetic Analysis

Runs RAxML rapid bootstrap on the annotated FASTA file, then a slow non-parametic bootstrap on the same file, and finally a best tree for the non-parametric slow bootstrap.

###Run RAxML for annotated alignments with 100 rapid bootstrap

replicates under the GTRGAMMA model. For every run, change x using random number

generator

raxml -s <input.fasta> -n <output tree name> -m GTRGAMMA /

-x 123476 -f a -# 100 -p 100 /

-o <name of output species in vcf>

####Run RAxML for filtd snps with 100 non-parametric bootstrap replicates under the

GTRGAMMA model.For every run, change x using random number generator

raxml -s <input.fasta> -n <output tree name> -m GTRGAMMA /

-b 123476 -p 123476 -# 100 /

-o <name of output species in vcf>

####Run RAxML to find best tree for non-parametric bootstrap replicate tree

raxml RAxML_<formernpbstree.tree> -t <rapid bootstrap tree> /

-n <output tree name>

Appendix 3

Appendix 3.1: Supplementary Tables and Figures for Chapter 3

Supplementary Table 3.1: Stocks used for behavioral analysis

Species	Species Group	Stock ID	Source	Origin
Spotted Species				
D. biarmipes	*suzukii*	14023-0361.11	NDSSC[1]	Spring Valley Cambodia (2011)
D. elegans	*elegans*	14027-0461.00	NDSSC	Hong Kong (2010)
D. fuyamai	*rhopoloa*	14029-0011.00	NDSSC	Brunei (2003)
D. lutescens	*takahashii*	14011-0271.00	NDSSC	Honshu, Japan (1976)
D. prostipennis	*takahashii*	14022-0291.00	NDSSC	Taiwan (1968)
D. pseudotakahashii	*takahashii*	14022-0301.01	NDSSC	Australia
D. subpulchrella	*suzukii*	DsubX	Turelli Lab	Chin Lab (2014)
D. suzukii	*suzukii*	WT4	Turelli Lab	Watsonville, CA (2009)
Spotless Species				
D. gunungcola	*elegans*	Dgun SK	Witkopp Lab	Sukarami, West Sumatra, Indonesia (1999)
D. lucipennis	*suzukii*	14023-0331.01	NDSSC	Wulai, Taiwan (1968)
D. mimetica	*suzukii*	14023-0381.01	NDSSC	Brunei (2002)
D. paralutea	*takahashii*	14022-0281.01	NDSSC	Unknown
D. takahashii	*takahashii*	14022-0311.10	NDSSC	Kagoshima, Japan (2005)

[1] NDSSC= National *Drosophila* Species Stock Center. Species were obtained when the stock center was at the University of California, San Diego

Supplementary Table S3.2 Summary of results for all evolutionary models for LR analysis

Mating Parameter	Brownian Motion			Orstein-Ulenbeck			Pagel's Lambda		
	AIC[1]	Likelihood[2]	P Value[3]	AIC[1]	Likelihood[2]	P Value[3]	AIC[1]	Likelihood[2]	P Value[3]
Courtship Frequency	29.94	-11.97	0.119	25.21	-8.60	0.713	28.54	-10.27	0.619
Copulation Frequency (60 min)	60.75	-27.38	0.454	61.18	-26.59	0.715	62.75	-27.38	0.454
Copulation Frequency (10 days)	61.82	-27.91	0.002	60.57	-26.29	0.012	61.67	-26.84	0.004

[1]Akaike Information Criterion values
[2]Likelihood values for the given model
[3]*P* value for the association between spot type and treatment effect under the given model

Supplementary Table S3.3. Summary statistics for courtship observations

	Courtship Frequency				Courtship Latency			
	Light		Dark		Light		Dark	
Species	N[1]	Proportion	N[1]	Proportion	N[2]	Mean[4] (seconds)	N[2]	Mean[3] (seconds)
Spotted Species								
D. biarmipes	22	0.95	31	0.82	21	107.57 ± 27.93	27	375.00 ± 41.02
D. elegans	25[4]	0.88[4]	29[4]	0.48[4]	22	172.23 ± 28.94	14	1022.71 ± 133.29
D. fuyamai	29	0.66	29	0.62	19	426.26 ± 90.01	18	357.33 ± 46.30
D. lutescens	21	0.95	25	0.64	20	309.55 ± 49.89	16	759.81± 155.74
D. prostipennis	20	0.90	27	0.23	19	356.00 ± 52.85	5	209.40± 55.15
D. pseudotakahashii	28	0.71	30	0.53	20	738.85 ± 132.66	16	682.63 ± 180.16
D. subpulchrella	43	0.40	41	0.32	17	632.06 ± 78.70	13	1243.38 ± 124.60
D. suzukii	26	0.83	30	0.84	22	436.00 ± 123.83	25	552.00 ± 77.60
Spotless Species								
D. gunungcola	39	0.56	40	0.40	22	677.9 1± 59.20	16	791.44 ± 55.01
D. lucipennis	27	0.81	30	0.60	22	174.95 ± 54.28	18	729.22 ± 102.24
D. mimetica	23	1.00	23	0.65	23	177.83 ± 26.28	15	368.73 ± 28.84
D. paralutea	23[5]	0.87	25	0.52	20	361.80 ± 73.07	13	336.08 ± 93.26
D. takahashii	18	1.00	18	0.94	18	119.31 ± 30.54	17	108.31 ± 31.77

[1] Number of pairs observed.
[2] Number of males that courted among the pairs observed.
[3] Mean ± standard error of the mean. Only males that courted in the 60-minute observation period were included.
[4]Values that differed significantly between treatments are shaded. Significance values are specified in Table 3.1

Supplementary Table 3.4. Summary statistics for copulation observations

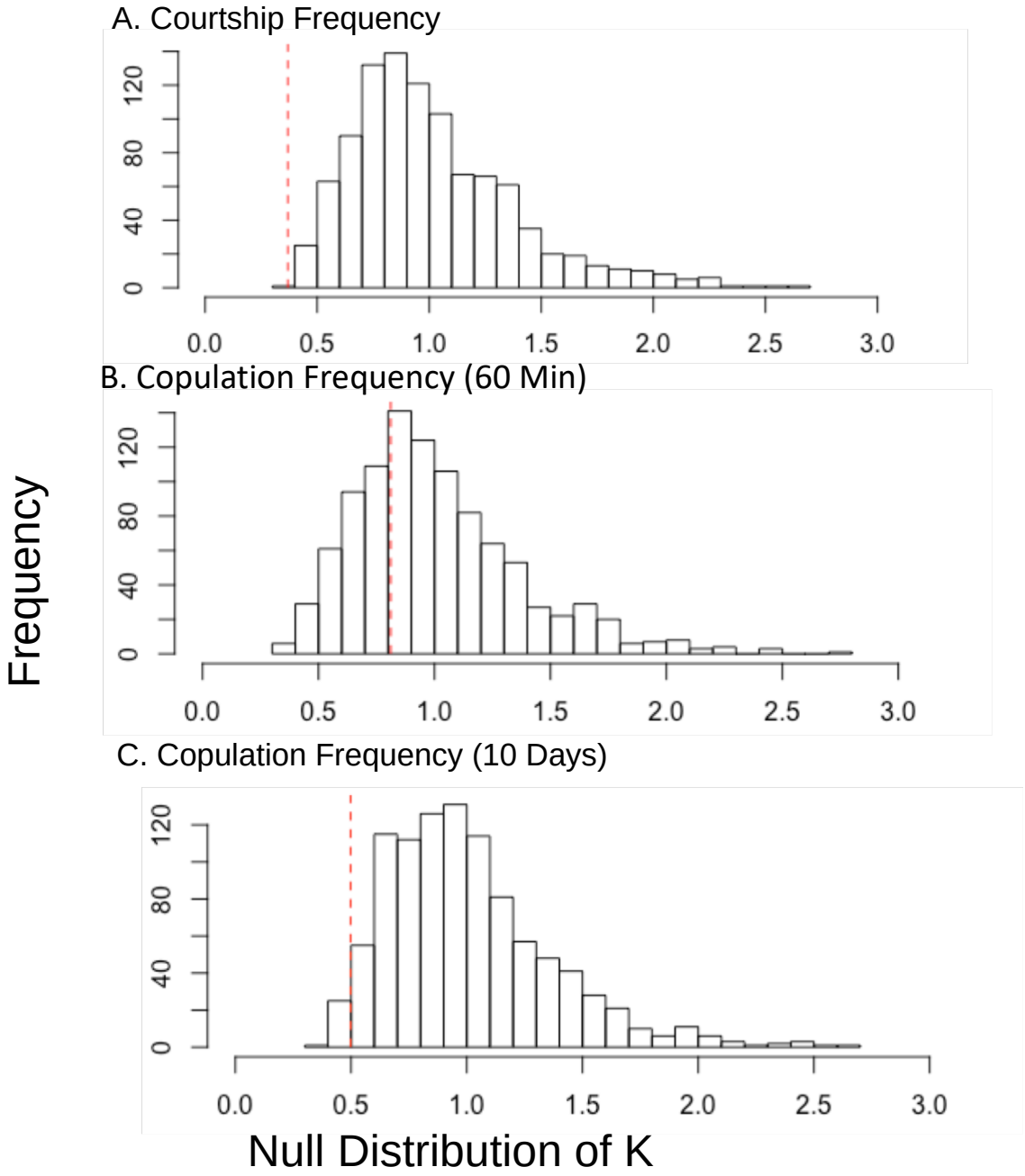
	Copulation Frequency				Courtship Duration[2]				Copulation Duration			
	Light		Dark		Light		Dark		Light		Dark	
Species	N[1]	Proportion	N[1]	Proportion	N[2]	Mean [3](seconds)	N[2]	Mean [3](seconds)	N[2]	Mean[3] (seconds)	N[2]	Mean [3] (seconds)
Spotted Species												
D. biarmipes	21	0.80	27	0.70	17	342.71 ± 76.26	19	1274.5625 ± 199.66	17	900.48 ± 72.99	19	808.86 ± 29.04
D. elegans	22	0.91	14	0.07	20	228.50 ± 26.45	1	336.00	20	1289.10 ± 72.42	1	1209.00
D. fuyamai	19	0.68	18	0.22	13	836.85 ± 145.60	4	673.80 ± 136.12	13	793.69 ± 73.82	4	618.00 ± 83.45
D. lutescens	20	0.70	16	0.13	14	608.07 ± 145.36	2	759.81 ± 772.50	14	1886.86 ± 67.69	2	1892.00 ± 5.00
D. prostipennis	18	0.00	5	0.00	0	NA	0	NA	0	NA	0	NA
D. pseudotakahashii	20	0.25	16	0.25	5	688.6 ± 161.40	4	406.00 ± 142.27	5	1780.60 ± 93.39	4	1473.50 ± 115.47
D. subpulchrella	17	0.76	13	0.00	13	851.85 ± 105.10	0	NA	13	1308.42 ± 130.53	0	NA
D. suzukii	22	0.73	25	0.00	16	1427.69 ± 180.66	0	NA	16	2909.69 ± 203.45	0	NA
Spotless Species												
D. gunungcola	22	0.59	16	0.13	13	761.31 ± 132.23	2	956.00 ± 74.00	13	1074.85 ± 78.74	2	1178.00 ± 119.00
D. lucipennis	22	0.68	18	0.00	15	327.07 ± 51.58	0	NA	15	210.13 ± 18.04	0	NA
D. mimetica	23	0.91	15	0.93	21	284.76 ± 22.45	14	369.93 ± 44.67	21	1124.19 ± 48.21	14	937.36 ± 93.27
D. paralutea	20	0.10	13	0.00	2	1816.00 ± 831.00	0	NA	2	1641.50 ± 181.50	0	NA
D. takahashii	18	1.00	17	0.88	18	651.53 ± 160.98	15	702.07 ± 174.89	18	1215.27 ± 63.51	15	1037.69 ± 52.89

[1] Number of pairs observed.
[2] Number of pairs that copulated in the 60 minute observation period among the pairs observed.
[3] Mean ± standard error of the mean. Only pairs that in the 60 minute observation period were included.
[4]Values that differed significantly between treatments are shaded. Significance values are specified in Table 3.1

Supplementary Table 3.5. Summary statistics for larval presence after ten days

	Light		Dark	
Species	N[1]	Proportion	N[1]	Proportion
Spotted Species				
D. biarmipes	22	1.00	31	0.97
D. elegans	25	0.84	29	0.04
D. fuyamai	29	0.66	29	0.38
D. lutescens	21	0.90	23	0.15
D. prostipennis	20	0.90	27	0.0
D. pseudotakahashii	28	0.79	25	0.20
D. subpulchrella	39	0.33	38	0.00
D. suzukii	26	0.96	30	0.00
Spotless Species				
D. gunungcola	29	0.80	35	0.63
D. lucipennis	27	0.63	30	0.60
D. mimetica	23	0.91	20	0.80
D. paralutea	23	0.74	25	0.80
D. takahashii	18	0.94	16	0.88

[1] Number of living pairs observed. All pairs placed from light into dark after mating survived and produced larvae; their progeny are included in the proportion of larval presence in the light.
[2]Values that differed significantly between treatments are shaded. Significance values are specified in Table 3.1



Supplementary Figure S3.1. Observed phylogenetic signal plotted to a Brownian null distribution. Observed values of Blomberg's K were plotted over a null distribution generated from simulated data in a Brownian motion framework. A K value of 1 represents a Brownian motion process. The red dashed line designates the observed K value for each $\log_2$ treatment effect A. courtship frequency; B. copulation frequency in the first 60 minutes of observation; and C. copulation frequency as measured by the presence or absence of larvae in vials after ten days. From 1,000 simulations from a Brownian motion distribution of possible K values, the expected mean value of K is approximately one. The K value from the actual courtship frequency data fall significantly under expected mean values under a stochastic process. The K value for copulation after 60 minutes does not fall significantly under the expected mean K value from a stochastic process. The K value for copulation frequency after 10 days falls significantly under a value expected from a stochastic process.