Evolution of Sexual Signals in the *Drosophila saltans* Species Group

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Evolution of Sexual Signals in *Drosophila saltans* Species Group

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Abstract

The complex courtship signaling of three of the four species groups of the *Sophophora* subgenus (e.g., *melanogaster*, *obscura*, and *willistoni*) has been studied. In this dissertation, I examined the complex courtship signaling of the fourth species group, *saltans*, for the first time. In the first chapter, I assessed through what modalities courtship signals were communicated in *Drosophila saltans* (*sensu stricto*). No single modality ablation eliminated mating, and all ablations affected mating, thus courtship was multimodal. In the second chapter, I examined if the role of two modalities (i.e., vision and audition) during mating were correlated in nine species of the *saltans* species group. In particular, I investigated if the two modalities were assessed in an integrated manner (i.e., positively correlated), or if there was a tradeoff between the role of two modalities (i.e., negatively correlated). The role of vision and audition varied from playing no role to being necessary for mating success and were not correlated when considering independent contrasts. In Chapter Two, I also described the auditory courtship signal of each species of the group and found variation in song production with one species producing an elaborated song element and two species producing reduced songs. Furthermore, song production (reduced or elaborated) was not associated with the role of audition in mating success. In the third chapter, I examined if the two song types (i.e., pulse and beep) in the elaborated song of *D. sturtevanti* communicated one or multiple messages and if the messages were unique or redundant. The song of *D. sturtevanti* conveyed multiple redundant messages. In summary, I found that courtship signaling was complex by being multimodal in *D. saltans* (*sensu stricto*) and containing both multiple, and redundant messages in the auditory modality of *D. sturtevanti*. Also, I found that apparent signal complexity of song was not associated with the role of song during courtship.

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Introduction

A signal is energy generated by a behavior or action of one organism (sender) that induces a behavior in another (receiver) and is evolutionarily selected because it is beneficial to the sender and/or the receiver (Wilson 1975). In nature, signals must be filtered through both the environment and the receiver, potentially complicating how messages are sent and received. Signaling environments are often noisy, placing efficacy-based selection pressures on the signals traveling through them (reviewed in Hebets & Papaj, 2005). Receiver sensory constraints may also limit signal transmission, placing sensory detection and internal processing efficacy-based selection pressures on signals (reviewed in Hebets & Papaj, 2005). To overcome transmission and reception constraints, signals are often complex. Two ways in which signals can be complex are by being multimodal, containing more than one signal in more than one modality, or by being comprised of multiple signals within a single modality (reviewed in Hebets and Papaj 2005).

During courtship many messages need to be communicated to ensure accurate recognition and assessment of potential mates. One role of courtship signaling is communication of species identity (Ryan, 1990), which is necessary for choosing a compatible mate with whom reproduction will result in fertile offspring (Andersson, 1994; Mendelson & Shaw, 2012). Another role of courtship signaling is communication of mate quality, which allows females to distinguish between high and low quality males (Andersson, 1994). Considering the various messages that need to be communicated, courtship communication is often complex, occurring as an arrangement of behaviors in which the sender and receiver use multiple sensory channels to send and receive signals (Higham & Hebets, 2013). When signals are complex, both the signaler and receiver benefit from greater signal detectability ("how easily the signal can be perceived as distinct from environmental noise") and discrimination (a learned or innate predisposition to

respond differently to various stimuli; reviewed in Rowe, 1999). Furthermore, complex signals may evolve to communicate one message more efficaciously through a variable environment, or to convey multiple messages more efficiently (e.g., those necessary for sexual communication; reviewed in Hebets & Papaj, 2005).

Courtship communication in the fruit fly, *Drosophila melanogaster*, occurs through chemical, tactile, visual, and acoustic stimuli (reviewed in Greenspan & Ferveur, 2000). Many other *Drosophila* species have similarly multimodal courtship (reviewed in Markow & O'Grady, 2005). Not only is the display often multimodal, but single modalities can be comprised of multiple signals (reviewed in Markow & O'Grady, 2005).

Drosophila is a large genus made up of over 1,600 described species (O'Grady and DeSalle, 2018), a few hundred of which have described mating displays (reviewed in Markow and O'Grady, 2005), making it ideal for addressing large-scale questions about the evolution of mating behavior. The mating behavior and sexual signals of three of the four species groups in the *Sophophora* subgenus (e.g., *melanogaster*, *obscura*, and *willistoni*) have been studied (reviewed in Markow & O'Grady, 2005). In this dissertation, I focus on the fourth group, *saltans*, the mating behavior of which has been largely unexplored. The *saltans* species group is a Neotropical clade comprised largely of sympatric species (de Campos Bicudo, 1973), and most closely related to the *willistoni* species group.

This dissertation incorporates methods from animal behavior, evolution, and phylogenetics and focuses on the mating behavior and sexual signals of the *saltans* species group to address the following questions:

1. What sensory modalities do males and females rely on to send and receive signals during courtship?

2. How does the role of hearing and vision in mating success vary across species group and is their role correlated?
3. How does courtship song vary across species group and is the production of song associated with its role in mating success?
4. Does a two-part signal (song) convey multiple or redundant messages?

Chapter 1 : Dissection of signaling modalities and courtship timing reveals a novel signal in *Drosophila saltans* courtship *

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Abstract

Courtship signaling, necessary for the recognition of potential mates, is often complex and uses many modalities with multiple signals or components. *Drosophila* courtship is comprised of chemical, tactile, visual and acoustic stimuli. Ablation of single sensory channels, either signal production or reception, can determine the roles of individual modalities in overall reproductive success. Adding measures of courtship timing, particularly courtship latency, the time for the male to initiate courtship, and courtship duration, the time from courtship initiation until the female accepts the male for copulation, allows me to identify the stage of courtship at which a signal acts. This study focuses on *Drosophila saltans*, a member of the *saltans* species group. Little is known about sexual behavior of species in this group, part of the *Sophophora* subgenus. I find that the ablation of any one signal in *D. saltans* does not eliminate mating success, and instead all modalities examined contribute to mating success. Thus courtship is multimodal. In addition to examining the signals and signal reception common to most *Drosophila* species, I also examine the role that the midtarsi play in courtship. The removal of the female's midtarsi significantly reduces mating occurrence. Measuring courtship latency and courtship duration, as well as the occurrence of courtship and copulation, allows me to determine if a signal plays a role in activating the male to initiate courtship or stimulating the female to mate. Using timing data, I discern that the absence of midtarsi in the female does not affect the male's ability to identify the female as a potential mate, but the male may be unable to sufficiently stimulate the female without midtarsi to copulation.

Introduction

Courtship behavior is comprised of one or more signals that ensure accurate recognition and assessment of potential mates. A signal is any stimulus that, once emitted, benefits both the signaler and receiver and has evolved under selection for the purpose of communicating information (Maynard Smith & Harper, 2004). One role of courtship signaling is communication of species identity (Ryan, 1990), which is necessary for choosing a compatible mate with whom reproduction will result in fertile offspring (Andersson, 1994; Mendelson & Shaw, 2012). Another role of courtship signaling is communication of mate quality. Examining the role of signals in mate choice is necessary for understanding how individuals choose appropriate mates.

Courtship communication is often multimodal, occurring as an arrangement of behaviors in which the sender and receiver use multiple sensory channels to send and receive signals (Higham & Hebets, 2013). Multimodal displays may increase the effectiveness of signal transfer by conveying redundant signals or by conveying multiple signals expeditiously through multiple sensory channels (Partan & Marler, 2005). By eliminating individual courtship signals or sensory channels used to receive the signals, one can test the roles of individual signals in a display to determine if they are redundant (their absence does not change reproductive success), are essential (absence eliminates reproductive success), or play a synergistic role (absence does not eliminate reproductive success but may affect the speed at which mating occurs). Through ablation of signals and their reception, the roles of signals may be individually determined.

In most species of *Drosophila*, courtship communication occurs through chemical, tactile, visual, and acoustic stimuli (reviewed in Markow & O'Grady, 2005). For example, in *Drosophila melanogaster*, courtship begins when the male and the female come into contact, usually on a food source (Ewing, 1983). At this stage, and throughout the entirety of courtship,

visual signals may be important for either the male or the female (Greenspan & Ferveur, 2000). Generally, the male initiates communication by approaching the potential female mate and tapping her abdomen with his foretarsi (Spieth, 1974), which contain chemoreceptors (Stocker, 1994). By "tasting" cuticular hydrocarbons (CHCs) that function as pheromones (Ferveur, 1997), the male gains information about whether or not the other individual is a female, if she is a conspecific, and if she has recently mated (Cobb & Jallon, 1990). In addition to perceiving CHCs through gustatory receptors on the foretarsi, the male may also detect CHCs and other pheromones through olfactory receptors on the antennae (Stocker, 1994). After receiving olfactory input, the male may break off courtship if anti-aphrodisiac signals are received (e.g., Cobb & Ferveur, 1996) or he may continue courtship and proceed by sending courtship signals through other sensory channels. A male continuing courtship will vibrate his wing(s) to create a species-specific courtship song (e.g., Liimatainen et al., 1992; Ritchie et al., 1999) that is received by the female through her aristae, the sound reception organ (Cook, 1973a, 1973b). The male will closely follow the female, lick the tip of her abdomen with his proboscis (also containing gustatory and olfactory receptors, Stocker, 1994) and periodically bend the tip of his abdomen to meet hers to attempt to copulate. When the female is receptive, she will slow down locomotion and spread her wings in order to allow the male to mount and copulate. Female courtship signaling has been described to be limited to rejection signals with the exception of the final acceptance signal, though female behaviors remain understudied (Dukas & Scott, 2015).

The importance of a particular sensory modality and the associated signal(s) varies across the *Drosophila* genus (Ewing, 1983; Spieth, 1974). For example, vision is necessary for male reproductive success in *Drosophila nebulosa* but not in *Drosophila willistoni*, which are from the same species group (Gleason et al., 2012). Vision is necessary for males of both *Drosophila*

subaquinaria and the closely related *Drosophila recens* (Giglio & Dyer, 2013). Acoustic signals also vary in the role they play in courtship success. Species-specific courtship song increases the rate at which mating occurs in *D. melanogaster* and *Drosophila simulans* (and to a lesser extent in *Drosophila sechellia*) but is not necessary for mating in these species (Ritchie et al., 1999). Variation in courtship song contributes to reproductive isolation in *D. melanogaster* and *Drosophila lini* and their respective sibling species because females use species-specific song components to discriminate against heterospecifics (Ritchie et al., 1999; Wen et al., 2011). In *Drosophila montana* absence of courtship song inhibits mating completely (Liimatainen et al., 1992).

To understand the role of isolated signals in a multimodal courtship repertoire, one signal may be ablated at a time and the subsequent effect on courtship and mating success examined (e.g., Gleason et al., 2012; Hebets & Uetz, 1999; Liimatainen et al., 1992). Signal transmission can be ablated by preventing the production of the signal or by obstructing the reception of the signal. If a signal is essential to elicit courtship or copulation, the ablation will eliminate courtship and/or copulation. Alternatively, single signals may not be necessary but may facilitate courtship and copulation. This latter aspect may be missed by focusing solely on the *occurrence* of courtship and/or copulation, as has been done in many studies (e.g., Benelli et al., 2012; Giglio & Dyer, 2013; Gleason et al., 2012; Mayr, 1950; Narda, 1966; Robertson, 1983). Measuring courtship latency (the time it takes courtship to start) and courtship duration (time from the start of courtship to the start of copulation) may help to better understand the stage of courtship at which a signal acts. An increase in courtship latency after a signal ablation has different implications than an increase in courtship duration. Long courtship latency means that the male is unable to detect a female signal efficiently or be sufficiently stimulated to initiate

courtship. In this case, either the male cannot receive a signal or the female cannot send a signal. Long courtship duration can mean that the male is unable to sufficiently stimulate the female to copulation acceptance or that the male cannot receive the female's acceptance signal. Courtship latency and duration must be assessed to disentangle at what stage of courtship progression a specific sensory modality plays a role.

In this study, I focused on *D. saltans,* a member of the *saltans* species group that has observed courtship behavior similar to what was described above. The *saltans* species group is a Neotropical clade comprised largely of sympatric species (de Campos Bicudo, 1973), and most closely related to the *willistoni* species group. The mating behavior and sexual signals of the other species groups in the *Sophophora* subgenus (the *melanogaster*, *obscura,* and *willistoni* groups) have been studied extensively (reviewed in Markow & O'Grady, 2005) but the *saltans* group has not received the same attention. Within the *saltans* group, species vary greatly in their courtship song (Chapter two), thus this group is a good model to examine the importance of sensory modalities and use of sexual signals. Understanding the sexual behavior of *D. saltans* (*sensu stricto*) will allow me to start filling in the gap of our understanding of sexual behavior in the *Sophophora* subgenus and allow for the examination of shifts in signals and their associated roles across the subgenus.

I examined the relative importance of individual sensory modalities in *D. saltans.* I hypothesized that due to the multimodal nature of signaling in *D. saltans*, ablation of a single signal or its reception will not cause elimination of mating. I found this to be case for *D. saltans*. even though removing two sensory modalities together, olfaction and hearing (olfaction cannot be isolated because the hearing sensory organ is located at the tip of the olfactory sensory organ), eliminates mating. In addition, through our experiments I discovered a new behavior involving

the midtarsi. Removal of the female's midtarsi significantly affected mating success. By exploring timing data (courtship latency and courtship duration) I found evidence that the new behavior may mediate an interaction between the male and female that significantly increases the probability of mating.

Methods

Fly Culturing

I maintained *D. saltans* (*Drosophila* Species Stock Center stock number: 14045-0911.00) cultures in 24 mm d x 94 mm h vials containing standard cornmeal-molasses *Drosophila* food at 24°C with 12:12 light:dark cycle. The stock culture was maintained with 15–30 flies of both sexes. Subcultures were standardized to generate the flies for our experiments by being started with ten potentially gravid females and one male. These flies were removed after 2–3 weeks. Virgin experimental flies were collected under light CO_2 anesthesia within 4 hours of eclosion. Virgin flies were housed in single-sex groups of up to 10 individuals in small food vials (16.5 mm d x 95 mm h) with cotton plugs.

Behavioral Assays

Individual virgins were removed from single-sex group vials at 7–9 days post eclosion and were assigned to the manipulation treatments as described below. Post manipulation, flies recovered for 24–48 hours before behavioral assays were performed. In each behavioral assay, a single male and a female were aspirated into a new, small food vial. The cotton was pushed into the vial to restrict the flies to approximately 1 cm^3 space. A single trial consisted of observations of all possible treatments simultaneously (control female with control male, manipulated female with control male, control female with manipulated male and both sexes manipulated). The observer watched the flies for an hour or until copulation was completed. The proportion of

males that courted and proportion of pairs that mated were calculated, as well as the courtship latency and courtship duration for all pairs.

Vision

To determine the general effect of light on mating success, pairs of virgin males and females were placed in small food vials in a standard 12:12 light:dark (light treatment; N=87) cycle or in a continuous dark (dark treatment; N=95) cycle for seven days. Females were aspirated first into small vials and then randomly assigned a treatment. For the light treatment, males were introduced into the vials assigned to a normal photoperiod (12:12 light:dark) in a lit, 24° C room. For the dark treatment, males were introduced into the vials under a red light and kept in a 24° C, continuously dark incubator. Seven days later, all vials were scored for the presence of larvae. Only vials with both parents alive at the end of the seven-day incubation were used in analysis.

To test the specific effects of vision on each sex, individuals were blinded (N= 20). Flies were aspirated and immobilized in a truncated pipette tip. Experimental individuals were blinded by covering their ommatidia with a dot of paint from a non-toxic gold metallic Sharpie® paint marker, while control individuals received a dot of paint on the back of their head to control for the presence of paint. Individuals were group housed by treatment (control or experimental treatment) in single-sex groups of up to 10 individuals in new, small food vials. Behavioral assays proceeded as described above.

Wing removal

To determine the effect of the production of song on mating success, wings (the song production organ) were removed (N= 15). Flies were anesthetized with light CO_2 and separated into either a wing treatment (control) or wingless treatment. The wings were removed from the

wingless treatment individuals by severing the wing close to the body with a dissecting probe. Individuals were group housed by treatment in single-sex groups of up to 10 individuals in new, small food vials. Behavioral assays proceeded as described above.

Aristae removal

To determine the effect of song reception on mating success, I removed the aristae (sound perception organ; N= 15). Flies were aspirated and immobilized in a truncated pipette tip. Aristae were removed from half of the individuals by pinching the aristae at the base between a razor blade and synthetic rubber eraser. Control individuals were held in the pipette tip for an equivalent amount of time as required to remove the aristae. Behavioral assays proceeded as described above.

Olfaction

To determine the effect of olfaction on courtship and copulation, we removed antennae (N= 16). Flies were aspirated and immobilized in a truncated pipette tip. Antennae were removed from half of the individuals by cutting them off with a small razor blade. Aristae removal (described above) was used as the control for this group in order to decouple the effects of olfaction and audition because antennae cannot be removed without removing aristae as aristae are distal to antennae. Behavioral assays proceeded as described above.

Gustation

Gustation is inhibited by the removal of foretarsi, the location of chemoreceptors for cuticular hydrocarbons in *D. melanogaster* (Stocker, 1994; N= 13). *D. saltans* flies were anesthetized with light CO_2 and separated into treatments with and without tarsi. Micro-dissection scissors were used to remove the five tarsal segments of the foretarsi from both front

legs for treatment lacking foretarsi. Control individuals were anesthetized for the same amount of time as required to perform the procedure. Behavioral assays proceeded as described above.

Midtarsi

In preliminary trials on the effects of foretarsi removal, we use midtarsi removal as a control for the manipulation. In these trials, we found a significant effect of midtarsi removal. Thus, we tested midtarsi removal separately (N= 15). The procedure and the assay was the same as for foretarsi.

Data Analysis

Data analyses were completed in R Studio (R version 3.1.0 (2014-04-10)—"Spring Dance"). A Fisher's Exact Test was used to test for the effect of light on mating success. For all other behavioral assays I compare the control to each treatment group (female ablated, male ablated, and both ablated) using a Fisher's Exact Test.

For the timing data (courtship latency and courtship duration) those that did not court within the 60-minute observation period were removed from analysis for courtship latency. Those that did court, but did not copulate, were scored with courtship duration of 3600 seconds minus the courtship start time (in seconds) as an underestimate of the likely duration of courtship if flies were watched indefinitely. Duration data were highly skewed because many pairs failed to court or mate, thus data were log transformed for examination. An ANOVA was used to test for an effect of male treatment and female treatment on the log transformed data. I present the findings of the ANOVAs using the log-transformed data because both the data and the residuals of the log transformed data approach a normal distribution.

Results

Vision

Single pairs held in constant darkness (N= 95) were less successful at producing progeny than those held in a 12:12 hour light:dark cycle (N= 87; Figure 1.1; two tailed Fisher's exact test: $P < 0.0001$). I concluded that mating was reduced in the dark because egg laying was not inhibited by constant darkness; larvae were produced in vials in which mated females were transferred to constant darkness (data not shown). The effect may be due to the males inability to see and not the females; when males were blind (N=20) mating success was reduced but not eliminated (Figure 1.2A; Fisher's Exact Test: $P = 0.0225$). However, when females were blind the reduction in mating success was not significant (Figure 1.2A; Fisher's Exact Test: NS). When both sexes were blind, there was a significant reduction of mating success (Figure 1.2A; Fisher's Exact Test: $P = 0.0095$) presumably because the males were blind. The reduction in mating success was not caused by a reduction in courtship occurrence (Supplementary Table 1.1; Fisher's Exact Test: NS). Also, of the males that courted, courtship latency and courtship duration were significantly longer when males were blind (Table 1.1, Supplementary Figure 1.1). Courtship latency and courtship duration were not affected when females were blind (Table 1.1, Supplementary Figure 1.1).

Wing Removal

Males and females use their wings differently during courtship. Males vibrate their wings to produce courtship song and wing vibration may produce visual (e.g., display of *D. subobscura*; Markow and O'Grady, 2005) as well as auditory cues (Chapter two). Females spread their wings to signal receptivity (i.e., conspicuous wing spreading of female of *D. montana*; Hoikkala et al., 1998; Ritchie et al., 1998; Saarikettu et al., 2005), which serves as a visual signal of acceptance to the males and allows the male to mount. Wing removal in both

males and females (N=15) significantly affected mating success. Wingless males had a significant decrease in mating success (Figure 1.2B, Fisher's Exact Test: $P = 0.0025$). Removal of the female's wings also significantly decreased mating success (Figure 1.2B, Fisher's Exact Test: $P = 0.05$). Furthermore, when both sexes were wingless, mating success was significantly reduced (Figure 1.2B, Fisher's Exact Test: $P < 0.001$). The reduction in mating success was not caused by a reduction in courtship occurrence (Supplementary Table 1.1, Fisher's Exact Test: NS). Courtship latency was not affected by the absence of wings in either males or females (Table 1.1, Supplementary Figure 1.2) indicating that courtship was initiated normally. However, of those that did court, courtship duration was significantly longer when females and males were wingless (Table 1.1, Supplementary Figure 1.2).

Aristae Removal

The aristae are the auditory reception organs in *Drosophila* (Ferveur, 1997; Stocker, 1994) thus the removal of aristae allowed me to isolate and examine the effect of hearing. Aristaeless females (N=16) had a significant decrease in mating success (Figure 1.2C, Fisher's Exact Test: $P = 0.0025$). In contrast, aristaeless males had no significant decrease in mating success (Figure 1.2C, Fisher's Exact Test: NS). When both sexes had aristae removed, mating success was significantly reduced (Figure 1.2C, Fisher's Exact Test: $P = 0.0025$) likely because the females were unable to hear. The reduction in mating success was not caused by a reduction in courtship occurrence (Supplementary Table 1.1, Fisher's Exact Test: NS). Courtship latency was not affected by the removal of the aristae in males or females (Table 1.1, Supplementary Figure 1.3). Also, of those that did court, courtship duration was significantly longer (Table 1.1, Supplementary Figure 1.3) when females lacked aristae. Courtship duration was not affected when males lacked aristae (Table 1.1, Supplementary Figure 1.3).

Olfaction

The third antennae segment is an olfactory organ of all *Drosophila* (Cook, 1973b). Because antennae cannot be removed without removing the aristae (thereby eliminating hearing), aristae were removed in the control (N= 16). Comparisons were made between pairs in which controls had hearing ablated and treatments had olfaction and hearing ablated. No males or females without antennae copulated regardless of which sex was ablated; however, because the control individuals, which lacked aristae, mated at a very low rate (6%), sample sizes need to be far larger than feasible to detect specific effects on copulation caused specifically by lack of olfaction. Although I cannot measure the effect of olfaction on copulation, unlike the previously examined senses, olfaction significantly reduces the occurrence of courtship. Male lacking antennae had a significant reduction in courtship occurrence (Figure 1.2D, Fisher's Exact Test: $P = 0.05$); the same was not true when the female's antennae were removed (Figure 1.2D, Fisher's Exact Test: NS). Additionally, when both sexes had antennae removed, courtship occurrence was significantly reduced (Figure 1.2D, Fisher's Exact Test: $P = 0.05$) potentially because the males were unable to smell. Latency of courtship was not affected by the removal of the antennae in males or females (Table 1.1, Supplementary Figure 1.4).

Gustation

The foretarsi, five distal segments of the front leg, of *Drosophila* are lined with gustatory receptors (Carlson, 1996). Males that had foretarsi removed (N=13) had a significant reduction in courtship occurrence (Supplementary Table 1.1, Fisher's Exact Test: $P = 0.0016$) and copulation occurrence (Figure 1.2E, Fisher's Exact Test: $P = 0.0048$). Removal of the female's foretarsi did not affect courtship occurrence (Supplementary Table 1.1, Fisher's Exact Test: NS) or copulation occurrence (Figure 1.2E, Fisher's Exact Test: NS). When both sexes had foretarsi

removed, courtship occurrence was significantly reduced (data not shown, Fisher's Exact Test: $P = 0.0149$) as well as copulation occurrence (Figure 1.2E, Fisher's Exact Test: $P = 0.0472$) likely because the males were unable to taste. Alternatively, the reduction could be mechanical and due to the males inability to mount the female as easily. Courtship latency and courtship duration were not affected when males or females had foretarsi removed (Table 1.1, Supplementary Figure 1.5).

Midtarsi

Preliminary trials to examine the effect of gustation (foretarsal removal) on courtship and copulation were done using the removal of midtarsi as a control (data not shown). I was surprised by the effect of the midtarsi removal on behavior and thus performed separate experiments to examine the effects of each set of legs separately. I almost missed the midtarsi behavior because it was subtle and had not been considered in the assessment of courtship behavior in *Drosophila.* Observations of courtship behavior of the control treatment of *D. saltans* progress similarly as described in the introduction of this chapter; after a brief interaction between the male and the female (which includes the male tapping the female's abdomen with his foretarsi) the male follows closely behind the female, alternating vibrating his wings and licking the tip of the female's abdomen. Male attempts to mount at this point are often prevented by the female kicking him with her hind legs, causing the male to resume following, singing, licking, and circling. When the male is on the female's side with his head proximal to her abdomen, she may extend her middle leg to touch his foretarsi with her midtarsi. This seems to be a signal initiated by the female, but full ethogram analysis of *D. saltans* courtship behavior is needed to demonstrate this conclusively. When the midtarsi of the female is ablated, the male approaches her from the side and taps his foretarsi where her midtarsi would be if they were

intact. The male then continues courting the female, often even more aggressively, circling around her while he vibrates his wings with intermittent abdomen licking and side-tapping.

Examining the role of midtarsi in courtship is unique to this study. Although many studies have explored multimodal courtship in *Drosophila* species (reviewed in T. A. Markow & O'Grady, 2005), recent studies have not examined the role of the midtarsi in courtship. Midtarsi removal affected the sexes differently. When the midtarsi were removed from the males (N=15), there was no significant effect on copulation occurrence (Figure 1.2F, Fisher's Exact Test: NS) whereas when midtarsi were removed from the females, copulation occurrence was significantly reduced (Figure 2F, Fisher's Exact Test: $P = 0.0209$). Also, when both sexes had midtarsi removed, copulation occurrence was significantly reduced (Figure 1.2F, Fisher's Exact Test: $P = 0.0209$), likely because the females had no midtarsi. The removal of midtarsi in males or females did not affect whether or not courtship occurs (Supplementary Table 1.1, Fisher's Exact Test: NS). Courtship latency was not affected when the midtarsi were removed from females (Figure 1.3, Table 1.1), but courtship duration was significantly increased (Figure 1.3, Table 1.1). Courtship latency and courtship duration were not affected when only males had midtarsi removed (Figure 1.3, Table 1.1).

Discussion

Courtship is Multimodal

In each experiment I removed either signal production or signal reception in *D. saltans* courtship. The removal of any one signal or its reception did not completely eliminate mating success or courtship (Table 1.2) indicating that *D. saltans* courtship was multimodal with multiple sensory modalities influencing courtship success. Thus signals may be redundant. The largest effect of the manipulations on mating success was on copulation: normal production and

reception of signals made copulation more likely. Every ablation had an effect indicating that the previously described sensory modalities for *Drosophila* courtship communication from *D. melanogaster* are all used by *D. saltans.*

Courtship occurrence was only altered by the ablation of the male antennae, which involves the removal of both olfaction and audition. In all other cases courtship was initiated, though it was delayed when the male was blind (Table 1.2) indicating that the male was unable to receive stimulating signals from the female. Thus the female's initial signals to the male are visual. This is reflected also in the reduction of mating success when pairs were kept in the dark; though mating was not completely eliminated, mating was inhibited by darkness.

The courtship signaling of *D. saltans* was similar to most other *Drosophila* relying on gustatory, olfactory, tactile, acoustic and visual signals (e.g., Ewing, 1983; Giglio & Dyer, 2013; Gleason et al., 2012; Spieth, 1974). Rarely is solely a single sensory modality necessary for courtship success though there are species for which olfaction (e.g., *D. nebulosa*, Gleason et al., 2012), or vision (e.g., multiple species, Grossfield, 1971) or courtship song (e.g., *D. pallidosa*, Doi et al., 2001) is required for mating success. Our experiments were not designed to test species-specific signal recognition, but given the multimodal nature of *D. saltans* courtship it is likely that discrimination against heterospecifics involves assessing multiple signals and is not inhibited by single aberrant signals.

Our tests were no-choice experiments, which allowed us to measure effects on courtship progression in individual pairs. Increased courtship duration implies that a male needed to work harder to gain a female response, or that males failed to receive a signal from the female, leading them to continue courting. Thus, most signals as tested here may be reflecting mate quality redundantly. If single signals were necessary for mate recognition, failure to receive them would

eliminate mating. This may be a possibility with olfactory signals, though I could not study their reception without eliminating hearing as well, because copulation was completely eliminated in the absence of olfaction. Currently nothing is known about pheromones produced by *D. saltans*, or its relatives. In other species groups pheromones, particularly cuticular hydrocarbons, which are largely gustatory signals, play a large role in reproductive isolation (e.g., *melanogaster* group, Cobb & Jallon, 1990) or sexual selection by female choice (e.g., *D. serrata*, Chenoweth & Blows, 2005).

Ablation of signals and their reception by physical manipulation is potentially damaging to the fly in unanticipated ways, but lacking the genetic resources of *D. melanogaster*, I was unable to use genetic ablation. Such mutations have been used to determine that the elimination of a single modality in *D. melanogaster* does not prevent mating (Markow, 1987) though elimination of both hearing and olfaction abolished mating success, implying a synergistic interaction (Rybak et al., 2002). Given that our approach has similar effects, the use of physical manipulations seems to be equivalent to that of genetic manipulations. Relying on genetic manipulations, however, would have caused me to miss the midtarsi behavior.

Different Effects on the Sexes

The effects of a manipulation on the sexes were considered different when the ablation of a body part in one sex did not alter mating success while the same ablation on the other sex had a detrimental effect on mating (Table 1.2). This implies that males and females need to receive different types of signals for courtship to progress, as has been seen in other species (Gleason et al., 2012). In no cases did altering the female change the male's propensity to court her. Females were always attractive to the male. Through our manipulations, I was not able to alter female pheromone production, though male failure to initiate as often when chemosensory

reception (through olfaction) was altered implies that female pheromones are instrumental in stimulating male courtship.

Wing removal was the only ablation that had the same effect when removed from the female as when removed from the male. The wing generates signals in different modalities for each sex. For the male they are used to produce an acoustic signal. Lack of male wings is paralleled by the reduction in mating when females cannot hear (lack of aristae). Lack of vision for females does not affect mating success implying that male wings are not used for an important visual signal. However, lack of female wings was paralleled by the reduction in mating when males could not see (lack of vision). In female courtship behavior wings are used in a visual signal of acceptance; when males cannot see, mating success is reduced. In *D. melanogaster*, males need vision to track the movement of females and to follow closely behind during courtship (reviewed in Greenspan & Ferveur, 2000; Spieth, 1974). This may not be the case for *D. saltans*, considering blind males attempted mounting often and failed because females had not spread their wings (Odu, pers. obs.). However, when a female spread her wings, which allows easier access for male mounting, the blind male often did not attempt to mount and therefore did not succeed in mating.

Removal of male aristae did not affect mating success indicating that female wings are not producing an acoustic signal of importance. Although females of some *Drosophila* species produce auditory signals with wing vibrations (Cook, 1980), *D. saltans* females do not (Colyott, pers. obs.). The reduction in copulation occurrence when females' wings were ablated is therefore inferred to be caused by elimination of a visual signal.

The differential effect of signaling modalities between males and females is probably a ubiquitous characteristic of *Drosophila* courtship because each sex signals in different

modalities. For instance, although most *Drosophila* males produce an acoustic signal (reviewed in Markow & O'Grady, 2005), most females do not produce acoustic signals, with a few exceptions (e.g., Donegan & Ewing, 1980). For *D. melanogaster* males, lack of aristae does not affect a male's ability to produce normal courtship song (Burnet et al., 1977), thus inability to hear affects courtship more when females are deficient than when males are deficient. In competition experiments using genetic mutants, visually defective females are as successful as wild-type females, although visually defective males are never successful when competing with wild-type males (Markow, 1987), likely because males need to be able to follow females, whereas females are not similarly restricted.

Midtarsi: a Potential Tactile Signal?

Females lacking midtarsi mate less frequently than intact females. Males court females lacking midtarsi as often as they court females with midtarsi with no change in courtship latency, implying that the male still receives necessary signals to initiate courtship. However, when females lack midtarsi, courtship duration is increased, meaning that the reduced number of males that achieve copulation have to court for longer to achieve copulation. When females lack midtarsi, males court as vigorously as with control females (Colyott, pers. obs.). One male courting a female lacking midtarsi was so vigorous that he stood on top of the female, unable to achieve mating because the female had not spread her wings to facilitate mating (Colyott, pers. obs.).

The observed interactions between the female and male centered around the female midtarsi and are potentially part of a two-way conversation between the male and female. All other signals involved in courtship are one-way signals from one individual that causes a change in behavior in the other individual. Because I had observed the females reaching out with their

legs toward the males, the midtarsi may convey a tactile signal to the male that is an active encouragement signal preceding the wing spreading posture. Or the female may need a tactile response from the male to progress to acceptance. Regardless of the nature of the communication, in the absence of the female midtarsal signal, males may continue courtship more aggressively not knowing to proceed to the next stage thereby delaying copulation. Although observations of the role of midtarsi in courtship have not been noted in other well-studied *Drosophila* species, this behavior is probably not restricted to *D. saltans* as similar behaviors were noted in the distantly related *Drosophila malerkotliana* (*melanogaster* group). A *D. malerkotliana* male uses his foretarsi to tap the midtarsi of the other individual; following the midtarsi tap, courtship progresses if the individual is a conspecific female or breaks off if the individual is a heterospecific female or a male (Narda, 1966). This behavior of the *D. malerkotliana* male may be analogous to the initial foretarsi tap of other species where the male uses his foretarsi to tap the body of the female. For a *D. malerkotliana* male, absence of foretarsi does not prevent the progression of courtship but the male fails to distinguish male and female targets as well as heterospecific and conspecific females (Narda, 1966). A male that taps a female that lacks midtarsi proceeds with courtship in the same way that he continues if he lacks foretarsi.

Given that the midtarsi-associated behavior of *D. malerkotliana* results in the interruption of courtship when the target individual is the wrong species or sex, whereas in *D. saltans* midtarsi are associated with the continuation of courtship of the opposite sex, these are probably behaviors with different messages. Few discrete female signals have been described for *Drosophila* species, though the most common one is wing spreading by the females, a visual signal indicating receptivity required before males will mount females (reviewed in Markow &

O'Grady, 2005). The midtarsi signal by females of *D. saltans* may be an additional signal to encourage courtship, though not of final acceptance, which is the wing spreading signal. This may be a further mechanism through which females can control the dynamics of courtship, as has been suggested for *D. melanogaster* (Dukas & Scott, 2015). The midtarsi behavior should be examined in additional species because it may be present but not yet detected.

Timing Data

By using no-choice tests, I likely underestimated the effect of signal or reception, which would likely be much higher in choice tests (Coyne et al., 2005). However, the use of no-choice tests permitted timing measures that are not possible in a competitive assay and allowed me to determine where courtship breaks down. Because males initiated normally courtship independent of the female ablation, none of our changes affected the recognition of the female as a mating target. In nearly all manipulations, except the removal of antennae, failure to copulate was a failure in progressing from courtship to copulation. To definitively determine where courtship breaks down requires building ethograms, such as has been done with genetic mutants (Markow, 1987). However, ethogram analysis is exceedingly time consuming thus the use of timing data along with the occurrence of courtship and copulation provides information about when sensory signals are used. Use of timing data is recommended for understanding the role of different sensory modalities in courtship success.

Conclusions

I found that the removal of no single modality eliminated courtship or copulation indicating that *D. saltans* courtship is multimodal. I also described a courtship behavior that should be considered in future studies of *Drosophila* courtship signaling. Lastly, I suggest that future studies should consider measuring courtship latency and courtship duration as well as the

occurrence of both to understand the role that courtship signals play in the progression of courtship.

Figures

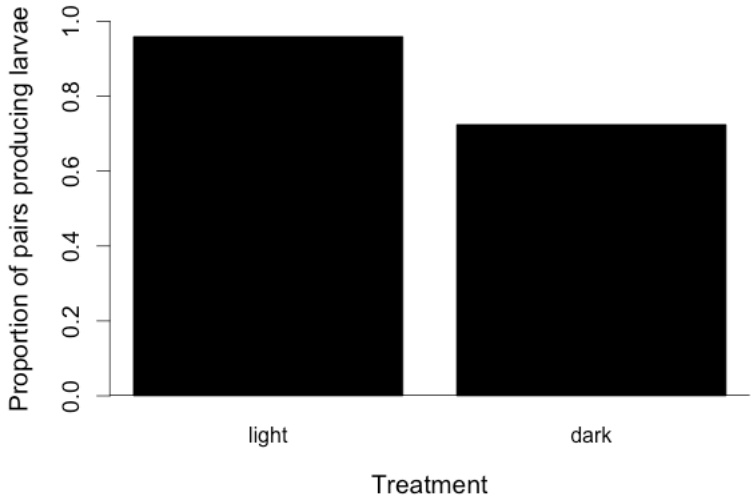


Figure 1.1. Effect of light on mating in *D. saltans*.Mating pairs were left in the light (N=87; 12 hr light: 12 hr dark) or in the dark (N=95; continuous darkness). After seven days, the vials were scored for presence of larvae. Pairs in continuous darkness produced offspring significantly less often than pairs in a normal light: dark cycle (two-tailed Fisher's Exact Test: $P < 0.001$).

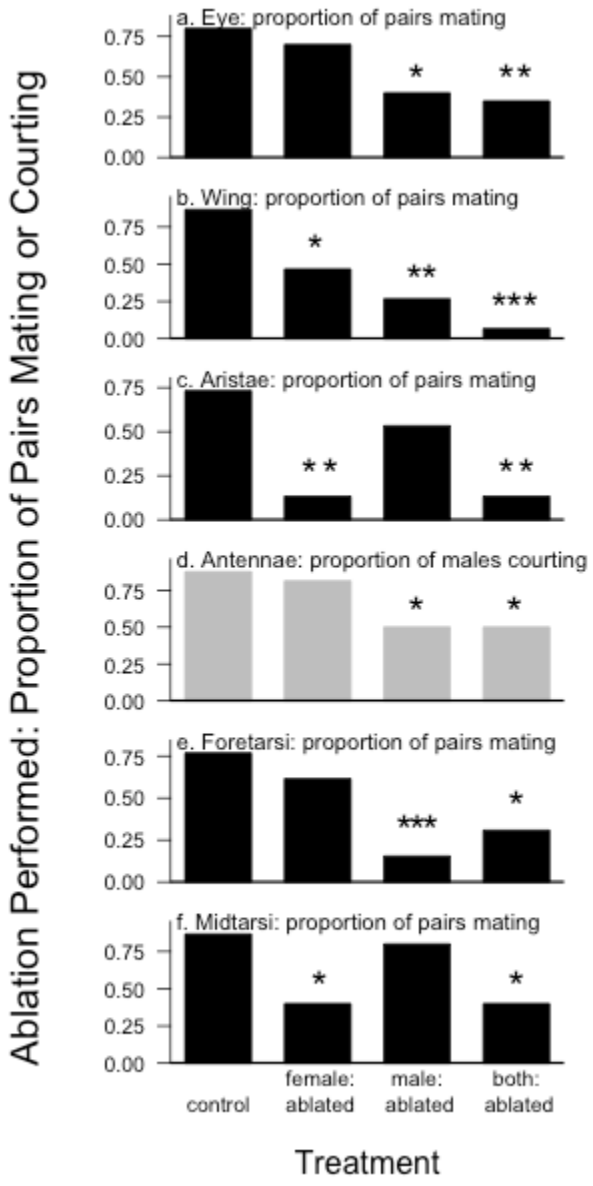


Figure 1.2. Proportion of pairs mating or courting in ablation experiments.Mating trials were conducted with control pairs, ablated females only, ablated males only, or both sexes ablated. Pairs were observed for an hour or until copulation ended and the proportion that courted and copulated were recorded. A Fisher's Exact Test was used to compare each treatment to control. Significance level of test is indicated on bar plots (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.005$) A. Male blindness inhibits copulation (N=20 per treatment). B. Absence of wings inhibits copulation (N=15 per treatment). C. Female inability to hear inhibits copulation (N=15 per treatment). D. Male inability to smell inhibits courtship (N=16 per treatment). E. Male inability to taste inhibits courtship and copulation (N=13 for each treatment). F. Copulation inhibited when females have ablated midtarsi (N=15 for each treatment).

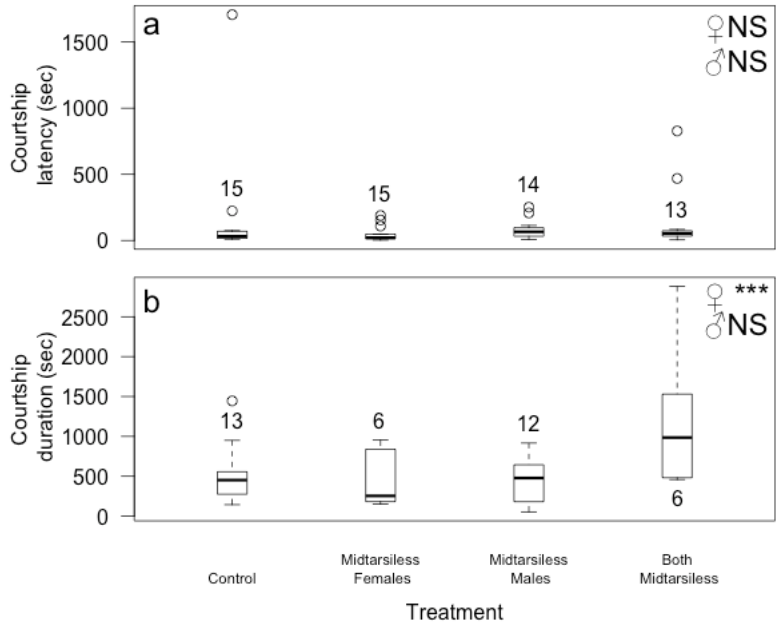


Figure 1.3. Effects of midtarsi on courtship latency and courtship duration.Mating trials were conducted with control pairs, midtarsi ablated females, midtarsi ablated males, and both sexes midtarsi ablated. Pairs were observed for an hour or until copulation ended and the time it took males to court (A. courtship latency), and courting males to copulate (B. courtship duration) was recorded. An ANOVA was used to test for an effect of male treatment and female treatment on log transformed timing data. The effect of female treatment and male treatment are in the upper right corner of plot (NS: not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$). For pairs that did not copulate data were scored as described in the methods before performing ANOVA analyses. To construct the boxplots this data was left out for better visualization of the recorded data.

Tables

Table 1.1. Summary of ANOVA results for timing data.

	Courtship latency				Courtship duration			
	Female effect		Male effect		Female effect		Male effect	
Ablation	F-value	P-value	F-value	P-value	F-value	P-value	F-value	P-value
Eyes	$F_{1,58}$=	0.182	$F_{1,58}$=	**0.007**†	$F_{1,58}$=	0.176	$F_{1,58}$=	**0.010**
Wings	$F_{1,56}$=	0.148	$F_{1,56}$=	0.088	$F_{1,56}$=	**0.014**	$F_{1,56}$=	< **0.001**
Aristae	$F_{1,56}$=	0.701	$F_{1,56}$=	0.153	$F_{1,56}$=	< **0.001**	$F_{1,56}$=	0.408
Antennae‡	$F_{1,41}$=	0.454	$F_{1,41}$=	0.709	–	–	–	–
Foretarsi	$F_{1,34}$=	0.573	$F_{1,34}$=	0.235	$F_{1,34}$=	0.983	$F_{1,34}$=	0.116
Midtarsi	$F_{1,55}$=	0.469	$F_{1,55}$=	0.067	$F_{1,55}$=	< **0.001**	$F_{1,55}$=	0.889

†Significant *P*-values are bold ($\alpha = 0.05$). ‡For the antennae, the proportion of the control individuals that courted and copulated was too low for comparisons to be made with ablated individuals.

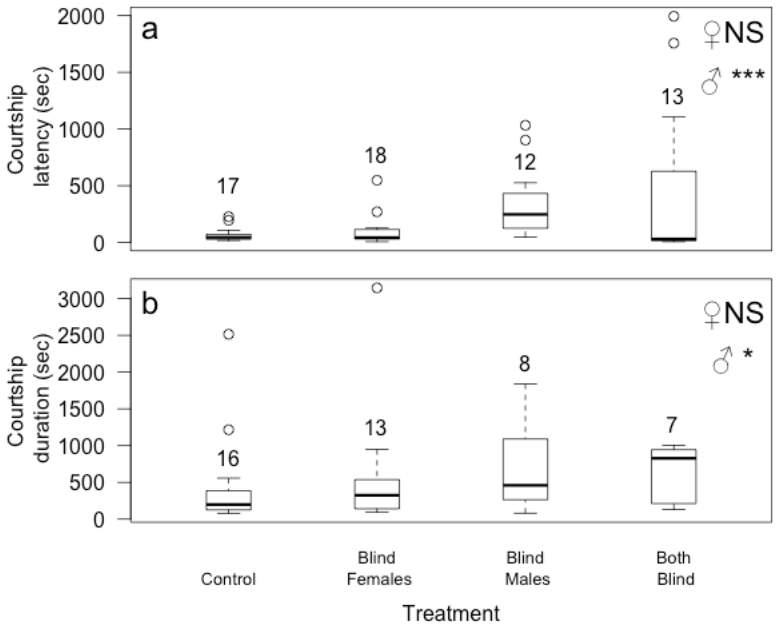
Table 1.2. Summary of results for sensory modality use.

Ablation	Courtship occurrence†		Copulation occurrence		Courtship latency		Courtship duration	
	female	male	female	male	female	male	female	male
Eyes	NS	NS	NS	↓	NS	↑	NS	↑
Wings	NS	NS	↓	↓	NS	NS	↑	↑
Aristae	NS	NS	↓	NS	NS	NS	↑	NS
Antennae‡	NS	↓	-	-	NS	NS	-	-
Foretarsi	NS	NS	NS	↓	NS	NS	NS	NS
Midtarsi	NS	NS	↓	NS	NS	NS	↑	NS

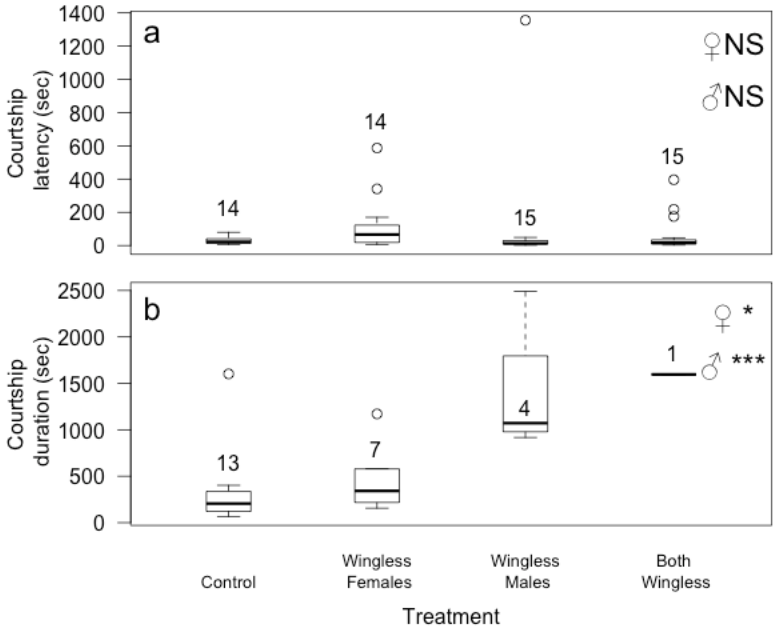
†Abbreviations: NS: no significant change; ↓: significant decrease; ↑: significant increase.
‡For the antennae, the proportion of the control individuals that courted and copulated was so low that comparisons could not be made with ablated individuals.

Supplementary Figures

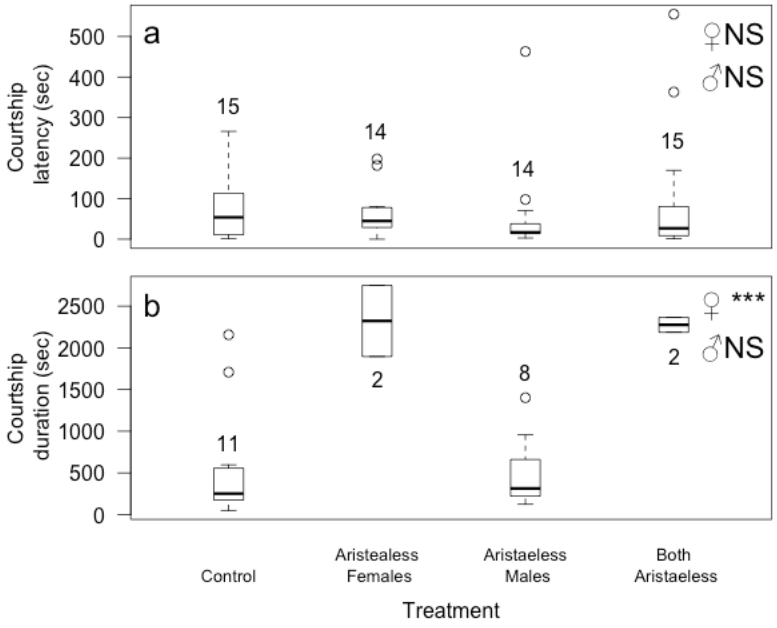
For the following figures (supplementary 1.1-1.5) mating trials were conducted with control pairs, ablated females, ablated males, and both sexes ablated. Pairs were observed for an hour or until copulation ended and the time it took males to court (a. courtship latency), and courting males to copulate (b. courtship duration) were recorded. An ANOVA was used to test for an effect of male treatment and female treatment on log transformed data. The number of courting (a. courtship latency) and copulating pairs (b. courtship duration) for each treatment appears in the figures. The effect of female treatment and male treatment are in the upper right corner of plot (NS: not significant; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$; ND: not enough data to perform analysis). For pairs that did not copulate data were scored as described in the methods before performing ANOVA analyses. To construct the boxplots this data was left out for better visualization of the recorded data.



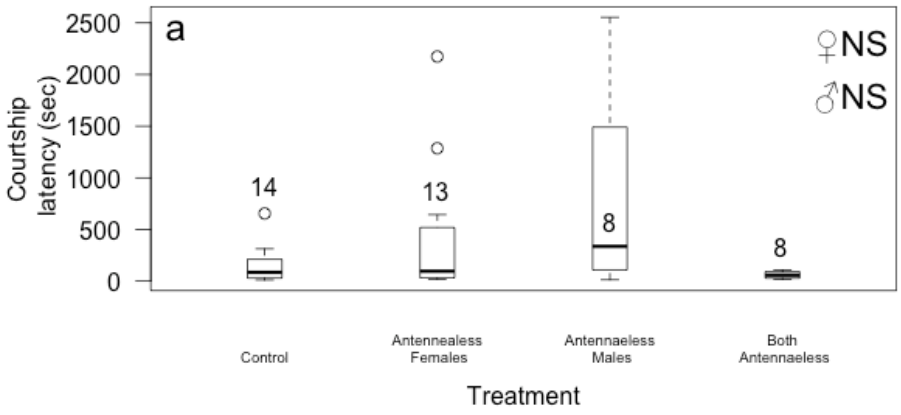
Supplementary Figure 1.1. Effects of vision on courtship latency and courtship duration.



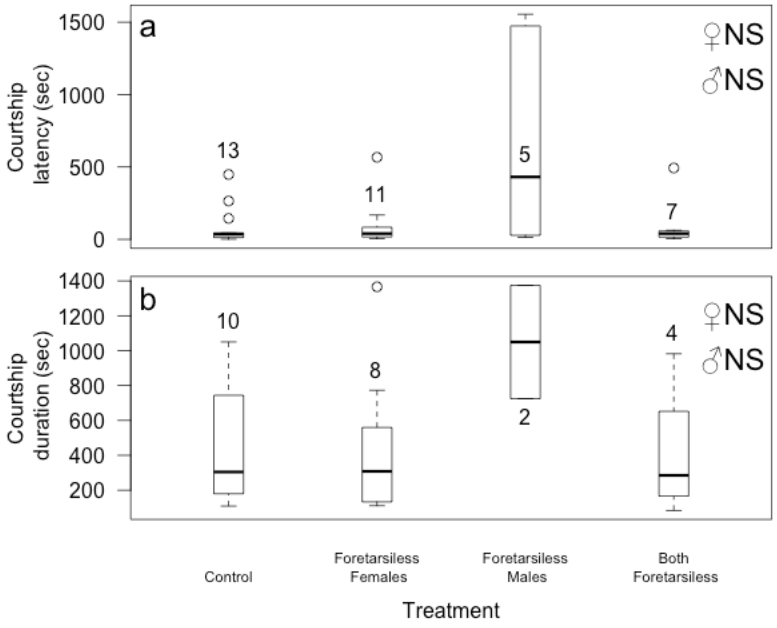
Supplementary Figure 1.2. Effects of wings on courtship latency and courtship duration.



Supplementary Figure 1.3. Effects of hearing on courtship latency and courtship duration.



Supplementary Figure 1.4. Effects of antenna on courtship latency and courtship duration.Only one control (lacking aristae) pair mated so I was unable to assess the effect on courtship duration.



Supplementary Figure 1.5. Effects of foretarsi on courtship latency and courtship duration.

Supplementary Tables

Supplementary Tables 1.1. Courtship occurrence for ablations.

Ablation†	N control courtship occurrence	N ablated female courtship occurrence	N ablated male courtship occurrence	N ablated female and male courtship occurrence
Eyes (N=20)	17	18	13	13
Wings (N=15)	15	15	15	15
Aristae (N=15)	15	14	14	15
Antennae (N=16)	14	13	**8 ***	**8 ***
Foretarsi (N=13)	13	11	5	7
Midtarsi (N=15)	15	14	14	14

†Number of pairs in which courtship occurred in the control and treatments for each ablation. A Fisher's Exact Test compared each treatment to control. Significant results (* $P < 0.05$) are in bold.

Chapter 2 : Role of hearing in mating success is not associated with song production or role of vision in the *Drosophila saltans* species group

Introduction

The complexity and variability of courtship song along with its role in sexual selection and reproductive isolation in species of *Drosophila* makes it ideal for the study of the evolution of complex signals. Courtship songs of species of *Drosophila* are evolutionarily complex (Chang and Miller, 1978; Ewing and Miyan, 1986; Ritchie and Gleason, 1995) sexual signals that are often multicomponent or comprised of multiple signals (von Schilcher, 1976; Clemens et al., 2015). Song is associated with slowing female locomotion (Trott et al., 2012) and increasing female receptivity (Kyriacou and Hall, 1982). Characteristics of song are the target of female choice (Hoikkala et al., 1998; Ritchie et al., 1998) with differentiation in courtship song contributing to reproductive isolation between some species because females use species-specific song components to discriminate against heterospecifics (Liimatainen et al., 1992; Ritchie et al., 1999; Yukilevich et al., 2016).

Characteristics of courtship song that influence female receptivity and communicate mate quality vary among *Drosophila* species. In *Drosophila melanogaster* the timing of pulses, as measured by the interpulse interval (IPI), is associated with species recognition (von Schilcher, 1976; Ritchie et al., 1999). A short IPI (i.e., fast) pulse song is also associated with male mating success in *Drosophila pseudoobscura* and *Drosophila persimilis* (Williams et al., 2001). In contrast, female *Drosophila montana* (*virilis* group) prefer males that sing with high carrier frequency (i.e., the reciprocal of the interval in seconds between the peaks of a pulse; Ritchie et al., 2001). Female *D. montana* that mate with males with the preferred high carrier frequency obtain the indirect benefit of producing fitter offspring (Hoikkala et al., 1998).

Courtship song is one signal among a suite of signals in a multimodal courtship display characteristic of *Drosophila*. Males may produce multiple signals during courtship in various

sensory modalities including chemical, tactile, visual, and acoustic stimuli (e.g., Spieth, 1974; Ewing, 1983; Gleason et al., 2012; Giglio and Dyer, 2013). Some species, like *D. melanogaster,* have multimodal courtship communication (reviewed in Greenspan and Ferveur, 2000) with the channel(s) used depending on sex and stage of courtship progression. Removal of male vision and/or olfaction reduces male courtship, but female olfaction and audition are required for female receptivity (reviewed in Greenspan and Ferveur, 2000). Vision in males contributes to the successful orientation of the male towards the female, and male chemoreception contributes to how long the male pursues the female in courtship (Agrawal et al., 2014). Therefore, *D. melanogaster* uses all modalities during courtship. Conversely, in species which instead rely heavily on only one or two modalities, courtship looks different. For example, males of *Drosophila nebulosa* use their wings to fan an anal droplet at females during courtship. In this species, vision is necessary for males to orient, whereas olfaction is necessary for females; other modalities play little to no role in mating success (Gleason et al., 2012). Other species of *Drosophila* (e.g., *Drosophila suzukii*) require vision for mating (Roy, 2019) and lack an auditory component to courtship (reviewed in Ewing, 1983).

Multimodal communication may benefit the sender and receiver by increasing signal efficiency by allowing the communication of multiple signals in a short period of time or signal accuracy by allowing a redundant signal to be sent multiple times (reviewed in Candolin, 2003; Hebets and Papaj, 2005). Two signals in a multimodal display may be nonredundant, and thus communicate different messages of quality, attractiveness, or motivation to the receiver. In this case, natural selection may favor integrated evaluation of the two signals, resulting in a positive relationship between signals (e.g., integrated evaluation of song and plumage ornamentation in birds-of-paradise due to strong, consistent selection on the two signals containing nonredundant

messages; Ligon et al., 2018). Alternatively, a tradeoff may occur between two modalities due to a costliness associated with maintaining the modalities, resulting in a negative relationship between signals (e.g., tradeoff between vision and olfaction found across 62 species of *Drosophila*; Keesey et al., 2019).

Sexual signal production is often associated with the use of the associated sensory modality during courtship in species of *Drosophila*. For example, frontal wing display is often paired with reliance on the associated visual modality in the receiver (Markow and O'Grady, 2005; Roy, 2019). Similarly, an absence of a behavior, such as the lack of song observed in *D. subobscura* or *D. suzukii*, is associated with a reduced or lost role of the auditory modality during courtship (Ewing, 1983; Markow and O'Grady, 2005). Often, species of *Drosophila* that do not produce auditory signals rely heavily on the visual sensory channel during courtship. For instance, *D. suzukii* requires vision for successful mating (Roy and Gleason, 2019). Other examples of species that may have lost the auditory channel and allocated all energetic costs to the visual channel include *D. subobscura* of the *obscura* group (Markow and O'Grady, 2005).

The songs of the *saltans* species group have been largely unexplored, making the species group an outlier within the subgenus *Sophophora*. The songs of the *melanogaster* (6 species; reviewed in Markow and O'Grady 2005), *obscura* (8 species; reviewed in Markow and O'Grady 2005), and *willistoni* (6 species; Ritchie and Gleason, 1995) groups have been described qualitatively and quantitatively. Describing and analyzing the courtship song of the *saltans* species group fills in the missing gap within the *Sophophora* group and extends our ability to evaluate evolution of complex signals. To evaluate the ancestral song state, the patterns of song loss and gain and at what scale song types, elements, and structures are homologous, this gap must be filled and song must be evaluated across the *Sophophora* group. To this end, I record

songs of multiple individuals of the available species from the *saltans* species group. I describe and analyze songs across the *saltans* group, and find extensive variation in song types, elements, and structure. There are two ancestral songs, primary and secondary (distinguished by wing orientation during production) in the *saltans* group. I find two species that have reduced songs and one species that has an elaborated song element.

The variability in song production, paired with the novel song element found in the *saltans* group, led me to hypothesize that song production might be associated with role of hearing during courtship. If there is an association between signal production and signal use, I expect to find that as signal production is reduced or elaborated, signal use during courtship will decrease or increase, respectively. I expect to find that the acoustic modality contributes highly to mating success in species that have a novel song. Furthermore, I expect to find that the acoustic modality does not contribute to mating success in species that have a reduced song.

To characterize the contribution of song during courtship, I ablate the auditory modality (i.e., remove male wings, sound production organ, and remove female aristae, sound reception organ) and analyze how mating success is affected. Visual and acoustic communication are linked morphologically in *Drosophila,* as the wings can produce both visual and auditory signals. To disentangle the effect of audition and vision on mating success, I perform light/dark experiments (i.e., assess the role of light in mating success) in tandem with wing removal experiments (i.e., assess the role of audition in mating success). Examining the role of both the auditory channel and the visual channel allows me to also determine if the two signal modalities are sent and evaluated in an integrated manner (i.e., are positively correlated) or trade off (i.e., are negatively correlated). I do not find that the two modalities are significantly correlated. I do

not find an association between contribution of song to mating success and song production or contribution of vision in the *saltans* group.

Methods

Species Included

I used nine species from the *saltans* species group obtained from the *Drosophila* Species Stock Center in this study. The species included four of the five described subgroups, *saltans*, *sturtevanti*, *cordata*, and *ellipitica* (Table 2.1). Species from the fifth described subgroup, *parasaltans*, were not available from the stock center. The outgroup used was *D. willistoni*, a representative of the closest species group, *willistoni* (O'Grady et al., 1998; Gao et al., 2011; O'Grady and DeSalle, 2018). All song data for *D. willistoni* was from Ritchie and Gleason (1995). The number of pairs that mated for treatments (wingless females, wingless males, aristaeless females, and aristaeless males) and controls for *D. willistoni* was from Gleason et al. (2012). These numbers were used to calculate the trait effects (Equation 2.1) for *D. willistoni*.

Fly Culturing

Species cultures were maintained in six oz. square bottom, polypropylene bottles containing standard cornmeal-molasses *Drosophila* food at 24°C with 12:12 hour light:dark cycle. Experimental cultures were maintained by transferring approximately 15–40 flies onto new food vials (24 mm d x 94 mm h) every or every other week. I collected virgin males and females under light CO_2 anesthesia within four hours of eclosion.

Courtship Song Recording and Analysis

Virgin males were housed individually and wing-ablated virgin females were housed in single-sex groups of up to 10 individuals, both in small food vials (16.5 mm d x 95 mm h). Female wings were removed during collection by severing the wings close to the body with a

dissecting probe. Individual songs were recorded using one sexually mature, virgin male (7–11 days old) and one wing-ablated, immature, virgin female (0–3 days old) placed in an acoustically-transparent chamber inside an Insectavox (Gorczyca, 1987). Digital recording was started with the initiation of courtship song and continued for five minutes. Songs were digitally filtered (high pass: 100 Hz, low pass: 1000 Hz) with Audacity 2.2.2 (Audacity Team) and analyzed in Spike 2 (CED, Cambridge, UK) using established procedures (Ritchie and Kyriacou, 1994). All *D. willistoni* song data were from Ritchie and Gleason (1995) and unlike data collected for the *D. saltans* species, each data point represented the mean of a strain, with seven strains represented (Guana, Atlixco, Belize II, Caño Mora, Belize VI, Lima B, and L' Habitatué).

Pulse song measurements were made using Spike 2 (CED, Cambridge, UK). Mean IPI was calculated by marking the peak of each pulse in the song, measuring the time between each set of pulses, and producing a histogram of IPI measurements using Spike 2. Songs that produced multiple, distinct peak histograms were characterized as having multiple IPIs. A non-parametric Mann Whitney U test compared pulse song measurements for each pair of species because the data were not normal and could not be log transformed to normality. For non-pulse song carrier frequency was measured in Audacity 2.2.2. using fast Fourier transforms with a Hanning window of 2048 (i.e., data was analyzed in windows that contained 2048 data points). Mean carrier frequency for a song was calculated as the mean of three bouts of the trait of interest with one bout examined at the beginning, middle, and end of the song (similar to the methods of Li et al., 2012).

Because I did not have access to *D. willistoni* song recordings, the oscillogram of *D. willistoni* was produced in R 3.3.0 (R Core Team, 2017) interfaced through RStudio v1.1.383 (RStudio, Inc.) using the R packages "seewave" and "TuneR". Carrier frequency, pulse duration,

and IPI from Ritchie and Gleason (1995) were used to synthesize the song that was used to produce the oscillogram. All other oscillograms were produced by taking screenshots of song recordings.

Single Sex Ablation Effect Behavioral Assays

Prior to behavioral assays, virgin males and females were collected and housed in single-sex groups of up to 10 individuals in small food vials with cotton plugs. One day before the experiment, virgin males and females, 7–9 days post-eclosion were modified (i.e., aristae were removed, or wings were removed, or eyes were covered) using the methods of Chapter One. To observe courtship and mating behavior, a single male and female were aspirated into a new, small food vial and restricted to a space of approximately 1 cm^3. A single trial consisted of observations of one control and two treatments simultaneously (i.e., control female with control male, ablated female with control male, control female with ablated male). The observer watched the flies until copulation was completed, or in the event that copulation did not occur, for one hour. The proportion of males that courted and the proportion of pairs that mated were calculated for all control and treatment groups. A Fisher's Exact Test was used to compare the mating success of the control group (i.e., control female with control male) to the treatment group (i.e., ablated female with control male, control female with ablated male).

Unlike the wing and aristae removal tests, separate effects of vision on each sex were tested for only a subset of the species (*D. austrosaltans*, *D. prosaltans*, and *D. saltans*). These three species were chosen because they shared a subgroup designation and differed in the role light played in mating success. In addition comparing the number of successful mating pairs in each group, both courtship latency (i.e., time it took males to court) and courtship duration (i.e., time spent courting until copulation) were calculated for all pairs. Those that did not court or

mate within the 60-minute observation period were removed from analysis for courtship latency and courtship duration. The data were highly skewed because many pairs failed to court or mate; thus, data were log-transformed for examination. A *t*-test was used to test for an effect of male treatment and female treatment on the log-transformed data.

Light Effect Behavioral Assays

To determine the effect of light on mating success, pairs of virgin males and females that were 7–10 days old were placed in small food vials on a standard 12h:12h light:dark cycle (light treatment) or in continuous darkness (dark treatment) for seven days. After seven days, all vials were scored for presence of larvae. Only vials with both parents alive at the end of the trial were used in analyses. A Fisher's Exact Test was used to compare the number of vials that produced larvae in each treatment. Species were classified according to Grossfield (1971) into one of three classes: class I species mate equally well in the light and dark (light independent), class II species have light facilitated mating, and class III species are dark repressed and no mating occurs in the dark (light dependent).

Effect of Wings, Aristae, and Light

The $\log_2$ fold change between the number of mating occurrences in the control and treatment groups was calculated by taking log base two of the proportion of control pairs that mated over the proportion of treatment pairs that mated:

$$log_2\ fold\ change\ (trait\ effect) = log_2 \frac{proportion\ of\ control\ pairs\ mated}{proportion\ of\ treated\ pairs\ mated} \quad [2.1]$$

Because the log of zero is undefined, all instances where zero pairs courted or mated were replaced with 1/100. Replacing zeros with 0.01 or 0.001 is common practice when calculating $\log_2$ fold change (John Kelly, pers. comm.). In this case, replacing zeros with 0.1, 0.01, 0.001, or

0.0001 does not alter the ultimate result of whether or not there is a significant relationship between two traits.

A $\log_2$ fold change of zero meant that control and ablated individuals mated equally well. A negative value meant that the modality that was tested hindered mating success (i.e., ablation of the trait removed the hinderance). A positive value meant that the modality contributed to successful mating (i.e., more mating occurred when the modality was used than when it was removed). The value associated with $\log_2$ fold change described the level of difference between the number of pairs that mated when comparing the control to ablated individuals. The $\log_2$ fold change was a continuous variable that was mapped on topologies to make phylogenetic comparisons. Ancestral state reconstruction was performed to assess trait evolution.

Phylogenetic Analysis

To examine song phenotype and the use of auditory and visual sensory channels in a phylogenetic context, and to perform phylogenetically-independent contrasts using sensory modality use data, I first needed a phylogeny on which to map the traits. I updated a previous phylogeny (O'Grady et al., 1998) by adding a species (*D. dacunhai*) and an additional gene (the *per* gene). I was provided with the new gene sequences, old gene sequences, and an alignment that were used to produce the phylogeny (Supplementary Table 2.1; Gleason, pers. comm.). Genes used included COI, COII, *Adh*, *per*, and ITS1. Given that the nuclear genome is large, and *per* and *Adh* are on different chromosomes, it is likely that *per*, *Adh*, and ITS1 are unlinked (or at least not closely linked). Therefore, I used at least 3 (and probably 4) unlinked loci.

Maximum likelihood methods for phylogenetic analyses were performed using Paup* 4.0a (build 165; Swofford, 2002). The Generalized time-reversable substitution model plus gamma distributed rate variation among sites (GTR+G model) of nucleotide substitution was

chosen as a best fit model under the Akaike information criterion (AIC) using MrModelTest2 (Nylander, 2015) implemented in Paup*. Maximum likelihood analyses were performed using the GTR+G model (Hasegawa et al., 1985) site setting in Paup*. Topologies were produced by carrying out several individual and combined analyses (i.e., nuclear, mitochondrial, single nuclear gene) in Paup*. All topologies were rooted using *D. willistoni*. Initial heuristic searches were conducted using random stepwise addition and tree-bisection-reconnection branch swapping and bootstrapping with 100 replicates in Paup*. Further phylogenetic analyses were performed in BEAST2 (Bouckaert, 2014) and *BEAST2 (Heled and Drummond, 2010) to infer gene trees and consensus trees respectively, using Bayesian Inference. Default parameters except for the relaxed log normal clock model (a molecular clock model was rejected) and an estimated Hasegawa-Kishino-Yano (HKY; one transition and one transversion rate) site model were set in BEAUti and loaded into both BEAST2 and *BEAST2.

Due to incongruencies among the maximum likelihood (Supplementary Figure 2.1) and Bayesian Inference (Supplementary Figure 2.2) topologies, and to perform downstream analyses without biasing results, a set of plausible trees was obtained by performing an Approximately Unbiased (AU) test (Shimodaira, 2002). The topologies with the highest likelihood values were obtained by performing the AU test on a set of backbone constrained trees (N=135) in PAUP* with 1000 bootstrap replicates. The trees with the highest likelihoods and non-significant AU *P*-values (Supplementary Table 2.2) were used.

Topologies of the top six trees produced (Supplementary Figure 2.3) were then loaded into BEAST2 to produce trees with branch lengths so they could later be used along with trait data to perform ancestral state reconstruction for correlation analyses.

Ancestral State Reconstruction

R 3.3.0 (R Core Team, 2017) was used for all analyses of behavioral assay data. Trees produced in Paup* 4.0 with branch lengths added in BEAST2 were loaded into R along with the calculated, continuous trait effect data. The package 'phytools' (Revell, 2012) was used to estimate and map the ancestral states of the continuous behavioral characters onto the various topologies.

Independent Contrast Analyses

The package "ape" (Paradis, 2004) was used to calculate phylogenetically independent contrasts. The "base" package (R Core Team, 2017) was then used to perform correlation analyses using the phylogenetically-independent data. The top six topologies were used to ensure that differences in topology did not change the results of the correlation analysis (i.e., all correlation analyses were performed six times).

Results

Inferring Relationships

Topologies were produced in Paup*, BEAST2, and *BEAST2. To perform downstream analyses without biasing results, a set of topologies (N=6) with the highest likelihood values were obtained by performing an AU test (Shimodaira, 2002). These trees represented the most plausible relationships given the data (Supplementary Figure 2.3). When each tree topology (Tree 2.2- Tree 2.6) was compared to the best tree topology (Tree 2.1; Supplementary Figure 2.3) using the AU test, ten comparisons resulted in non-significant *P*-values, indicating that the likelihood values for the two trees were not significantly different (Supplementary Table 2.2). We chose six of the ten with the highest likelihood values to perform downstream analyses. The topology of Tree 2.2 was used in all figures because it matched the combined analysis tree

produced using Bayesian inference. The only difference between Tree 2.2 and Tree 2.1 was the relationship among species within the *saltans* subgroup; the relationships among subgroups were the same (See Supplementary Figure 2.3).

Species Group Song Description

To characterize song complexity, I named and described the associated measurements of the various song types, elements, and structures that occurred in the *saltans* species group (Table 2.2). Seven of the nine species examined here had two song types in their courtship display: primary and secondary. In these species, I defined primary song as the song most often performed first during courtship (Table 2.2). Primary song could be distinguished from secondary song by the orientation of the males' wings during the production of song. The production of primary song occurred when the male vibrated both wings at an approximately 20º (40º to each other for *D. willistoni*; Ritchie and Gleason 1995) angle to the midline of his thorax (Figure 2.1). Primary song was often produced as the male approached the female from behind, and chased her around the chamber. Secondary song often occurred at a later time in the song or directly after primary song, but did not occur in every individual. During production of secondary song, the male extended and vibrated one wing at an approximately 70º (>40º for *D. willistoni*; Ritchie and Gleason 1995) angle to the midline of his thorax (Figure 2.1). The wing extended was usually the one that was closest to the female's head. Secondary song often occurred while the male circled the female and when he moved from behind her to in front of her.

Song elements were defined visually by their appearance on an oscillogram, and by the associated sound. Three different song elements were described: pulses (see Figure 2.2), beeps, and rasps (see Figure 2.3). A pulse element was defined using the definition from Ewing and

Bennet-Clark (1968) as a “discrete unit of sound that consisted of one or more cycles and has no harmonics.” A series of pulse elements was referred to as a pulse train, a group of pulses that were characterized by a relatively consistent amount of time occurring between adjacent pulses (Table 2.2). Pulse trains appeared structured as singlets (i.e., all pulses in train occurred at a consistent rate) and as doublets (i.e., pulses have two distinct interval measurements; intradoublet intervals are shorter intervals between adjacent pulses, while interdoublet intervals are longer intervals between adjacent doublet pulses; see Figure 2.2). A beep element was characterized as a tone song comprised of a complex sinusoidal wave that progressed into a series of sinusoidal wave sound cycles without pulse structure, (similar to tone song of *D. silvestris*; Hoy et al., 1988) that are often structured with higher amplitude sound cycles that fade into lower amplitude cycles (see Figure 2.3). A rasp element was a short train, (~75 ms) of fast pulses (IPI ~6 ms) that were variable in amplitude across the element (see Figure 2.3).

For pulse songs, at least 70 (primary) and 30 (secondary) pulses were examined per song. Pulse song was characterized by the time (in milliseconds) between the peak of two adjacent pulses (i.e., the interpulse interval, IPI). To compare IPI across species of the group, overall mean IPI was calculated for species that had doublet (or in the case of *D. lusaltans,* singlet and doublet) song structure by taking a weighted mean of all present IPI measurements (i.e., interpulse, intradoublet, and interdoublet). The weighted mean was calculated by multiplying and averaging the mean timing of the intervals by the number of times the interval measurement was made. For species that only produced singlet song, overall mean IPI was the mean IPI of singlet song. For each species, I tested for a correlation between mean temperature during song recording and overall mean IPI. For the three species that resulted in significant correlations (*D.*

neocordata, *D. prosaltans* and *D. sturtevanti*), I corrected overall mean IPI to a common temperature of 23ºC (the mean temperature across all recordings).

Like pulses, both beeps and rasps occurred in trains. I measured the total number of beeps per song and the average number of beeps per train, as well as the average carrier frequency. The rasp IPI was measured as well as the average carrier frequency of rasps in a song.

Individual Species Song Description

***saltans* subgroup**

The primary and secondary songs of *D. austrosaltans, D. prosaltans* and *D. saltans* were similar in structure with some significant differences in timing of pulses (Figure 2.4; Table 2.3). Primary songs had doublet pulses and were the fastest pulse songs of all the species measured (Figure 2.5; Table 2.3). Primary song comparisons in this subgroup were significantly different from each other except for the comparison of *D. austrosaltans* with *D. prosaltans* and comparison of *D. austrosaltans* with *D. saltans* (Figure 2.5; Supplementary Table 2.3). Secondary songs in this subgroup were quieter (Figure 2.4) and composed of singlet pulses with a longer IPI when compared to primary songs (Table 2.3). Secondary song comparisons were significantly different from each other except for the comparison of *D. prosaltans* with *D. saltans* (Supplementary Table 2.4).

The primary song of *D. lusaltans*, in contrast, had both singlet and doublet pulse trains in no particular arrangement throughout primary song. Mean IPI of primary song was significantly longer than the other species in this subgroup (Supplementary Table 2.3). Secondary song was qualitatively similar to that of the sibling species, but had a significantly longer IPI (Table 2.3; Supplementary Table 2.4).

***ellipitca* subgroup**

D. emarginata was the one species in the *saltans* group that did not sing a primary song (Figure 2.4), as defined by the position of the wings when singing (Figure 2.1). The secondary song of *D. emarginata* was characterized as a rasp, similar to a pulse song, but with a very short IPI (Table 2.3). The rasp element of *D. emarginata* was a short train, (~75 ms) of fast pulses (IPI ~6 ms) that were variable in amplitude across the element (Figure 2.3).

***cordata* subgroup**

D. neocordata was the one species of the *saltans* group that did not produce a secondary song. The only song produced by *D. neocordata* was a primary pulse song that was structured as singlet pulses only (Figure 2.4). Of all species in the *saltans* group, *D. neocordata* had the longest and most variable IPI (Figure 2.5; Table 2.3).

***sturtevanti* subgroup**

The primary and secondary songs of *D. dacunhai* and *D. milleri* shared a singlet pulse structure (Figure 2.4) with significant differences in timing (Table 2.3). Primary songs had significantly longer mean IPI than the *saltans* subgroup, aside from *D. lusaltans*, for which there was no difference when compared to *D. dacunhai* (Figure 2.5; Supplementary Table 2.3). The primary song of *D. milleri* was significantly longer than that of *D. dacunhai* (Figure 2.5; Supplementary Table 2.3). Secondary songs were quieter (Figure 2.4) and composed of singlet pulses with a longer IPI than primary songs. Secondary song IPI was shorter than that of *saltans* subgroup (Supplementary Table 2.4). Mean IPI of secondary song of *D. dacunhai* was significantly longer than *D. milleri* (Supplementary Table 2.4).

Males of the *D. sturtevanti* species produced a singlet primary pulse song that often appeared in two forms. Standard pulse song was in every song (N= 17) and almost always made up the majority of IPIs in a song (Mean IPI = 51.13 ms; Median number of IPIs ± SD = 405 ±

321.827). Fast pulse song, pulse song that was twice as fast as standard pulse song, did not appear in every song (appeared in 10 songs) and except for one song, made up a fewer number of IPIs (Mean IPI = 25.05 ms; Median number of IPIs ± SD = 76 ± 314.416) than standard song IPIs. Standard pulse song had a significantly longer IPI than that of both *D. dacunhai* and *D. milleri* (Supplementary Table 2.3). The unique secondary song was described as a 'beep' because of the way it sounded (supplementary mp3). The duration of a beep was about 50 to 70 ms with individual wave sound cycles of ~4 ms (Figure 2.3). Males were variable in the total number of beeps they produced in courtship (N = 17; min = 0, max = 68, mean = 26.59, standard error = 5.35). When they produced beeps, males varied in the mean number of beeps that occurred per beep train (N = 15; min = 3.25, max = 7, mean = 5.29, standard error = 0.26).

willistoni outgroup

The outgroup produced a primary and secondary pulse song and, in some strains, produced a rasp independently or in association with a primary pulse song (Ritchie and Gleason, 1995). Rasp song was described as a short burst of song (less than 200 ms) that had a much shorter IPI than pulse song, which had an IPI of 30 ms (Ritchie and Gleason, 1995). Primary song occurred before and lead into secondary song, as was found in the species above. Primary song was produced early in courtship with both wings held at a 40 ° angle to each other while secondary song was produced later in courtship with one wing extended at an angle >40 ° (Ritchie and Gleason, 1995).

Overall Song Structure Description

There was variability in song types, song elements, and song structure across this group. Primary song was present in all *saltans* species examined but one, *D. emarginata* (Figure 2.4). Similarly, all species had a secondary song except for *D. neocordata*. All secondary songs of the

group had a lower amplitude than the primary songs of the group except for the secondary songs of *D. emarginata* and *D. sturtevanti*. There was variability in song elements that appeared during song production with most species producing one or two pulse songs. Two other elements (rasps and beeps) were produced during song in this group and both appeared in secondary songs. Species within the *saltans* subgroup shared overall song structure (Figure 2.4) with some species differing in the timing of pulses in both primary and secondary song (Table 2.3). Similarly, two species within the *sturtevanti* subgroup (*D. dacunhai* and *D. milleri*) shared overall song structure (Figure 2.4) with the two species differing in the timing of pulses in both primary and secondary song (Table 2.3). The third species in the *sturtevanti* subgroup, *D. sturtevanti,* differed from the other two in both overall song structure (Figure 2.4) and primary song timing (Table 2.3).

Sensory Channel Use

The effects of the use of acoustic and visual signaling on mating success were measured across the group. The $\log_2$ fold change between the level of mating that occurred in the control and the treatment conditions (Equation 2.1) was calculated for our manipulations to understand the role a sensory channel played in mating success. The $\log_2$ fold change value was placed on the tips of the tree and an ancestral state reconstruction was performed to evaluate how sensory channel use has evolved across this group.

Male aristae

The ablation of male aristae removes the male's ability to hear. In the *saltans* species group, the presence of male aristae had a negative one- to positive five- log2 fold change effect on mating but none of these effects was significant (Figure 2.6A; Fisher's Exact Test: NS) therefore male aristae played no role in mating success.

Acoustic reception: Female aristae

Female aristae are reception organs for acoustic and vibratory signals (Cook, 1973b, a). Female aristae removal is often used as a proxy of courtship song importance as it removes the ability of the female to hear courtship song. The presence of female aristae had a zero- to six-$\log_2$ fold change effect on mating success. For most *saltans* species, female aristae had a positive effect (i.e., positive $\log_2$ fold-change) on mating success (Figure 2.6B). The exceptions were *D. austrosaltans* and *D. neocordata* for which female aristae did not play a role in mating; ablated females and control females mated at the same rate (Table 2.4; Fisher's Exact Test: $P = 1.0$ and 0.10). For *D. prosaltans*, *D. lusaltans*, and *D. emarginata*, mating was eliminated (i.e., no mating occurred) when female aristae were ablated. In *D. milleri*, *D. dacunhai*, *D. sturtevanti*, and *D. willistoni* significantly more mating occurred in the control females when compared to the ablated females (Table 2.4).

Acoustic and potential visual signal: Male wings

Because males use wings to produce courtship song, the ablation of male wings is an acoustic channel ablation often used as a proxy of courtship song importance. Male wings may also serve as a visual signal to females (e.g., frontal wing display of *D. subobscura*; Markow and O'Grady, 2005). The presence of male wings had a zero- to six- $\log_2$ fold change effect on mating success (Figure 2.6C). For most species, male wings had a statistically significant positive effect on mating success. However, male wings played no role in mating success of *D. dacunhai* (Table 2.4; Fisher's Exact Test: $P = 0.72$).

Female wings

Female wings may convey visual and/or auditory signals to males of their willingness (e.g., wing spreading in *D. montana*; Liimatainen et al., 1992) or lack of willingness (e.g.,

buzzing in *D. melanogaster* and *D. simulans*; reviewed in Greenspan and Ferveur, 2000) to mate. Female wings had a zero- to one- $\log_2$ fold change effect on mating in the *saltans* species group (Figure 2.6D). When comparing the two groups (control and wingless female) only two species differed significantly in mating success, *D. saltans* (Table 2.4; Fisher's Exact Test: $P = 0.05$) and *D. sturtevanti* (Table 2.4; Fisher's Exact Test: $P = 0.04$). Thus in general, removing female wings did not affect mating success.

Visual signals: Mating in light and dark

The number of pairs mating in the light were compared to the number of pairs mating in the dark to examine the role of vision during courtship. The presence of light had a zero- to six- $\log_2$ fold change effect on mating in the *saltans* species group (Figure 2.6E). For most *saltans* species, light had a positive significant effect on mating (Figure 2.6E). The exceptions to this were *D. emarginata* (Table 2.5; Fisher's Exact Test: $P = 0.15$) and *D. dacunhai* (Table 2.5; Fisher's Exact Test: $P = 0.07$), which were light-independent. The outgroup species, *D. willistoni*, was also light-independent (Table 2.5; Fisher's Exact Test: $P = 0.56$). This means *D. emarginata*, *D. dacunhai*, and *D. willistoni* are class I species according to Grossfield (1971); pairs mated equally well in the light and dark. In *D. austrosaltans*, no mating occurred in the dark, qualifying the species as dark-repressed or class III. The rest of the species qualified as class II, or as having light-facilitated mating.

In light:dark experiments, one cannot discern if vision was important for the male, the female, or both. To understand to which sex and at what stage of courtship vision was important, vision must be individually ablated for the sexes. Single sex vision ablations were completed for *D. austrosaltans*, *D. prosaltans*, and *D. saltans*. In the three species that were studied, light facilitated mating success in *D. prosaltans* and *D. saltans*, and light was necessary for *D.*

austrosaltans mating success (Table 2.5). For all three species, mating success was affected only when male vision was ablated and not when female vision was ablated (Table 2.6). Vision played a role in the initiation stage of courtship (Table 2.7) then subsequently affected mating success (Table 2.6). Male *D. austrosaltans* (Fisher's Exact Test: $P < 0.05$) and *D. prosaltans* (Fisher's Exact Test: $P < 0.05$) initiated courtship less often than controls when vision was ablated (Table 2.6). I did not find the same to be true for *D. saltans*: blind males courted as often as males with vision (Table 2.6; Fisher's Exact Test: $P > 0.05$). Both *D. austrosaltans* (Fisher's Exact Test: $P < 0.05$) and *D. saltans* (Fisher's Exact Test: $P < 0.001$) males that courted when vision was ablated took longer to initiate courtship than control males (Table 2.7), but this was not the case for *D. prosaltans* (Table 2.7; Fisher's Exact Test: $P > 0.05$). All three species had a reduction in the number of pairs that mated successfully when males were blind (Table 2.6).

Song Phenotype and Role of Song

I categorized the song of *D. sturtevanti* as elaborated due to the novel secondary beep element and the songs of *D. neocordata* and *D. emarginata* as being reduced due to the loss of a song type. The songs of the remaining species were categorized as standard relative to the overall pattern of songs within this group because they all contained two pulse songs.

I evaluated individual species to assess if role of song in mating success and song phenotype met the expectations of being associated with one another because there was not enough power to test the hypothesis statistically. Expectations were as follows: standard song phenotype will facilitate mating but will not be necessary, in a species with reduced song phenotype, song will play a reduced or no role in mating success, and elaborated song phenotype will be necessary for mating success.

I did not find an association between the role of song in mating and the song phenotype. For some species that produced standard song, song was necessary (e.g., *D. prosaltans* and *D. lusaltans*), but for others song facilitated mating (e.g., *D. austrosaltans*, *D. saltans*, *D. milleri*, and *D. dacunhai*). In one species that had reduced song, *D. neocordata*, male wings were necessary for mating to occur, but the effect of female aristae could not be determined due to low mating proportion of controls. For the other species that had reduced song, *D. emarginata*, song was necessary for mating (i.e., mating was eliminated both when male wings were removed and when female aristae were removed). In the species that had an elaborated song element, *D. sturtevanti*, song facilitated mating but was not necessary. There was a significant reduction in mating when female aristae were removed and when male wings were removed. For the most part, individual *saltans* species do not match the expectations laid out to support an association between role of song in mating and the song phenotype.

Correlation Analyses

Correlation analyses using independent contrasts were performed to assess if the effects of any two traits were correlated with one another. I expect two traits to be correlated if both traits are produced and evaluated in an integrated manner (positive correlations) or if there is a tradeoff between the use of two sensory modalities (negative correlation). Two traits will be positively correlated if the evaluation of the two are integrated in the receiver (e.g., visual and acoustic signals of birds-of-paradise; Ligon et al., 2018) or if they both ablated the same sensory channel and therefore represented the same trait use. We found that two traits were positively correlated: male wings and female aristae (Table 2.8; Tree 2 – $F_{1,8} = 12.83$, $P = 0.007$, $R^2 = 0.62$). Although no other pairs of traits were correlated, the correlation of female aristae and vision trended towards a negative correlation (Table 2.8; Tree 2 – $F_{1,8} = 4.12$, $P = 0.08$, $R^2 =$

0.34) and female aristae and male aristae trended towards a positive correlation (Table 2.8; Tree 2 – $F_{1,8} = 4.61$, $P = 0.06$, $R_2 = 0.37$). The statistical significance of the correlation analyses did not change depending on topology though *P*-values varied (Table 2.8).

Discussion

Sexual signals and the role they play in mating success are variable among species. Understanding the variability that exists among species across a species group (diverged ~22.3 million years ago; Gao et al., 2011) and ultimately across a subgenus (*Sophophora*; diverged ~57.3 million years ago; Gao et al., 2011), will illuminate at what rate the song types, elements and structures evolve and how signal phenotype and signal use relate. In this study, I examined whether the role of song during courtship was associated with the phenotypic variability of song and/or was correlated with the role of vision during courtship. Specifically, I approached this study with three goals: 1. Describe the courtship songs of the *saltans* group, 2. Understand how courtship songs are evolving in a phylogenetic context and 3. Understand the role of audition and vision in conspecific mating.

Song Variation

I found variation in song type, element and structure. Even in the species that shared overall song structure (i.e., *D. austrosaltans*, *D. prosaltans*, and *D. saltans*, and *D. milleri* with *D. dacunhai*), there was never overlap in IPI for both primary and secondary song. The differentiated pulse timing may be enough for females to identify a conspecific male. For instance, in the sibling species *D. melanogaster* and *D. simulans*, females differentiate between conspecific and heterospecific males by assessing IPI of pulse song (Ritchie et al., 1999).

I characterized a rare song structure, doublet pulse structure of primary song, in the *saltans* group. Because I was not equipped to use high speed video recording and analysis, I was

unable to determine how the song structure was produced, but hypothesize it may occur because of the interference of one wing with another during the production of a pulse, resulting in two pulses instead of one produced with each wing beat. The interference could be caused by asynchronous wing beats similar to what occurs in the lesser wax moth (Jang and Greenfield, 1996). The doublet pulse structure has been found and described outside of the *saltans* group, but the doublet structure found within the *saltans* group appeared to differ from what has been described elsewhere. Ewing and Miyan (1986) described a doublet pulse structure that occurred in at least two lineages within the *repleta* species group. They hypothesized that due to the timing and oscillating amplitude of the doublet pulses in primary song and the timing of the pulses in secondary song, that secondary song was simply primary song without the intervening pulse. This does not appear to be the case in the *saltans* species. Among *saltans* species, there was no oscillating amplitude; amplitude did vary slightly among pulses but did not alternate from high to low in the manner described in the *repleta* species. Also, for this to be the case, it would be expected that the time between the "quick" pulses (intrapulse interval) and the time between "slow" pulses (interpulse interval) would be equal to the time between adjacent secondary pulses, and this is not the case. Therefore, although the song structure of both *repleta* and *saltans* species were described as doublet, they may not be produced in a similar manner.

Accounting for wing position during song production allowed me to compare diverse song elements within the *saltans* group that I would not have been able to make otherwise. Considering wing position during song production allowed me to distinguish the loss of song that occurred in *D. emarginata* and *D. neocordata* as two distinct losses instead of one (see Figure 2.4). Consideration of wing position during song production also allowed me to characterize the novel 'beep' found in *D. sturtevanti* as a secondary song despite the structure of the element

being so different from that of other secondary songs described within this group. Despite the structure of the beep element being so dissimilar to the other *saltans* species described, the tonal beep appeared to be quite similar in structure to the sine song of *melanogaster* species (Kyriacou and Hall, 1980)

The diversity of song elements across lineages of *Drosophila* made it difficult for Markow and O'Grady (2005) to examine courtship song at a broad scale while preserving the diverse characteristics associated with song. To examine songs that have no homology in associated characteristics, Markow and O'Grady (2005) simplified all song characteristics to the number of song types typically described for a group or subgroup. I propose the inclusion of wing(s) position during song production as a resolution to the issue of homology. The addition of wing(s) position during song production when describing song will allow for the inclusion of more characteristics beyond number of song types sung. I also propose consideration of when the song occurs during courtship. Combining wing position and placement in the courtship sequence, homology of at least song type can be considered, and comparisons on a broad scale can be made possible despite the variation in elements across lineages.

Song Production and Role in Mating Success

Song production and role of song in mating success were not associated. I expected that song phenotype (i.e., reduced, elaborated, or standard) would be associated with the effect of song (i.e., none, necessary, or facilitates) during courtship. Although this was not statistically evaluated, I found that in the two species that song was reduced, the role of song was necessary for mating success, and in the one species that song was elaborated, the role of song facilitated mating success but was not required. I also found that standard song, which was heterogeneous

across the species classified as standard, varied from having no effect on mating success to being necessary.

For the auditory modality to be necessary for mating success, one or more message(s) required for successful copulation must be contained in the sensory channel. Therefore, the reduced songs of *D. neocordata* and *D. emarginata* must retain essential messages necessary for copulation because mating success was eliminated when song was removed. Mate quality and species-specific messages have been associated with pulse song IPI (Ritchie et al., 1999; Saarikettu et al., 2005; Snook et al., 2005) and carrier frequency (Hoikkala et al., 1998; Ritchie et al., 1998) in other *Drosophila* species. In fact, in *D. montana*, a species that produces one song type, pulse song IPI and pulse song carrier frequency communicates species-specific and mate quality messages, respectively. It is possible that the pulse song of *D. neocordata* contains both of these messages as well considering its necessity for mating. However, the non-pulse song of *D. emarginata* appears to have a lower level of structural complexity compared to pulse song (and especially compared to the beep song ; see Figure 2.2 and 2.3). Studies are needed to understand what characteristics of *D. emarginata* song are being assessed by females during courtship.

For the auditory modality to facilitate mating and not be necessary, the messages contained in the modality must not be essential, or may be redundantly—and less efficiently—expressed in an available modality. Furthermore, the message(s) contained in another non-ablated modality may be additive with the messages present in the auditory modality, causing the reduction but not the elimination of mating success when the channel is removed. Therefore, the song of *D. sturtevanti* must act redundantly, additively or synergistically with other sensory modalities. Perhaps audition interacts with vision in *D. sturtevanti*, which was found to facilitate

mating as well. I did not measure the role of cuticular hydrocarbons (CHCs) in this study, which act in an additive manner with courtship song to produce mate choice outcomes in *D. melanogaster* (Rybak et al., 2002) and *D. montana* (Veltsos et al., 2011).

The use of male wings to produce both an auditory and potentially visual signal complicated the interpretation of results for at least a few species. In one species that had reduced song, *D. neocordata*, male wings were necessary for mating to occur but the effect of female aristae could not be determined due to low rate of mating in controls. Considering vision played a role in mating success, I cannot eliminate the possibility that male wings played a visual, rather than, auditory role. Auditory signals need to be further explored in *D. neocordata*.

Role of Vision and Audition in Mating Success

The role of the auditory channel was not correlated with the role of the visual channel during courtship. Although this relationship was not statistically significant, it trended towards a negative correlation. Perhaps with greater statistical power associated with a larger sample size I would find this negative relationship to be significant. For example, when Keesey et al. (2019) sampled 62 species of *Drosophila*, they found a tradeoff between resource allocation to visual and olfactory sensory systems. This tradeoff could be occurring between more than the two sensory modalities studied in the large sample.

Correlation Between Male Wings and Female Aristae

The role of male wings and female aristae during courtship were the only two trait effects that were significantly correlated. Both male wings and female aristae ablate courtship song through production (i.e., male wings) or reception (i.e., female aristae) of auditory signal. However, there were two species that differed in the role of these two traits in terms of mating success, meaning one ablation alone should not be used as a proxy for understanding the role of

song. In *D. austrosaltans*, male wings contributed to mating success, but female aristae did not. However, our ability to determine the contribution of female aristae to mating was limited by the low mating success of the control pairs, and thus auditory signal may still contribute to mating success in *D. austrosaltans*. Light was necessary for mating success in this species, but not for females because ablating female vision did not reduce mating. Vision was thus required only for males. Male wings may be serving as a non-auditory signal in this species, but the nature of signaling needs further investigation. In *D. dacunhai*, female aristae contributed to mating success, but male wings did not. Furthermore, lack of vision did not have an effect on mating success, eliminating the possibility that the male wings served as a visual signal. The aristae may be detecting vibrations other than those produced by male wings, as is the case for three species in the *melanogaster* group, *D. suzukii*, *D. melanogaster*, and *D. biarmipes,* which produce vibrations using locomotion, the abdomen, and thoracic muscles (Mazzoni et al., 2013). Vibrational signals need to be explored in *D. dacunhai*.

Study limitations

For phylogenetic analyses, five genes, and likely four unlinked loci, were used to understand the relationship among the *saltans* species. The gene trees produced here present a hypothesis about the relationship between the genes included in the study, and not necessarily the relationship among the species. Species trees and gene trees differ for many reasons and using a small number of loci will result in a limited ability to assess how the species are related. Even though, this data set is a good starting point and is our best chance at understanding how the *saltans* species are related to one another until we have a broader set of sequenced genes for the group.

I performed no-choice tests between conspecifics, which allowed me to have a broader understanding of the use of multiple sensory modalities as well as a robust description of one of the signals. Designing the study in this manner allowed me to test whether or not two modalities, or modality use and signal, were associated. However, the use of no-choice tests meant I was only examining non-competitive mating interactions. For example, I could not test if aristae facilitated mating when males were in competition with other males and heard the courtship song of competitors. Also, sensory modality use experiments were done only with conspecifics, which limited our ability to understand the role of sensory modality use in heterospecific interactions.

Drosophila courtship often involves olfaction and chemoreception, which were not measured in this study, but may give me greater insight into how the use of sensory modalities shift across the group. Nonetheless, our focus on vision and audition helped to disentangle the role of two sensory modalities that are linked in *Drosophila* courtship because both auditory and visual signals are often produced by the male wings. Measuring the role of both of these sensory modalities was necessary to help uncover the role of each specific sensory modality for mating success, and to assess how the two are correlated (or not) at the scale of the species group.

Conclusions

I found a great deal of variation in the production of song across a set of nine *Drosophila* species with two species having reduced song and one species having an elaborated song element. The role of audition and vision varied from having no effect to being necessary for mating success. I found that the contribution of song during courtship was not associated with song phenotype (loss or elaboration) in the *saltans* group. I also found that effect of song during courtship was not correlated with effect of vision during courtship in the *saltans* group. I suggest that broadening the scale of this investigation to include more species with a higher level of

variation in song loss and elaboration may be necessary to have enough power to detect the relationship if it does indeed exist.

Figures

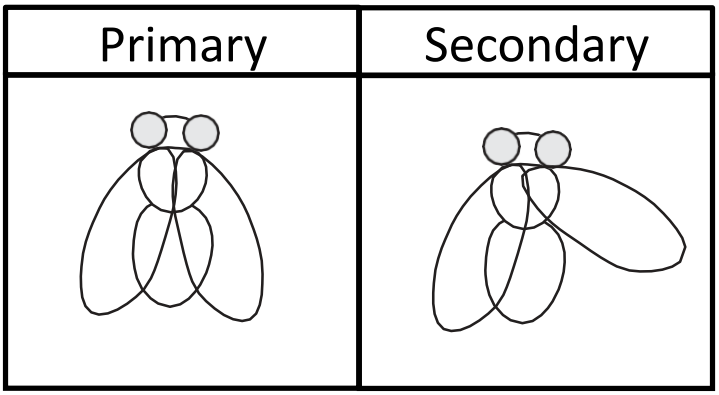


Figure 2.1 Song production. Species of the *saltans* species group produce one or two song types. Song types were defined by the designation of wing orientation during production of sound. Production of primary song occurred when the male vibrated both wings at an approximately 20º (40º to each other for *D. willistoni*) angle from the centerline of the male's thorax; secondary song was produced when the male extended and vibrated one wing (generally the one closest to the females head) at approximately 70º (>40º for D. willistoni) angle from the centerline of the male's thorax.

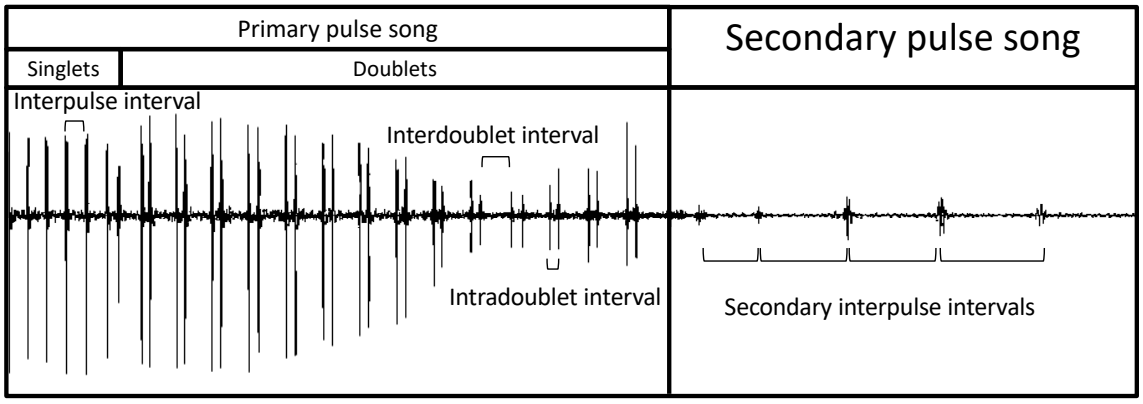


Figure 2.2. Oscillogram of pulse song. Primary pulse song appears as a series of singlet pulses at the beginning of the sonogram and then as a series of doublets. The interpulse interval of primary and secondary singlet song is labelled as well as the inter- and intradoublet intervals of primary, doublet song.

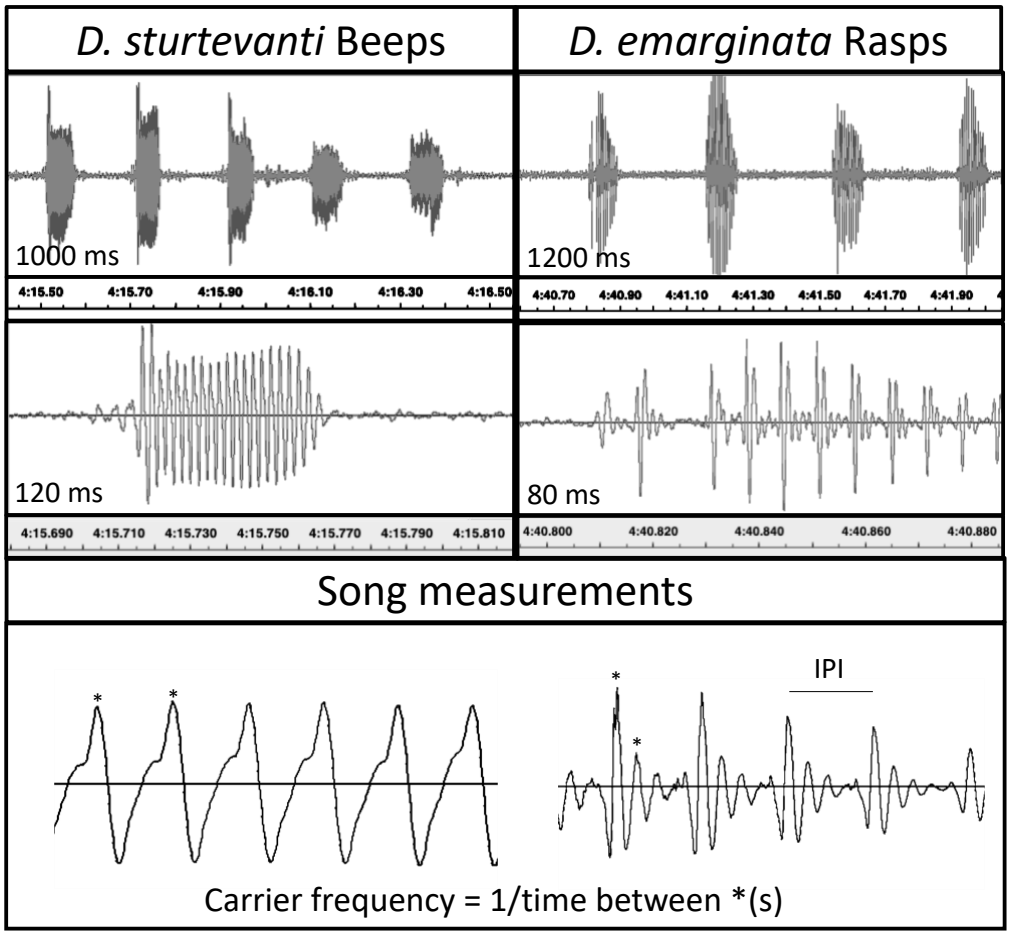


Figure 2.3. Oscillograms of beeps and rasps. The secondary songs of *D. sturtevanti* and *D. emarginata* are comprised of beeps (left panels) or rasps (right panels), respectively. The beep has a sinusoidal-like, cyclical, non-pulse structure, whereas the rasp has a pulse structure. The top panel has the longest time interval (1000–1200 ms). The time interval of the middle panel is much shorter (80-120 ms). The bottom panel illustrates measurements that were completed to describe the two elements. Asterisks mark a wave period with a beep (left) and within a rasp (right). Carrier frequency is the inverse of the time in seconds of a wave period.

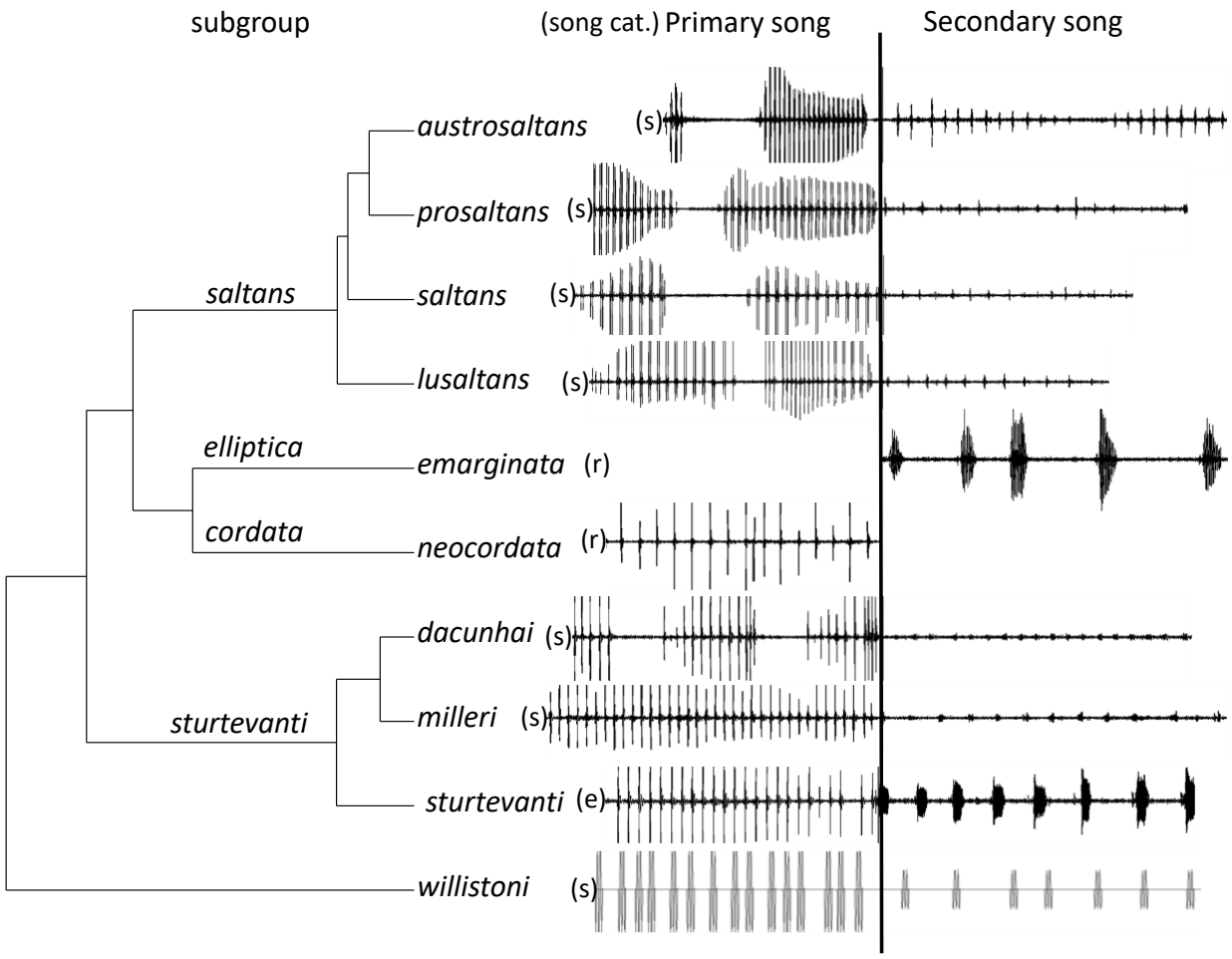


Figure 2.4. Song structure and production. Songs produced by male wing vibrations were recorded and analyzed qualitatively. Oscillograms shown are from actual song recordings, with the exception of that of *D. willistoni*, which was synthesized in R based on the song characters described in Ritchie and Gleason (1995). Songs were separated into two song types, primary (left of the line) and secondary (right of the line) and placed next to species on phylogeny to assess how song structure varies across the species group. Each song was categorized (s, standard; e, elaborated; r, reduced) and the letter associated with the song categorization was noted next to each species song. The phylogeny used was updated from O'Grady et al. (1998) by adding a species (*D. dacunhai*), an additional gene (the *per* gene), and using a closer outgroup (*D. willistoni*). Subgroup designations were placed on the branches leading up to the species in this study that comprise the subgroup.

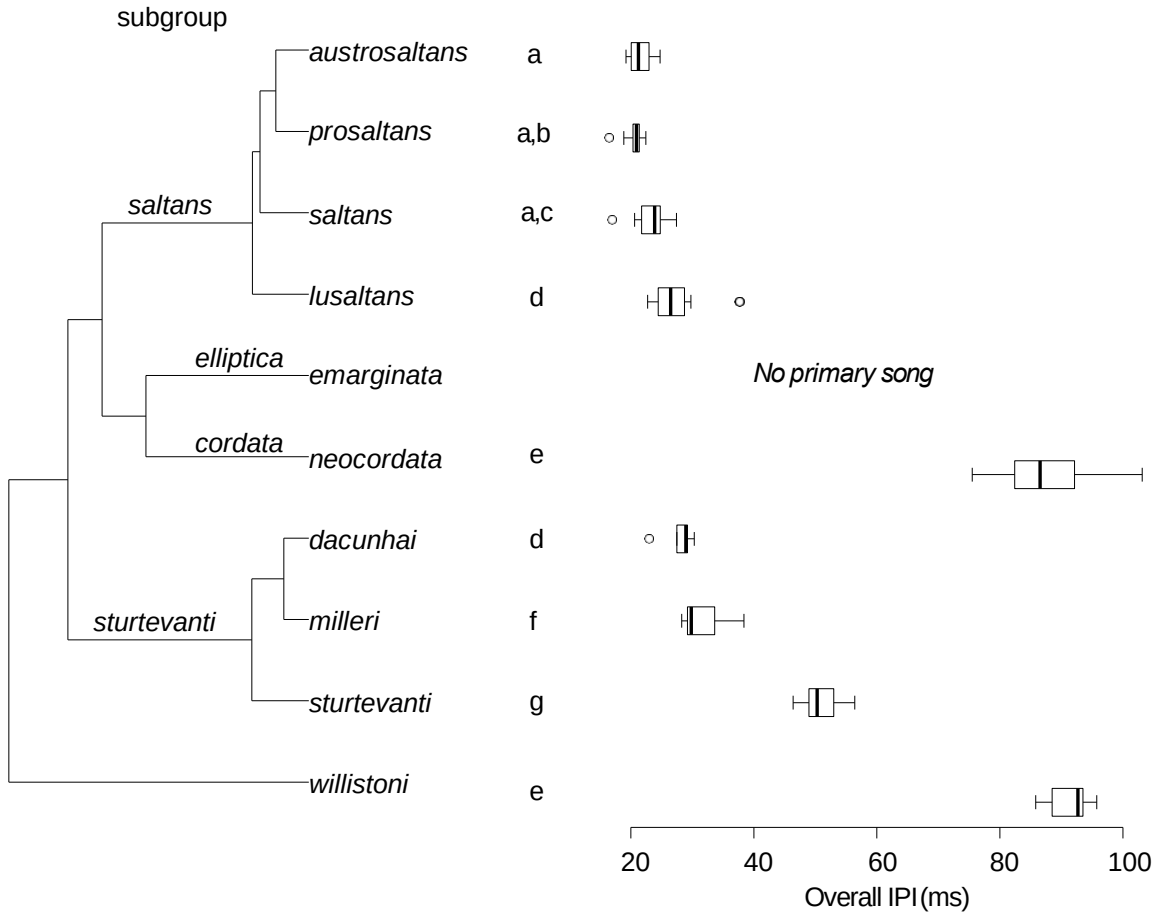


Figure 2.5. Primary song overall IPI. Overall IPI was measured by taking a weighted mean of the interpulse interval (time in ms between adjacent singlet pulses) and interdoublet interval (time in ms between adjacent doublet) measurements in the *saltans* subgroup. For the remaining species, overall IPI is the mean IPI of singlet song. In three species, *D. prosaltans*, *D. neocordata*, and *D. sturtevanti*, IPI was correlated with temperature. For these species IPI was corrected to the common temperature, 23º C, the mean temperature across all of the recordings. All species have a primary pulse song except for *D. emarginata*. Pairwise Mann Whitney U tests were done to compare the overall IPI of each pair of species. The letters next to the species name reflect whether or not two species differ from one another in overall IPI length ($\alpha = 0.05$). Species with the same letters do not differ significantly from one another ($P < 0.05$).

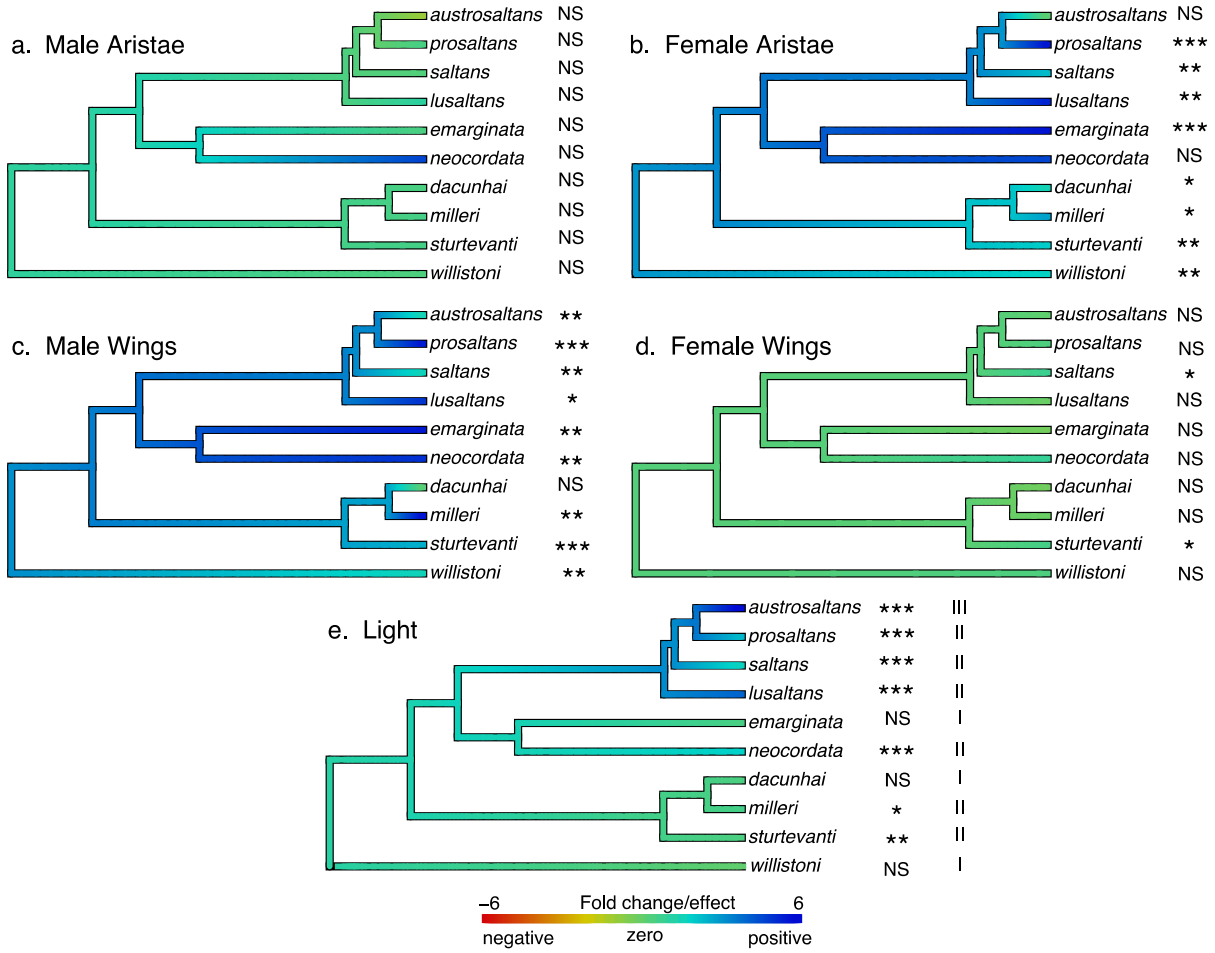


Figure 2.6. Effect of modalities on mating. The effects of a sensory modality were calculated as the $\log_2$ fold change between the level of mating that occurred in the control condition and the level of mating that occurred in the ablated treatment. The $\log_2$ fold change was placed on the tips of the phylogenies to perform an ancestral state reconstruction. A Fisher's Exact Test compared the number of control and treatment pairs that mated. Asterisks represent significant *P*-values ($P < 0.05$ *, $P < 0.01$ **, and $P < 0.001$ ***); NS: no significant difference. A. Male aristae (song reception organ) had no significant effect on mating. B. Female aristae (song reception organ) had a significant positive effect on mating for all species except for *D. austrosaltans* and *D. neocordata*. C. Male wings (song production organ) had a significant positive effect on mating for all species except for *D. dacunhai*. D. Female wings had a significant positive effect on mating for two species (*D. saltans* and *D. sturtevanti*). E. All visual signals used during courtship were removed by placing potential mating pairs in the dark and counting how many pairs produced offspring. Species were classified according to Grossfield (1971) into one of three classes: class I species mate equally well in the light and dark (light independent), class II species have light facilitated mating, and class III species do not mate in the dark (light dependent). The effect of light was not significantly different from continuous darkness for three species, *D. emarginata*, *D. dacunhai*, and *D. willistoni*. For the remaining species, light had a significant positive affect.

Tables

Table 2.1. Stocks used in study.

Group	Subgroup	Species	*Drosophila* Species Stock Center Number
saltans†	*saltans*	*Drosophila austrosaltans*	14045-0881.00
		Drosophila prosaltans	14045-0901.02
		Drosophila saltans	14045-0911.00
		Drosophila lusaltans	14045-0891.00
	sturtevanti	*Drosophila dacunhai*	14043-0854.00
		Drosophila milleri	14043-0861.00
		Drosophila sturtevanti	14043-0871.01
	cordata	*Drosophila neocordata*	14041-0831.00
	elliptica	*Drosophila emarginata*	14042-0841.09
willistoni‡	*willistoni*	*Drosophila willistoni*	

†Song description data obtained from recording and analyzing song of individuals from listed stocks. Behavioral data obtained from ablating sensory modalities, observing behavior, and analyzing mating success outcomes of pairs from listed stocks.

Table 2.2. Song terms and descriptions for *saltans* species.

Song type	differentiated by wing orientation during production
Primary	occurs when male vibrates both wings at an approximately 20º angle to the center of the male's thorax (see Figure 2.1)
Secondary	occurs when male extends and vibrates one wing at an approximately 70º angle to the center of the male's thorax (see Figure 2.1)
Song elements	differentiated visually by appearance on oscillogram and auditorily by sound
Pulse	discrete unit of sound consisting of one or more cycles with no harmonics (see Figure 2.2)
Beep	tone song consisting of a sinusoidal-like progression of sound cycles without pulse structure beginning with high amplitude sound cycles and fading into lower amplitude sound cycles (see Figure 2.3)
Rasp	train that is ~75 ms consisting of fast pulses with an IPI of ~7 ms; pulses are variable in amplitude across element (see Figure 2.3)
Song structure	differentiated by the patterning of the elements in the train
Train	series of elements that are characterized by a relatively consistent amount of time occurring between elements with no intervening elements. Songs consisted of primary singlet pulse trains, primary doublet pulse trains, secondary pulse trains, and beep trains
Singlet pulse train	all pulses in train occurred at an approximately consistent time interval (see Figure 2.2)
Doublet pulse train	a train that has two distinct interval measurements; intradoublet intervals are shorter intervals between adjacent pulses, while interdoublet intervals are longer intervals between adjacent doublet pulses (see Figure 2.2)

Table 2.3. Summary of primary and secondary song measurements.

			Primary Song					Secondary Song		
Subgroup	Species	N†	Structure	Overall IPI Mean ± SE (ms)	Intradoublet IPI‡ Mean ± SE (ms)	Interdoublet IPI§ Mean ± SE (ms)	Interpulse IPI¶ Mean ± SE (ms)	Structure	IPI Mean ± SE (ms)	Carrier frequency†† Mean ± SE (Hz)
saltans	*D. austrosaltans*	10	doublet	21.65 ±0.60	9.57±0.17	33.72±0.71	NA	singlet	121.00 ±1.60	–
	D. prosaltans	15	doublet	20.57 ±0.39	10.29±0.15	31.71±0.65	NA	singlet	102.27 ±2.20	–
	D. saltans	13	doublet	23.22 ±0.73	9.95±0.22	39.64±0.76	NA	singlet	102.65 ±4.93	–
	D. lusaltans	11	doublet + singlet	27.91 ±1.58	11.51±0.37	43.77±1.86	26.34 ±1.13	singlet	141.20 ±6.52	–
elliptica	*D. emarginata*	10	NA	NA	NA	NA	NA	rasp	6.27 ±0.25‡‡	692.00 ±101.22
cordata	*D. neocordata*	10	singlet	88.27 ±2.88	NA	NA	NA	NA	NA	NA
sturtevanti	*D. dacunhai*	6	singlet	27.99 ±1.08	NA	NA	NA	singlet	66.49 ±2.15	–
	D. milleri	10	singlet	31.57 ±1.04	NA	NA	NA	singlet	60.93 ±1.89	–
	D. sturtevanti	17	singlet standard; fast¶¶	51.13 ±0.72; 25.05 ±0.96	NA	NA	NA	beep	NA	442.42 ±15.29
willistoni	*D. willistoni*	7§§	singlet	91.17 ±1.44	NA	NA	NA	singlet	200.20 ±8.35	–

†Number of songs analyzed. ‡Measurement between two sequential pulses of a doublet song. §Measurement between a set of pulse pairs of a doublet song. ¶Measurement of singlet song for a species that sings both doublet and singlet song. ††Carrier frequency of secondary song was calculated for the two species that do not have a secondary pulse song. ‡‡Not the same as other secondary song IPIs. §§N for *D. willistoni* represents 7 strains. The average of each strain is used instead of the average of 7 individual songs of the same strain. ¶¶*D. sturtevanti* produced singlet pulse song that had distinct timing. Most song was singlet song that had a mean IPI of 51.13 and fewer pulses occurred at an IPI twice as fast at 25.05.

Table 2.4. Effect of modalities on mating.

Male aristae†							
Species	N trials	N control mated	N ablated mated	log_2 Fold change /Effect‡	95% CI log_2 Fold change§	P-value; Fisher's Exact Test¶††	
D. austrosaltans	14	6	11	-0.87	-1.83, 0.08	0.12	NS
D. prosaltans	20	11	7	0.65	-0.38, 1.69	0.35	NS
D. saltans	15	11	8	0.46	-0.35, 1.27	0.45	NS
D. lusaltans	20	9	4	1.17	-0.27, 2.61	0.18	NS
D. emarginata	16	9	6	0.59	-0.52, 1.69	0.10	NS
D. neocordata	14	4	0	4.84	1.78, 7.89	0.48	NS
D. dacunhai	11	9	6	0.59	-0.29, 1.46	0.36	NS
D. milleri	15	8	5	0.68	-0.56, 1.92	0.46	NS
D. sturtevanti	17	13	8	0.70	-0.12, 1.52	0.16	NS
D. willistoni ‡‡	20	12	8	0.51	-0.35, 1.52	0.34	NS
Female aristae†							
Species	N trials	N control mated	N ablated mated	log_2 Fold change /Effect	95% CI log_2 Fold change	P-value; Fisher's Exact Test	
D. austrosaltans	14	6	5	0.27	-1.07, 1.60	1.000	NS
D. prosaltans	20	11	0	5.78	2.91, 8.65	< 0.001	***
D. saltans	15	11	2	2.46	0.55, 4.37	< 0.01	**
D. lusaltans	20	9	0	5.49	2.59, 8.39	< 0.01	**
D. emarginata	16	9	0	5.82	2.93, 8.70	< 0.001	***
D. neocordata	14	4	0	4.84	1.78, 7.89	0.10	NS
D. dacunhai	11	9	3	1.58	0.14, 3.03	0.03	*
D. milleri	15	8	1	2.99	0.18, 5,81	0.01	*
D. sturtevanti	17	13	3	2.12	0.59, 3.64	< 0.01	**
D. willistoni‡‡	20	12	3	1.93	0.41, 3.59	< 0.01	**
Male wings†§§							
Species	N trials	N control mated	N ablated mated	log_2 Fold change /Effect	95% CI log_2 Fold change	P-value; Fisher's Exact Test	
D. austrosaltans	21	15	5	1.58	0.41, 2.76	<0.01	**
D. prosaltans	19	10	0	5.72	2.83, 8.60	<0.001	***
D. saltans	15	13	4	1.70	0.46, 2.94	<0.01	**
D. lusaltans	14	5	0	5.16	2.17, 8.15	0.05	*
D. emarginata	15	8	0	5.74	2.84, 8.63	<0.01	**
D. neocordata	21	8	0	5.25	2.33, 8.17	<0.01	**
D. dacunhai	16	10	8	0.32	-0.57, 1.22	0.72	NS
D. milleri	14	8	0	5.84	2.95, 8.73	<0.01	**
D. sturtevanti	13	13	2	2.70	0.86, 4.54	<0.001	***
D. willistoni‡‡	20	13	4	1.70	0.35, 3.05	< 0.01	**
Female wings§§							
Species	N trials	N control mated	N ablated mated	log_2 Fold change /Effect	95% CI log_2 Fold change	P-value; Fisher's Exact Test	
D. austrosaltans	21	15	13	0.21	-0.42, 0.83	0.74	NS
D. prosaltans	19	10	7	0.52	-0.53, 1.56	0.51	NS
D. saltans	15	13	7	0.89	0.06, 1.72	0.05	*
D. lusaltans	14	5	5	0.00	-1.43, 1.43	1.00	NS
D. emarginata	15	8	9	-0.17	-1.08, 0.74	1.00	NS
D. neocordata	21	8	4	1.00	-0.50, 2.50	0.31	NS
D. dacunhai	16	10	11	-0.14	-0.86, 0.59	1.00	NS
D. milleri	14	8	8	0.00	-0.93, 0.93	1.00	NS
D. sturtevanti	13	13	8	0.70	0.08, 1.32	0.04	*
D. willistoni‡‡	20	13	9	0.53	-0.31, 1.37	0.34	NS

†The ablations that were associated with the acoustic channel included female aristae, male wings, and male aristae. ‡A $\log_2$ fold change calculation was used to assess the extent of the effect of the modality on mating. §95% confidence interval for $\log_2$ fold change was calculated using equation for standard error of log relative risk. ¶A Fisher's Exact Test compared the number of control pairs that mated to the number of ablated individuals that mated. ††Asterisks were used to represent significant *P*-values ($P < 0.05$ *, $P < 0.01$ **, and $P < 0.001$ ***); NS: no significant difference. ‡‡The *D. willistoni* data is from Ritchie and Gleason 1995. §§The ablations that were associated with the visual channel included female wings and potentially male wings.

Table 2.5. Effect of light on mating.

Species	N trials (light, dark)	N light mated	N dark mated	Prop light mated	Prop dark mated	log_2 Fold change/ Effect†	95% CI log_2 Fold change‡	P-value; Fisher's Exact Tests§	Light dependency class¶
D. austrosaltans	22, 30	16	0	0.727	0.000	6.18	3.35, 9.02	<0.001 ***	III
D. prosaltans	56, 55	35	6	0.625	0.109	2.52	1.40, 3.65	<0.001 ***	II
D. saltans	98, 98	94	27	0.959	0.276	1.80	1.33, 2.26	<0.001 ***	II
D. lusaltans	81, 119	37	3	0.457	0.025	4.19	2.53, 5.83	<0.001 ***	II
D. emarginata	46, 114	15	24	0.326	0.211	0.63	-0.16, 1.42	0.15 NS	I
D. neocordata	82, 99	34	10	0.415	0.101	2.04	1.11, 2.96	<0.001 ***	II
D. dacunhai	70, 101	29	28	0.414	0.277	0.58	-0.03, 1.19	0.07 NS	I
D. milleri	51, 54	31	20	0.608	0.370	0.72	0.12, 1.31	0.02 *	II
D. sturtevanti	101, 144	66	63	0.653	0.438	0.58	0.24, 0.92	<0.01 **	II
D. willistoni¶	33, 30	26	21	0.788	0.700	0.17	-0.25, 0.59	0.56 NS	I

†A log_2 fold change calculation was used to assess the extent of the effect of light on mating. ‡95% confidence interval for log_2 fold change was calculated using equation for standard error of log relative risk. §A Fisher's Exact Test compared the number of control pairs that mated in the light to the number of individuals that mated in the dark. Asterisks were used to represent significant *P*-values ($P < 0.05$ *, $P < 0.01$ **, and $P < 0.001$ ***); NS: no significant difference. ¶Light dependency classifications according to Grossfield (1966). ¶Data from Ritchie and Gleason 1995.

Table 2.6. Vision ablation effect on courtship and copulation.

		Control		Female ablated‡		Male ablated‡	
Species	N trial †	N Court	N Mate	N Court	N Mate	N Court §	N Mate §
D. austrosaltans	15	13	11	13	10	**6***	**2****
D. prosaltans	15	13	10	11	9	**6***	**0*****
D. saltans	20	17	16	18	14	13	**8***

† Number of trials; each trial included all possible treatments (control female with control male, manipulated female with control male, control female with manipulated male and both sexes manipulated). ‡ The number of treatment (female ablated and male ablated) that courted and mated were compared to the number of control using a Fisher's Exact Test. § Significant results are designated in bold and asterisks were used to designate different levels of significance; *P*-values ($P < 0.05$ *, $P < 0.01$ **, and $P < 0.001$ ***).

Table 2.7. Vision ablation effect on courtship latency and duration.

		Control		Female ablated†		Male ablated†	
Species	N trial	Courtship latency ±SE (s)	Courtship duration ±SE (s)	Courtship latency ±SE (s)	Courtship duration ±SE (s)	Courtship latency ±SE (s) ‡	Courtship duration ±SE (s) §
D. austrosaltans	15	199.9±113.2	533.5±170.0	108.4±40.6	902.9±308.4	**706.1±276.6***	NA
D. prosaltans	15	491.5±211.4	1303.1±254.4	173.9±53.0	1095.0±329.5	553.8±250.4	no mating
D. saltans	20	65.5±14.5	427.1±155.9	91.2±30.7	572.1±226.0	**349.7±92.0****	726.9±243.9

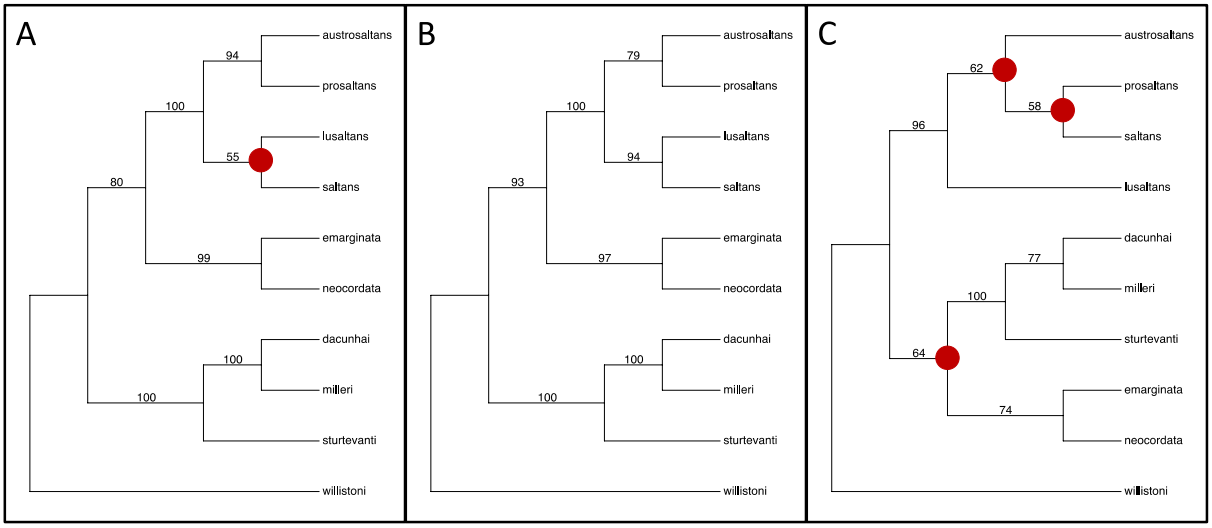
†Courtship latency and duration of the treatment (female ablated and male ablated) that courted and mated were log transformed and compared to the courtship latency and duration of the control using a t-test. ‡Significant results are designated in bold and asterisks were used to designate different levels of significance; *P*-values ($P < 0.05$ *, $P < 0.01$ **, and $P < 0.001$ ***). NA: no test was possible due to low number of individuals mated

Table 2.8. Correlation test p-values and results for top 6 topologies.

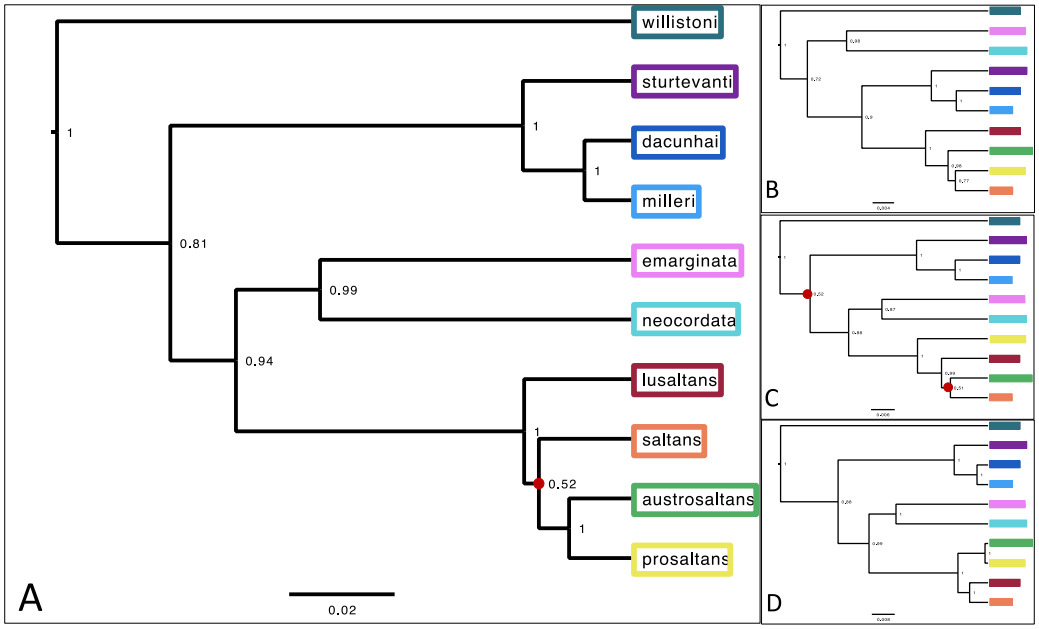
†	Tree 1	Tree 2	Tree 3	Tree 4	Tree 5	Tree 6
Light Female wings	*P*= 0.196 $F_{1,8}$= 1.996 R^2= 0.200	*P* = 0.205 $F_{1,8}$= 1.909 R^2= 0.193	*P* = 0.201 $F_{1,8}$= 1.945 R^2= 0.196	*P* = 0.226 $F_{1,8}$= 1.719 R^2= 0.177	*P* = 0.210 $F_{1,8}$= 1.855 R^2= 0.188	*P* = 0.187 $F_{1,8}$= 2.084 R^2= 0.207
Light Male wings	*P* = 0.628 $F_{1,8}$= 0.254 R^2= 0.031	*P* = 0.493 $F_{1,8}$= 0.516 R^2= 0.061	*P* = 0.663 $F_{1,8}$= 0.205 R^2= 0.025	*P* = 0.493 $F_{1,8}$= 0.516 R^2= 0.061	*P* = 0.519 $F_{1,8}$= 0.456 R^2= 0.054	*P* = 0.650 $F_{1,8}$= 0.223 R^2= 0.027
Light Female aristae	*P* = 0.154 $F_{1,8}$= 2.487 R^2= 0.237	*P* = 0.077 $F_{1,8}$= 4.117 R^2= 0.340	*P* = 0.174 $F_{1,8}$= 2.23 R^2= 0.218	*P* = 0.084 $F_{1,8}$= 3.891 R^2= 0.327	*P* = 0.086 $F_{1,8}$= 3.818 R^2= 0.323	*P* = 0.169 $F_{1,8}$= 2.286 R^2= 0.222
Light Male aristae	*P* = 0.341 $F_{1,8}$= 1.026 R^2= 0.114	*P* = 0.238 $F_{1,8}$= 1.624 R^2= 0.169	*P* = 0.382 $F_{1,8}$= 0.856 R^2= 0.097	*P* = 0.272 $F_{1,8}$= 1.393 R^2= 0.148	*P* = 0.265 $F_{1,8}$= 1.435 R^2= 0.152	*P* = 0.331 $F_{1,8}$= 1.072 R^2= 0.118
Female wing Male wing	*P* = 0.904 $F_{1,8}$= 0.016 R^2= 0.002	*P* = 0.944 $F_{1,8}$= 0.005 R^2= 0.001	*P* = 0.912 $F_{1,8}$= 0.013 R^2= 0.002	*P* = 0.977 $F_{1,8}$< 0.001 R^2< 0.001	*P* = 0.934 $F_{1,8}$= 0.007 R^2< 0.001	*P* = 0.910 $F_{1,8}$= 0.014 R^2= 0.002
Female wing Female aristae	*P* = 0.892 $F_{1,8}$= 0.020 R^2= 0.002	*P* = 0.884 $F_{1,8}$= 0.023 R^2= 0.003	*P* = 0.893 $F_{1,8}$= 0.019 R^2= 0.002	*P* = 0.959 $F_{1,8}$= 0.003 R^2= 0.0003	*P* = 0.878 $F_{1,8}$= 0.025 R^2= 0.003	*P* = 0.902 $F_{1,8}$= 0.016 R^2= 0.002
Female wing Male aristae	*P* = 0.356 $F_{1,8}$= 0.959 R^2= 0.107	*P* = 0.232 $F_{1,8}$= 1.673 R^2= 0.173	*P* = 0.355 $F_{1,8}$= 0.965 R^2= 0.108	*P* = 0.292 $F_{1,8}$= 1.273 R^2= 0.137	*P* = 0.230 $F_{1,8}$= 1.691 R^2= 0.174	*P* = 0.396 $F_{1,8}$= 0.806 R^2= 0.092
Male wing **Female aristae** ‡	***P* = 0.007** **$F_{1,8}$= 12.99** **R^2= 0.619**	***P* = 0.007** **$F_{1,8}$= 12.83** **R^2= 0.616**	***P* = 0.007** **$F_{1,8}$= 12.77** **R^2= 0.615**	***P* = 0.007** **$F_{1,8}$= 12.92** **R^2= 0.618**	***P* = 0.007** **$F_{1,8}$= 12.62** **R^2= 0.612**	***P* = 0.007** **$F_{1,8}$= 12.88** **R^2= 0.617**
Male wing Male aristae	*P* = 0.264 $F_{1,8}$= 1.444 R^2= 0.153	*P* = 0.262 $F_{1,8}$= 1.459 R^2= 0.154	*P* = 0.268 $F_{1,8}$= 1.415 R^2= 0.150	*P* = 0.251 $F_{1,8}$= 1.535 R^2= 0.161	*P* = 0.264 $F_{1,8}$= 1.442 R^2= 0.153	*P* = 0.249 $F_{1,8}$= 1.548 R^2= 0.162
Female aristae Male aristae	*P* = 0.068 $F_{1,8}$= 4.459 R^2= 0.358	*P* = 0.064 $F_{1,8}$= 4.608 R^2= 0.366	*P* = 0.066 $F_{1,8}$= 4.517 R^2= 0.361	*P* = 0.061 $F_{1,8}$= 4.747 R^2= 0.372	*P* = 0.062 $F_{1,8}$= 4.717 R^2= 0.371	*P* = 0.055 $F_{1,8}$= 5.061 R^2= 0.388

†Correlation analyses of trait effect were performed using phylogenetically independent contrasts of the top 6 trees (see Supplementary Figure A for topologies). ‡ Significant results are designated in bold.

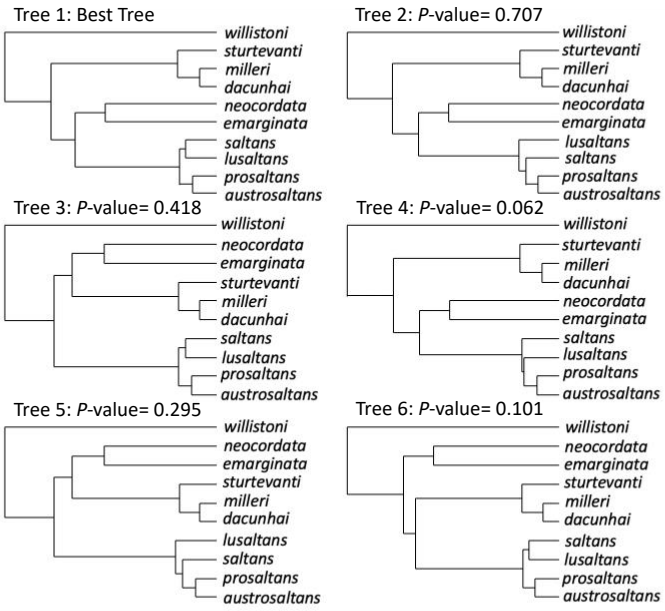
Supplementary Figures



Supplementary Figure 2.1. Maximum likelihood topologies produced in PAUP*.Trees were produced in PAUP* using maximum likelihood with 100 bootstrap replicates. The percentage of bootstrap replicates that contained the relationship were placed on the nodes. Nodes that had less than 70 percent support were identified with red circles. There was incongruency between topologies in the relationships among subgroups and the relationship among species within the *saltans* subgroup. A. Consensus tree using all data. B. Tree produced using nuclear data only. C. Tree produced using mitochondrial data only.



Supplementary Figure 2.2. Bayesian inference topologies produced in BEAST2 and *BEAST2. Consensus and gene trees were produced using Bayesian inference respectively using *BEAST2 and BEAST2. Each species is designated with a color to make interpreting the topologies along the right panel easier. Posterior probabilities are designated on the nodes with nodes that have a lower posterior probability than 0.70 being identified with red circles. There was incongruency between topologies in the relationships among subgroups and the relationship among species within the *saltans* subgroup. A. Consensus tree from *BEAST2 using all data B. Topology produced using mitochondrial data only. C. Topology produced using *Adh* data only D. Topology produced using *per* data only.



Supplementary Figure 2.3. Top six plausible trees. Tree 1 is the maximum likelihood tree. Tests for statistical differences in likelihoods of alternative topologies was assessed using the Approximately Unbiased (AU) test in PAUP*. The non-significant *P*-value associated with the various topologies showed that when each tree topology was compared to the best tree topology (Tree 1) the topology could not be rejected (i.e. the likelihood was not significantly different). All correlation analyses were done using all 6 topologies so as to not bias the results. Tree 2 was used in all figures that included phylogenies because it matched the combined analysis, Bayesian inference tree.

Supplementary Tables

Supplementary Table 2.1. Genbank sequence accession numbers.

Species†	COI	COII	ITS1	Adh	per
Drosophila austrosaltans	AF045107	AF045091	AF045363	AF045123	
Drosophila dacunhai					
Drosophila emarginata	AF045108	AF045092	AF045364	AF045124, AF045125, AF045126	
Drosophila lusaltans	AF045106	AF045090	AF045365	AF045122	
Drosophila milleri	AF045105	AF045089	AF045366	AF045121	
Drosophila neocordata	AF045104	AF045088	AF045367	AF045120	
Drosophila prosaltans	AF045103	AF045086	AF045368	AF045118, AF045119	
Drosophila saltans	AF045097	AF045081	AF045369	AF045113	
Drosophila sturtevanti	AF045098	AF045082	AF045370	AF045114, AF045115, AF045116	
Drosophila willistoni†	U51590	U51589		U95265	U51056

† pers. comm., Gleason

Supplementary Table 2.2. Comparison of likelihood values of top 6 backbone constrained topologies applying the Approximately Unbiased (AU) test.

Tree topology	Likelihood	Difference of likelihood from topology 1	AU *P*-values‡
1†	10665.25881	0.000	–
2	10665.46367	0.205	0.707
3	10668.93661	3.678	0.418
4	10669.20557	3.947	0.062
5	10669.39125	4.132	0.295
6	10671.06064	5.802	0.101

†Tree 1 is the maximum likelihood tree. Tests for statistical differences in likelihoods of alternative topologies was assessed using the Approximately Unbiased (AU) test in PAUP*. The non-significant *P*-value associated with the various topologies showed that when each tree topology was compared to the best tree topology (Tree 1) the topology could not be rejected (i.e. the likelihood was not significantly different).

Supplementary Table 2.3. Summary of Mann Whitney U test results for overall primary song IPI.

	N†	***aus***	***pro***	***sal***	***lus***	***neo***	***dac***	***mil***	***stu***	***wil***
***aus*‡**	10		W= 97¶ *P*= 0.24	W= 34 *P*= 0.06	W= 7 *P*< 0.001	W= 0 *P*< 0.001	W= 3 *P*< 0.01	W= 0 *P*< 0.001	W= 3 *P*< 0.001	W= 0 *P*< 0.001
pro	15	**NS§**		W= 29 *P*= 0.001	W= 0 *P*< 0.001	W= 0 *P*< 0.001	W= 0 *P*< 0.001	W= 0 *P*< 0.001	W= 0 *P*< 0.001	W= 0 *P*< 0.001
sal	13	**NS**	**		W= 28 *P*= 0.01	W= 0 *P*< 0.001	W= 8 *P*< 0.01	W= 0 *P*< 0.001	W= 0 *P*< 0.001	W= 0 *P*< 0.001
lus	11	***	***	*		W= 0 *P*< 0.001	W= 25 *P*= 0.46	W= 23 *P*= 0.02	W= 0 *P*< 0.001	W= 0 *P*< 0.001
neo	10	***	***	***	***		W= 60 *P*< 0.001	W= 100 *P*< 0.001	W= 160 *P*< 0.001	W= 22 *P*= 0.29
dac	6	**	***	**	**NS**	***		W= 10 *P*= 0.03	W= 6 *P*< 0.001	W= 0 *P*< 0.01
mil	10	***	***	***	*	***	*		W= 13 *P*< 0.001	W= 0 *P*< 0.001
stu	16	***	***	***	***	***	***	***		W= 0 *P*< 0.001
wil	7††	***	***	***	***	**NS**	**	***	***	

†Number of songs analyzed. ‡First three letters of the species name was used as an abbreviation. §On the bottom diagonal of the table, asterisks are used to represent significant *P*-values ($P < 0.05$ *, $P < 0.01$ **, and $P < 0.001$ ***); NS: no significant difference. ¶Test statistics and *P*-values are given on the top diagonal of the table. ††N for *D. willistoni* represents seven strains. The average of each strain is used instead of the average of seven individual songs of the same strain. Note: No tests were done for *D. emarginata* because they do not sing primary song.

Supplementary Table 2.4. Summary of Mann Whitney U test results for secondary pulse song IPI.

	N†	***aus***	***pro***	***sal***	***lu***	***dac***	***mil***	***wil***
***aus*‡**	10		W= 139¶ *P*< 0.001	W= 110 *P*< 0.001	W= 12 *P*< 0.01	W= 60 *P*< 0.001	W= 100 *P*< 0.001	W= 0 *P*< 0.001
pro	14	***§		W= 108 *P*= 0.23	W= 3 *P*< 0.001	W= 84 *P*< 0.001	W= 12 *P*< 0.001	W= 140 *P*< 0.001
sal	12	***	**NS**		W= 9 *P*< 0.001	W= 72 *P*< 0.001	W= 120 *P*< 0.001	W= 0 *P*< 0.001
lu	10	**	***	***		W= 60 *P*< 0.001	W= 100 *P*< 0.001	W= 1 *P*< 0.001
dac	6	***	***	***	***		W= 51 *P*=0.02	W= 0 *P*< 0.01
mil	10	***	***	***	***	*		W= 0 *P*< 0.001
wil	7 ††	***	***	***	***	**	***	

†Number of songs analyzed. ‡First three letters of the specific epithet of each species name was used as an abbreviation. §On the bottom diagonal of the table, asterisks are used to represent significant *P*-values (*P* < 0.05 *, *P* < 0.01 **, and *P* < 0.001 ***); NS: no significant difference. ¶Test statistics and *P*-values are given on the top diagonal of the table. ††N for *D. willistoni* represents seven strains. The average of each strain is used instead of the average of seven individual songs of the same strain. Note: No tests were done for *D. neocordata* because they do not sing secondary song and no tests were done for *D. emarginata* and *D. sturtevanti* because they do not have a pulse secondary song.

Chapter 3 : Complex song of *Drosophila sturtevanti* may communicate multiple signals redundantly

Introduction

Communicating messages in nature is complex because signals must be filtered through both the environment and the receiver. Signaling environments are often noisy, placing efficacy-based selection pressures on the signals traveling through them (reviewed in Hebets & Papaj, 2005). Receiver sensory constraints may also limit signal efficiency, placing sensory detection and internal processing efficacy-based selection pressures on signals (reviewed in Hebets & Papaj, 2005). When signals are complex (multimodal, or one modality containing multiple signals), both the signaler and receiver may benefit from increased signal detectability ("how easily the signal can be perceived as distinct from environmental noise") and discrimination (a learned or innate predisposition to respond differently to various stimuli; reviewed in Rowe, 1999). Complex signals may evolve to communicate one message more efficaciously through a complex environment, or to convey multiple messages more efficiently (e.g., those necessary for sexual communication; reviewed in Hebets & Papaj, 2005).

To understand the function of complex signals, I test three non-mutually exclusive hypotheses (reviewed in Hebets & Papaj, 2005; Johnstone, 1996). One hypothesis for the function of complex signaling is the redundant signaling hypothesis, which postulates that the same signal is sent in multiple forms to assure accuracy of the message conveyed (reviewed in Candolin, 2003; Hebets & Papaj, 2005). Redundant signals may be most important in complex signaling environments (e.g., due to complex microhabitat of leaf litter, wood/bark, soil and rocks, wolf spiders' communicate quality visually through foreleg tapping, waving, and arching and seismically through whole body vibrations; Gordon & Uetz, 2011). Another hypothesis is the multi-tasking hypothesis, (reviewed in Hebets & Papaj, 2005) which suggests that signals are energetically costly (i.e., may communicate quality) and constrain

one another. The third hypothesis is the multiple messages hypothesis which suggests each signal communicates a different message (e.g., species identity, mate quality) to the receiver. To test the three non-mutually exclusive hypotheses, I examine the degree to which multiple signals within a complex signal change together. Each of the three hypotheses is associated with a prediction about phenotypic covariance. Finding a positive relationship between two signals in a complex signal supports the redundant signaling hypothesis, a negative relationship supports the multi-tasking hypothesis, and no relationship supports the multiple messages hypothesis.

In *Drosophila* species, courtship song is evolutionarily complex and often composed of multiple signals (Chang & Miller, 1978; Ewing & Miyan, 1986; Ritchie & Gleason, 1995). Many species of *Drosophila* produce a song that is made up of more than one song type (reviewed in Markow & O'Grady, 2005) making *Drosophila* an ideal group in which to test the message content hypotheses. Often, the songs of *Drosophila* consist of two song types (Markow & O'Grady, 2005) that are produced through distinct wing orientations (described in Chapter Two). Many characteristics of song have been associated with communicating various messages during courtship. For instance, interpulse interval (IPI), average time in milliseconds between the peaks of two adjacent pulses, communicates species identity in *D. melanogaster* and sister species (Ritchie et al., 1999; von Schilcher, 1976). Another song characteristic, carrier frequency, reciprocal of the duration in seconds between the peaks of a pulse, communicates mate quality in *D. montana* (Hoikkala et al., 1998)

Although the message content hypotheses have not been directly tested using phenotypic covariance in any *Drosophila* species to our knowledge, it appears that song may have evolved to communicate multiple messages in *Drosophila melanogaster*. The song of *D.*

melanogaster, the most studied *Drosophila* species, consists of two song types: pulse song and a humming "sine" song. The timing of pulses is associated with species recognition (IPI;Ritchie et al., 1999; von Schilcher, 1976) and conspecific mate selection (preference for more pulse trains per unit time or longer pulse trains; Talyn & Dowse, 2004). Sine song, once thought to be associated with "priming" or stimulating females to copulate (von Schilcher, 1976), may play no function in courtship (Talyn & Dowse, 2004). Short timescale song features like IPI indicate species identity, while conspecific quality assessment occurs through the assessment of long timescale features like bout length (length of uninterrupted song consisting of pulse and sine song) in *D. melanogaster* (Clemens et al., 2015). Length of IPI communicates male quality in *D. pseudoobscura*, with fast (short) IPI being associated with higher fitness (Snook et al., 2005).

Song of species other than *D. melanogaster* may have evolved to communicate multiple messages as well. For example, the song of *D. montana*, of the *virilis* species group, consists of one pulse song. In this species, the IPI of pulse song plays a role in species recognition (Saarikettu et al., 2005), while the carrier frequency of pulses communicates male quality to the female (Hoikkala et al., 1998). Females prefer to mate with males with a high carrier frequency (Ritchie et al., 1998), and those females that mate with males possessing high carrier frequencies indirectly benefit by producing more progeny that survive from egg to adult (Hoikkala et al., 1998).

Assessing the effect of song on mating success, as above, was mostly performed using playback experiments. During playback experiments, the wings of males are removed, thereby muting them, and artificial song is played to assess female receptivity. Female receptivity is either assessed through wing spreading behavior (i.e., conspicuous wing spreading of female of

D. montana; Hoikkala et al., 1998; Ritchie et al., 1998; Saarikettu et al., 2005) or by successful copulation (Ritchie et al., 1999; Talyn & Dowse, 2004; von Schilcher, 1976). Although playback experiments are a powerful tool used in identifying the role of specific song components in courtship, it is difficult to apply in some species.

I used *Drosophila sturtevanti* for this experiment. Considering the ineffectiveness of playback experiments in this species (pers. obs. Colyott), I used the covariance framework outlined above to address questions about message redundancy. The use of *D. sturtevanti* allowed me to benefit from the fact that the two song types produced, primary pulse song and secondary beep song, were both easily quantifiable (see Chapter Two for more details). The beep of the secondary song was louder and therefore easier to capture in recordings and analyze than the secondary song of the other species described in Chapter Two. Also, the beep is a novel song component that sounds dissimilar to the songs of over ~100 other *Drosophila* species in which songs have been described (reviewed in Markow and O'Grady, 2005). Primary pulse song can be quantified by measuring the mean IPI and the number of IPIs present in song. Secondary beep song can be quantified by measuring the total number of beeps per song and per train (defined as a series of beeps with no intervening pulses; Figure 3.1). Beeps can also be quantified by measuring mean carrier frequency (Figure 3.1). Due to the high number of song recordings necessary for a study of this type, I also describe the novel song in greater detail than described in Chapter Two.

To study the covariation of two song types, there must be variation within each song type. To increase the variation and increase the probability of detecting covariation if present, I used two strains of *D. sturtevanti* that differed in both song types. The strains differed from one another in IPI, with one strain having a high IPI and the other a relatively low IPI. The strains

also differed from one another in the number of beeps present in song, with one strain beeping more than the other.

In this study, I quantified the primary and secondary song traits of *D. sturtevanti* to understand if primary and secondary songs communicate similar or different messages during courtship. I crossed two inbred strains of *D. sturtevanti* that differed in both primary and secondary song. I recorded and analyzed song from the parental lines, F1 generations, and advanced cross generations. To test hypotheses about message content, I examined the covariance of song traits of advanced cross generations. I found that IPI of pulse song and carrier frequency of beep song were positively correlated, and that total number of beeps per song was positively correlated with mean number of beeps per train and total number of IPIs per song. Neither IPI nor carrier frequency covaried with mean number of beeps per train, or total number of IPIs and beeps per song. In *D. sturtevanti*, song may have evolved to convey redundant information via multiple messages.

Methods

Fly Culturing

I maintained cultures of *D. sturtevanti* strains one and five (*Drosophila* Species Stock Center stock number: 14043-0871.01 and 14043-0871.05) in 6 oz square bottom, polypropylene bottles containing standard cornmeal-molasses *Drosophila* food at 24°C with 12:12 hour light:dark cycle. Cultures were maintained by transferring approximately 15–30 flies onto new large food vials (24 mm d x 94 mm h) weekly or biweekly.

Inbred Lines

Strains used in this experiment were inbred in the lab based on the assumption that they were not already inbred at the stock center. Founding virgin flies were collected under light CO_2

anesthesia from cultures within four hours of eclosion to produce inbred lines. One virgin male and one virgin female were placed in small food vials (16.5 mm d x 95 mm h) fitted with cotton plugs. Each generation of inbreeding was started in a new small food vial with one virgin male and one virgin female from the previous generation. Strain one of *D. sturtevanti* was inbred for 16 generations and *D. sturtevanti* strain five was inbred for seven generations at which point inbred lines were maintained in large vials (24 mm d x 94 mm h) with 15–30 flies. Approximately 15–30 flies were transferred to new food vials every or every other week. Multiple inbred lines for each strain were created and maintained.

Phenotyping

To select two inbred lines that differed in song traits, flies from inbred lines were recorded and analyzed. Virgin flies were collected from the inbred lines of the two strains (nine lines from each) to find two lines that were most differentiated in IPI and beep characters. Males were housed individually and wing ablated females were housed in single-sex groups of up to 10 individuals, both in small food vials. Female wings were removed to ensure all sound recorded was produced by male courtship and not by female grooming or rejection sounds. A sexually mature, virgin male (7–11 days old) and a wing ablated, immature, virgin female (0–3 days old) were placed in an acoustically transparent chamber (16.5 mm d x 10 mm h) inside the Insectavox (Gorczyca, 1987). Digital recordings started with the initiation of courtship song and continued for five minutes. Mean temperature inside the Insectavox was calculated using temperatures recorded at the beginning and end of the song recording with a digital thermometer. Songs were digitally filtered (high pass: 100 Hz, low pass: 1000 Hz) with Audacity 1.6.2 and analyzed in Spike 2 (CED, Cambridge, UK) using established procedures (Ritchie & Kyriacou, 1994). Pulse song was characterized by the total number of pulses that occurred in a song and mean IPI using

Spike 2. Mean IPI was calculated by marking the peak of each pulse in the song, measuring the time between each set of pulses, and producing a histogram of IPI measurements using Spike 2. Minimum and maximum IPI were set on the histogram and mean IPI was calculated for the measurements contained in the bounded histogram. Beep song was characterized by the total number of beeps per song.

All traits were examined to see if they were correlated with temperature and an alpha of 0.02 was used to adjust for multiple testing (0.05/3 = 0.02). Mean IPI and mean temperature during song recordings were correlated ($\propto = 0.02$, $F_{1,58} = 6.244$, $P = 0.015$, $R^2 = 0.097$) with a slope of -5.14. Mean IPI was adjusted to the common temperature of 25ºC by:
Adjusted mean IPI = -5.14 (25ºC – mean temperature during recording) + measured mean IPI [equation 1]. Total number of beeps per song was not correlated with temperature. A Tukey honest significant difference test was conducted to determine which lines were the most different from one another for both adjusted mean IPI and total numbers of beeps per song. No comparisons of any two fly lines resulted in $P < 0.05$ for either trait so two lines that differed in both mean IPI (Supplementary Figure 3.1; Tukey honest significant difference test comparing strain 1-7 to strain 5-11; difference = -10.66, adjusted $P = 0.239$) and total numbers of beeps per song (Supplementary Figure 3.2; Tukey honest significant difference test comparing strain 1-7 to 5-11; difference = 38.08, adjusted $P = 0.647$) were chosen to create advanced intercross flies.

Advanced Crosses

The chosen inbred line cultures (1-7 and 5-11) were used as the parental lines ($P_{1\text{-}7}$, $P_{5\text{-}11}$) to produce the F_1 and advanced intercross individuals (F_5, F_6, F_7, and F_8). One virgin female 1-7 was placed in a small food vial with one virgin male 5-11 to produce $F_{1(1\text{-}7)}$ flies. To produce the reciprocal cross, $F_{1(5\text{-}11)}$, one virgin female 5-11 was placed in a small food vial with one virgin

male 1-7 fly to produce F_1 flies. Three to five- virgin, F_1 flies were collected from each cross ($F_{1(1\text{-}7)}$ and $F_{1(5\text{-}11)}$) and 15-20 flies mixed together in 6 oz square bottom, polypropylene bottles. Flies were left in bottles for two weeks to mate and lay eggs and then adult flies were removed. After another two weeks, the flies emerging from the next generation (F_2) were flipped into new bottles. This process was repeated for each generation up to the eighth generation.

Phenotyping

Songs were recorded from flies of the various groups ($P_{1\text{-}7}$, $P_{5\text{-}11}$, $F_{1(1\text{-}7)}$, $F_{1(5\text{-}11)}$, F_5, F_6, F_7, and F_8). Virgin flies were collected, housed, and placed in an acoustically transparent chamber as above. Digital recordings were completed and filtered as above. We measured characteristics of the pulse song as above. A correlation analysis was done to determine the relationship between IPI and temperature ($\propto = 0.01$, $F_{1,359} = 47.86$, $P < 0.001$, $R_2 = 0.12$, $y = -3.19x + 130.21$) using an alpha of 0.01 was used to adjust for multiple testing (0.05/5 = 0.01).. The slope (-3.19) was used to correct all mean IPIs to the common temperature of 25ºC by: Adjusted mean IPI= -3.19 (25ºC – mean temperature during recording) + measured IPI. [equation 2]. All songs that did not have an associated temperature measurement were excluded from subsequent analyses (N=379). Number of IPIs was not correlated with temperature ($\propto = 0.01$, $F_{1,404} = 4.69$, $P = 0.031$, $R_2 = 0.01$).

I also measured characteristics of beep song. Beep song characters included the number of beeps per song, the mean number of beeps per train, and the mean carrier frequency. Carrier frequency of each beep train was measured in Audacity 2.2.2. using fast Fourier transforms with a Hanning window of 2048 (i.e., data was analyzed in windows that contained 2048 data points). The carrier frequency for an individual song was calculated by taking the mean of all individual train carrier frequencies. No beep measurements were correlated with temperature ($\propto =$

0.01; Total number of beeps per song: $F_{1,277} = 4.72$, $P = 0.031$, $R_2 = 0.02$; Mean number of beeps per train: $F_{1,221} = 2.28$, $P = 0.132$, $R_2 = 0.01$; Carrier frequency: $F_{1,221} = 5.12$, $P = 0.024$, $R_2 = 0.01$).

Data Processing and Analyses

All statistical analyses were performed using R v3.6.0 (R Core Team, 2017) interfaced through RStudio v1.1.383 (RStudio, Inc.). Data were processed prior to all analyses using the 'base' package of R.

Data processing

A custom bootstrap technique was used to assess how many IPI measurements were necessary to produce accurate mean IPI calculations (Supplementary Figure 3.3). The 20 songs with the highest number of IPI measurements (Range: 1605–4015) were resampled. A sample of a predetermined number of IPI measurements (10, 25, 50, 75, 125, 250, 500, 1000) were resampled 1000 times with replacement and a mean IPI was calculated for each sample. Confidence interval plots of the means calculated for the samples of each resampling group (10, 25, 50, 75, 125, 250, 500, 1000) were considered along with the actual measurement of the song to choose a minimum number of IPIs needed. A minimum of 75 IPI measurements was chosen for a mean IPI calculation because measuring 75 IPIs produced a standard deviation of 0.3 to 0.5 ms and led to the filtering of 47 songs, which seemed reasonable compared to other groupings (i.e., min. of 125 IPI, SD of 0.2 to 0.4, filtered 88 songs; min. of 50 IPI, SD of 0.4 to 0.6, filtered 27 songs). All songs that had less than 75 IPIs measurements were excluded (N= 47), leaving 418 songs that had more than 75 IPI measurements. Similarly, songs that had less than three train measurements were removed for analyses that included mean number of beeps per train.

Data analyses

Adjusted mean IPI was the only measurement that was normally distributed (Shapiro Wilk Normality Test; W= 0.993, *P*= 0.087). Transformations (square root, cube root, $\log_{10}$, arcsine) were done to try to normalize mean beeps per train, total beeps per song, and carrier frequency, but none was successful. The 'base' package of R was used to examine if song trait measurements differed between the various groups of flies ($P_{1\text{-}7}$, $P_{5\text{-}11}$, $F_{1(1\text{-}7)}$, $F_{1(5\text{-}11)}$, F_5, F_6, F_7, and F_8). A Wilcoxon rank-sum test was performed on non-parametric data while a Student's T test was performed on parametric data to examine potential differences in trait measurements among the groups. Tests were performed to assess differences between the parent generations ($P_{1\text{-}7}$, $P_{5\text{-}11}$), between F_1s ($F_{1(1\text{-}7)}$, $F_{1(5\text{-}11)}$), and between each parent and each advanced cross generation. A Tukey's test was performed to assess differs among the generations (F_5, F_6, F_7, and F_8). The 'Multcomp' package of R was used to perform a compact letter display procedure to produce the letters used to represent significant differences among generations on the boxplot.

To control for the differences among the generations of the advanced intercross flies, I calculated the deviation of each point within a generation from the generation mean. These deviations were used for all correlation analyses and PCA analysis. Both parametric (Pearson's) and non-parametric (Spearman's rank) correlation tests were conducted for all correlation analyses because no traits were normally distributed. The parametric results are presented here for ease of interpretation and because findings were the same as non-parametric tests.

Correlation analyses and PCAs were done using the deviation from generation mean data from the advanced crosses. The 'Hmisc' package of R was used to examine correlations among song traits. Data were processed individually for each correlation analysis (see caption of Table 3.2). For example, for the analysis of mean beeps per train and total beeps per song, songs that had less than 3 beep trains were removed because songs with less trains did not produce an

accurate mean beep per train measurement. The ‘ggpubr’ package of R was used to produce individual correlation plots. To assess whether or not data reduction resulted in a different outcome than the trait-by-trait correlation analyses, song traits were subjected to a Principal Component Analysis (PCA). The ‘stats’ package of R was used to perform the PCA. For this analysis, data were maximally processed to accommodate the test and included songs that had greater than 75 IPI measurements, a corrected mean IPI, and more than three beep trains (N= 199).

Results

Song Trait Variation

I analyzed 465 songs of *D. sturtevanti*; two parental strains ($P_{1\text{-}7}$, $P_{5\text{-}11}$), two F_1 crosses ($F_{1(1\text{-}7)}$, $F_{1(5\text{-}11)}$), and four advanced generation crosses (F_5, F_6, F_7, and F_8). Of the 465 songs, 463 produced pulse song and 406 produced beep song (Table 3.1).

Both parental lines used to produce advanced intercross flies had shorter IPIs (Figure 3.2) than when the lines were orginally recorded and analysed to choose inbred lines (Supplementary Figure 3.1) but $P_{1\text{-}7}$ remained the short IPI line and $P_{5\text{-}11}$ the long IPI line. The difference was likely due to such small sample sizes. The parental lines differed from one another in adjusted mean IPI. $P_{1\text{-}7}$ had a shorter (faster) IPI (Figure 3.2; $t_{8.47}$= -3.38, P= 0.009) than $P_{5\text{-}11}$. $F_{1(1\text{-}7)}$ did not differ from $F_{1(5\text{-}11)}$ (Figure 3.2; NS). $P_{1\text{-}7}$ had significantly shorter IPI than generation 7 (Figure 3.2; $t_{7.30}$= -3.64, P= 0.008) and generation 8 (Figure 3.2; $t_{12.72}$= -3.41, P= 0.005) but not from generation 6, while $P_{5\text{-}11}$ had a significantly longer IPI from generation 6 (Figure 3.2; $t_{7.20}$= 2.92, P= 0.022) but not from generations 7 or 8 (Figure 3.2; NS). Generation 6 had a significantly shorter (faster) IPI than both generation 7 (Supplementary Figure 3.1; Tukey’s

multiple comparison test: *P* adjusted = 0.047) and generation 8 (Supplementary Figure 3.1; Tukey's multiple comparison test: *P* adjusted = 0.054).

Both parental lines used to produce advanced intercross flies were similar in total number of beeps per song (Figure 3.3; Wilcoxon rank-sum test: NS) as when the lines were orginally recorded and analysed to choose inbred lines (Supplementary Figure 3.2; Tukey's multiple comparison test: NS). The parental lines (Figure 3.3; W=36.5, *P*= 0.141) and the F_1 crosses (Figure 3.3; W=18.5, *P*= 0.775) did not significantly differ from one another in total beeps per song. $P_{5\text{-}11}$ had significantly fewer beeps per song than generation 8 (Figure 3.3; W= 99.5, *P* = 0.027). Generation 8 had significantly more beeps per song than generation 6 (Tukey's multiple comparison test: *P* adjusted = 0.013) and generation 7 (Figure 3.3; Tukey's multiple comparison test: *P* adjusted < 0.001).

Data was filtered to only include flies that beeped. The parental lines (Figure 3.4; W=31.5, *P*= 0.406) and the F_1 crosses (Figure 3.4; W=11.5, *P*= 0.195) did not significantly differ from one another in mean beeps per train. Generation 8 had significantly more beeps per train than $P_{5\text{-}11}$ (Figure 3.4; W= 99.5, *P* = 0.027) and generation 6 (Figure 3.4; Tukey's multiple comparison test: *P* adjusted < 0.001).

Mean carrier frequency of beeps did not differ between the parental lines (Wilcoxon rank-sum test: W=13, *P*= 0.165), F_1s (Wilcoxon rank-sum test: W=18.5, *P*= 0.775), or generations (Table 3.1). Total IPIs did not differ between the parental lines (Wilcoxon rank-sum test: W=22, *P*= 0.805), F_1s (Wilcoxon rank-sum test: W=19, *P*= 0.836), or generations (Table 3.1).

When parental lines differed, they differed by design. Variation across the generations was not by design and was likely due to environmental variation (although temperature variation

was controlled for) or perhaps due to selection during mate choice or genetics (due to crossing over revealing previously hidden phenotypes).

Song Trait Correlations

Pearson's and Spearman's rank correlation tests were done on generation-corrected, advanced cross, song trait data. In all instances the Pearson's and Spearman's rank results were in agreement. Adjusted mean IPI was negatively correlated with mean carrier frequency of beeps (Figure 3.5; t_{301}= -7.09, R= -0.378, P< 0.001, R^2= 0.143, N= 303). Note that IPI is an inverse measurement and as the IPI measurement decreases, pulse rate becomes faster. The total number of beeps in a song was positively correlated with total number of IPIs (Figure 3.6; t_{436}= 13.654, R= 0.547, P< 0.001, R^2= 0.30, N= 438) and mean number of beeps per train (Figure 3.7; t_{240}= 7.805, R= 0.450 , P< 0.001, R^2= 0.203, N= 242). No other correlation analyses were significant (Table 3.2).

Principal Components Analysis

PCA of the five traits yielded two components with eigenvalues (i.e., values that describe how much variance there is in the data in a particular direction) greater than one, accounting for 59.0% of the total variance (Table 3.3). The two-component solution was selected following the common eigenvalue above one stopping rule (Kaiser, 1960) and as the most interpretable model compared to the one-, three-, four-, and five- component model. Three further extracted axes (not shown) cumulatively explained 41% of the total variation in song measures. Table 3.3 presents the component loadings for each behavior, as well as the eigenvalues and percent total variance explained. PC1 and PC2 align directly with the findings from the correlation analyses. Traits that reflected how much song was produced loaded on PC1 (Table 3.3). These traits (total beeps, total IPI, and mean number of beeps per train) were all positively correlated in the correlation

analyses and loaded in the same direction on PC1 indicating a positive relationship. Traits that reflected how quickly waves (carrier frequency; Hz or cycles per second) or pulses (adjusted mean IPI; mean time in ms between pulses) were produced loaded on PC2. Carrier frequency and adjusted mean IPI were negatively correlated and loaded in opposite directions on PC2. Again, note that IPI is an inverse measurement and as IPI decreases, pulse rate becomes faster. Therefore, as carrier frequency increases, pulse song becomes faster (lower IPI).

Discussion

In this study, I examined two signals (primary and secondary song as defined in chapter 2) of a complex signal. The major goal of this study was to determine if the two signals communicated similar or different messages during courtship. I examined the correlation of the traits to test hypotheses associated with message content. If two components do not covary, they contain different messages. If two components covary positively, they contain redundant messages, and if they covary negatively, the signals constrain one another. I quantified pulse song by measuring the number of pulses produced and the timing of those pulses (IPI). I quantified beep song by measuring the number of beeps per song, the mean number of beeps per train, and the mean carrier frequency.

Positive Relationship: IPI and Carrier Frequency

Adjusted mean IPI was negatively correlated with mean carrier frequency. However, IPI is an inverse measurement with respect to vigor (e.g., a fast pulse song has a short IPI). Flies that produced a fast (short) pulse song produced a beep song with a higher carrier frequency (more waves per cycle). Therefore, speed of IPI and carrier frequency were positively correlated, lending support to the redundant signaling hypothesis. Both song types may be communicating

male quality, similar to the fast IPI of *D. psuedoobscura* (Snook et al., 2005) and high carrier frequency of the pulse song of *D. montanta* (Hoikkala et al., 1998; Ritchie et al., 1998).

Positive Relationship: Number of IPIs and Beeps

Total number of IPIs was positively correlated with total number of beeps meaning flies that produced more pulses also produced more beeps. Therefore the relationship between number of IPIs and number of beeps also supported the redundant signaling hypothesis. Number of IPIs produced and number of beeps produced may both communicate male quality, with high quality males producing more IPIs and more beeps. To test if this was the case further experimentation is needed. The courtship song of males that varied in quality would need to be recorded and analyzed. One would expect high quality males to produce more IPIs and beeps and low quality males to produce fewer IPIs and beeps if both characters communicate male quality.

The amount of song produced during courtship may be associated with mate quality. In *D. melanogaster*, females preferred to mate with males that produced more song (Talyn and Dowse, 2004). Also, in *D. melanogaster*, amount of song was negatively correlated with female locomotion speed (Coen et al., 2014), with slow locomotion being a proxy for increased receptivity. To test if this is the case in *D. sturtevanti*, amount of song produced, latency to female receptivity, and male quality would need to be recorded. If the amount of song produced corresponded with male quality, males of higher quality would be expected to produce more song, and decrease the latency to female reception.

Flies that sang more beeps overall also sang more beeps per train. The positive covariation of these traits also supported the redundant signaling hypothesis. Number of beeps produced per song and per train may both communicate male quality, with high quality males producing more beeps per song and per train. In *D. melanogaster*, bout duration (song sang

uninterrupted by silence that is comprised of pulse and sine song) was most strongly negatively associated with female locomotion speed (Clemens et al., 2015). I did not measure bout duration in *D. sturtevanti*, but, based on our results, one might expect that an increased number of beeps per train would scale to an increased bout of song. This is assuming that number of beeps corresponds to duration of beeps which is a reasonable assumption to make considering that on average beep duration is rather consistent across flies. Therefore, the mean number of beeps per train may be associated with increased male quality, similar to increased bout length in *D. melanogaster* (Clemens et al., 2015). To test if this was the case, one needs to record the courtship song of males that varied in quality. The expectation being that high quality males produce more mean beeps per train and total beeps per song and low quality males produce fewer mean beeps per train and total beeps per song.

I found no phenotypic correlation between mean IPI and the total number of IPIs, total number of beeps or the mean number of beeps per train. I also found no phenotypic correlation between mean carrier frequency and the total number of IPIs, the total number of beeps, or the mean number of beeps per train. These findings support the hypothesis of multiple messages assuming each measurement made contains a signal. The correlation analyses as a whole suggest that mean IPI and beep carrier frequency convey a redundant message, while the total number of IPI, number of beeps per song, and mean beeps per train, together convey a different redundant.

Future Directions

A limitation to the methodology used in this study is that although I can test whether or not two signals of a complex signal communicate the same message or not, I cannot determine what messages are conveyed in individual signals. To further understand message content of

complex signals, further experimentation is needed. For instance, to assess if a signal communicates mate quality, the songs of high and low quality *D. sturtevanti* males need to be measured and compared. If a signal communicated mate quality, males of high quality would produce a different signal that males of low quality.

Another limitation to this study was that I was unable to assess the genetic architecture underlying the two song types. Understanding the genetic architecture underlying each song type and the extent to which the song types shared genetic control would help us to better understand how the song types have evolved, in concert or independently. If the two song types shared genetic control, we would expect them to be inherited together and if not, we would expect them to be inherited separately. Furthermore, understanding the number of loci contributing to each song type and the effect size of the loci would allow us to assess how the underlying genetic architecture has contributed to the variation of song overall. For instance, if the song was found to have many genes of small effect contributing to the phenotype (type I genetic architecture; Templeton 1981), similar to what was found in the *Laupala* genus of crickets, we might assess that song has evolved through an explosive radiation variation (Ellison and Shaw, 2013). Song in *D. melanogaster* and sister species was found to have a type I genetic architecture underlying the mean IPI species differences (Gleason and Ritchie, 2004). Whereas, if song traits were found to be controlled by few genes with large effect (type II architecture), we might expect variation and divergence to have evolved at a much slower rate.

Conclusion

I found that *D. sturtevanti* song conveys both redundant and multiple messages. More studies need to be done to understand the specific content of the messages Specifically, I would like to disentangle which signals communicate mate quality and serve as species

specific indicators . Although, understanding message content of specific song types or traits may be difficult without the ability to perform playback experiments.

Figures

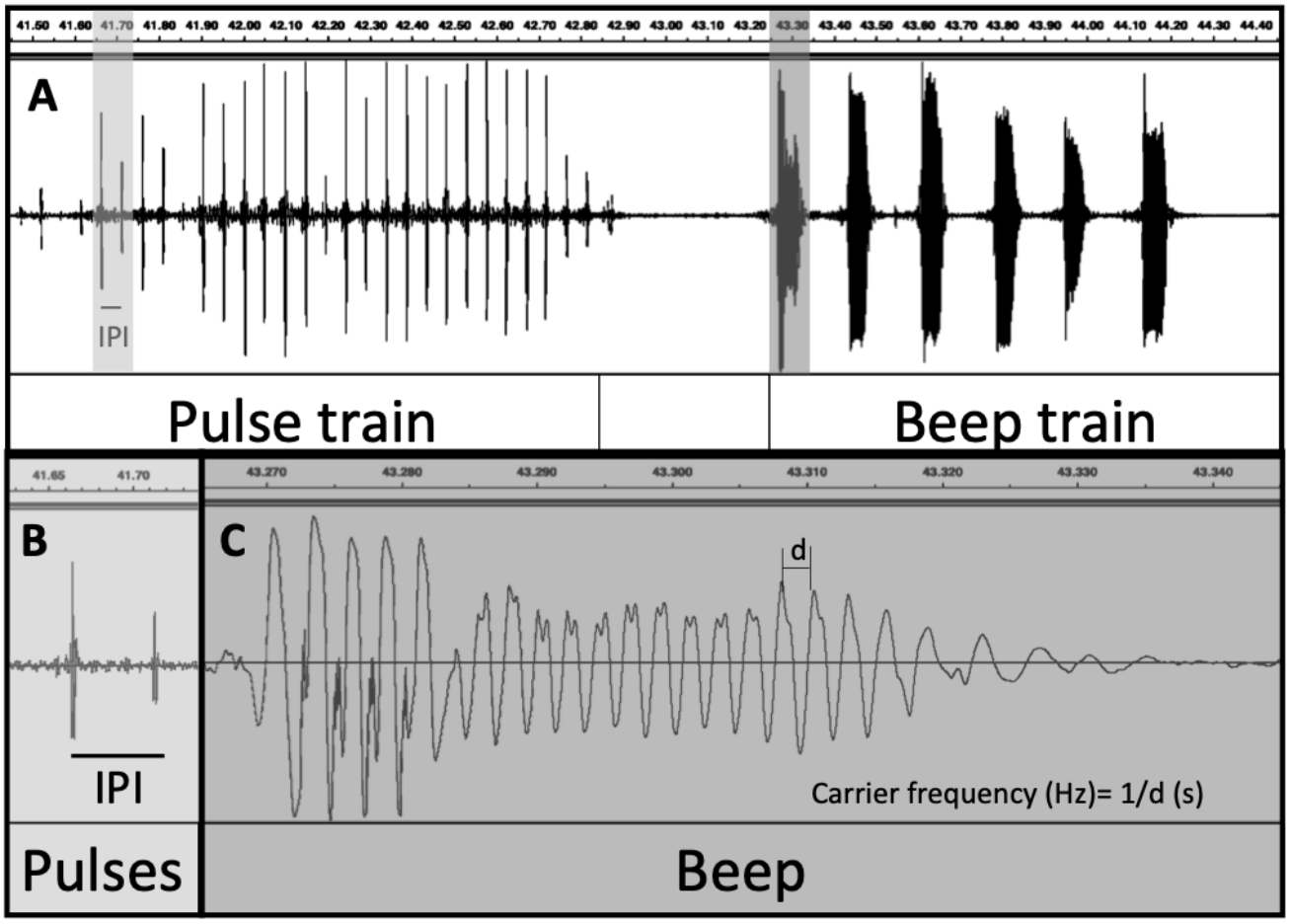


Figure 3.1. Sonogram of *D. sturtevanti* song. Song produced by *D. sturtevanti* male wing vibrations. A. Two song types produced by *D. sturtevanti*, pulse and beep song. The gray boxes indicate the portion of the song present in the bottom panels. B. Two pulses of a pulse train. Interpulse interval (IPI) is the time in milliseconds between two adjacent pulses. C. The beep is a tone song consisting of a complex sinusoidal wave that progresses into a series of sinusoidal wave sound cycles without pulse structure, starting with higher amplitude sound cycles that fade into lower amplitude cycles. Carrier frequency is the reciprocal of the time in seconds between the peaks of a pulse.

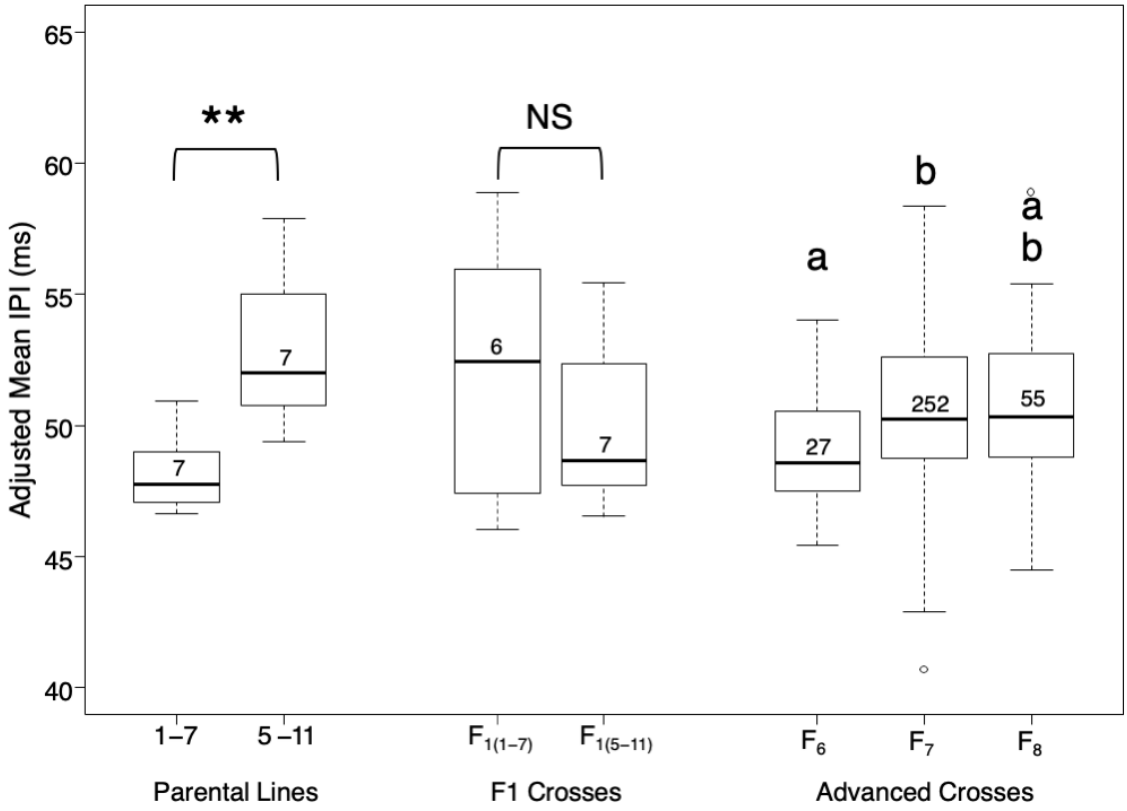


Figure 3.2. Adjusted mean IPI by parent line, F1 cross, and advanced cross generations. Songs were recorded and analyzed to examine how mean IPI varied among the parental lines, F1 crosses, and advanced intercross generations. Boxplots present the data associated with each group; the bold line is the median, the box is the interquartile range, whiskers denote the minimum and maximum, and open circles are outliers. The number of songs for each group is presented in the box associated with the group. T-tests were performed to compare the adjusted mean IPI of the parental lines and the F_1 crosses. Asterisks represent significant *P*-values ($P <$ 0.01 **); NS: no significant difference. Tukey's test was performed to compare differences among the advanced cross generations ($\alpha = 0.05$). A compact letter display procedure was used to produce the letters above the advanced cross generations. Generations represented by same letter do not differ significantly from one another. T-tests were performed to compare each parental line to each generation. Parent 1-7 is significantly different from generation 7 ($t_{7.30}$= -3.64, *P*= 0.008) and generation 8 ($t_{12.72}$= -3.41, *P*= 0.005). Parent 5-11 is significantly different from generation 6 ($t_{7.20}$= 2.92, *P*= 0.022).

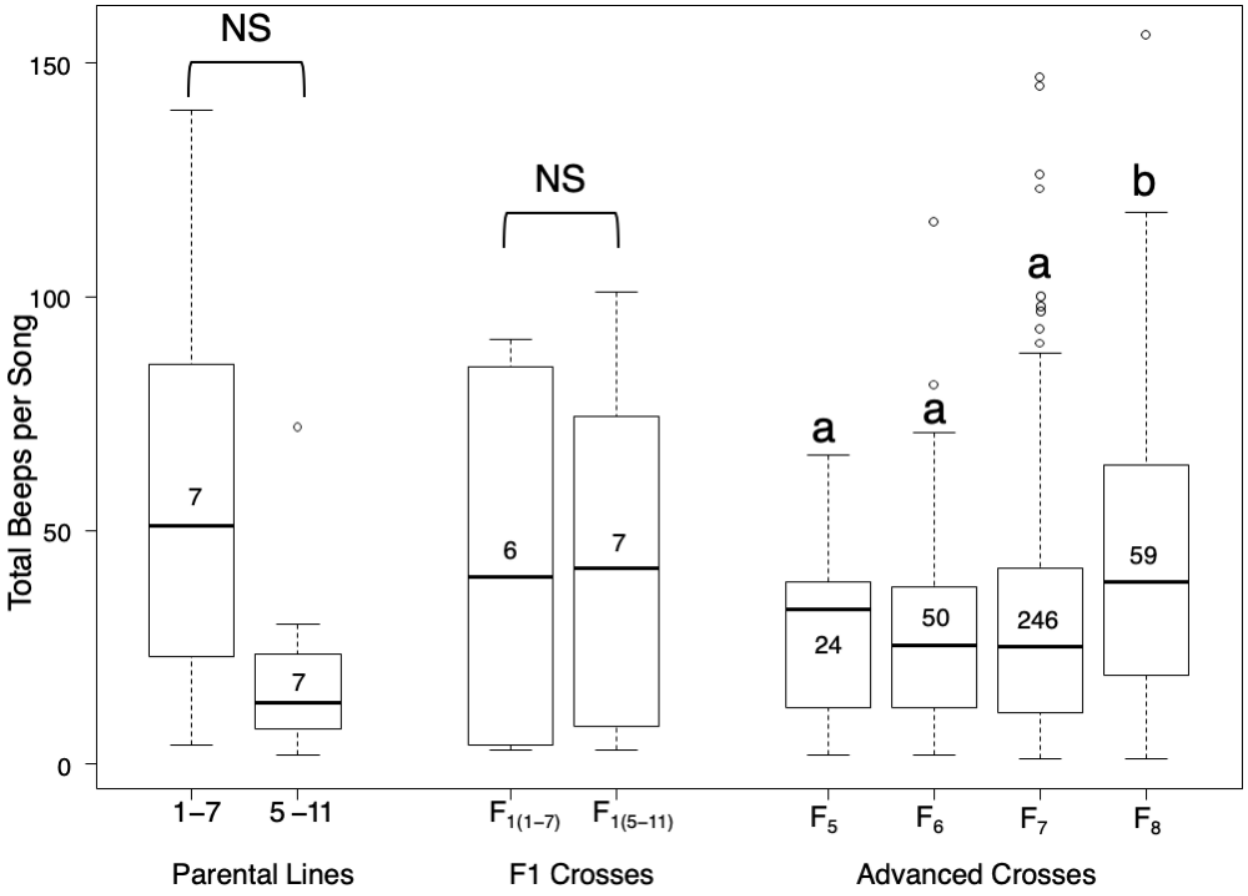


Figure 3.3. Total beeps per song by parent line, F1 cross, and advanced cross generations. Songs were recorded and analyzed to examine how total number of beeps varied among the parental lines, F1 crosses, and advanced intercross generations. Boxplots were used to present the data associated with each group; bold line is median, box is interquartile range, whiskers are minimum and maximum, and open circles are outliers. The number of songs for each group is presented in the box associated with the group. Wilcoxon rank-sum tests were performed to compare the number of beeps per song of the parental lines and the F_1 crosses. NS represents no significant difference. Tukey's test was performed to compare differences among the advanced cross generations ($\alpha = 0.05$). A compact letter display procedure was used to produce the letters above the advanced cross generations. Generations represented by same letter do not differ significantly from one another. Pairwise Wilcoxon rank sums tests were performed to compare each parental line to each generation. Parent 5-11 is significantly different from generation 8 (W= 99.5, *P*= 0.027).

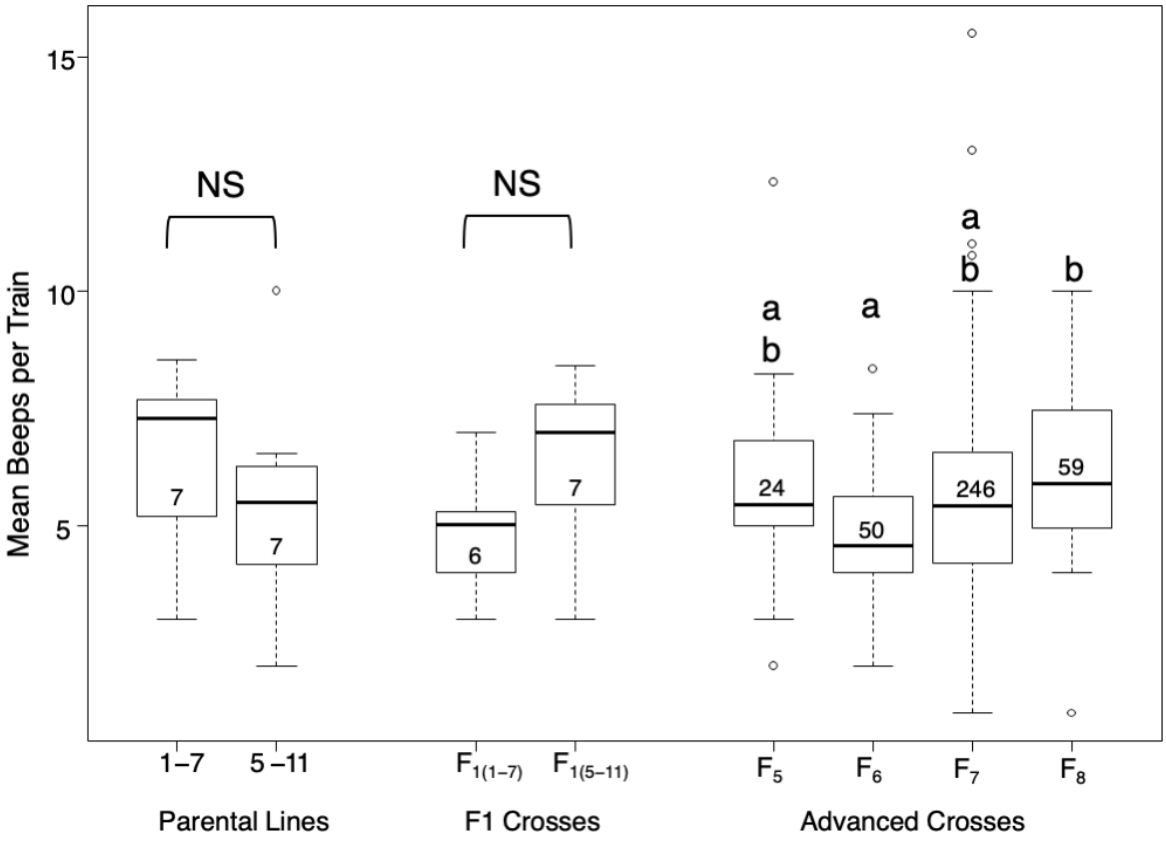


Figure 3.4. Mean beeps per train by parent line, F1 cross, and advanced cross generations. Songs were recorded and analyzed to examine how mean number of beeps per train varied among the parental lines, F1 crosses, and advanced intercross generations. Boxplots were used to present the data associated with each group; bold line is median, box is interquartile range, whiskers are minimum and maximum, and open circles are outliers. The number of songs for each group is presented in the box associated with the group. Wilcoxon rank-sum tests were performed to compare the mean number of beeps per train of each pair of groups. NS represents no significant difference. Tukey's test was performed to compare differences among the advanced cross generations ($\alpha = 0.05$). A compact letter display procedure was used to produce the letters above the advanced cross generations. Generations represented by same letter do not differ significantly from one another. Parents 1-7 and 5-11 were not significantly different from any generation.

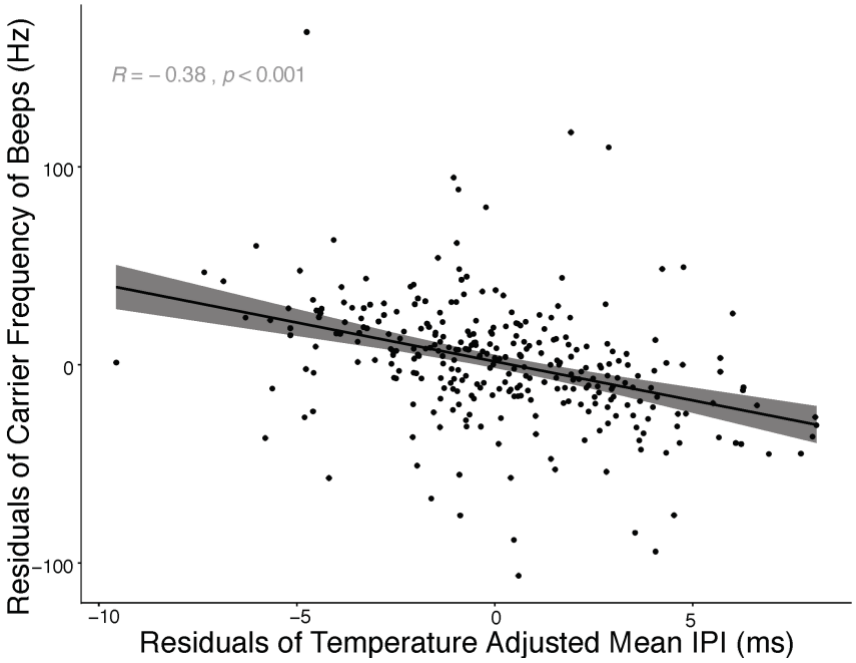


Figure 3.5. Correlation analysis of mean carrier frequency of beeps and adjusted mean IPI. A Pearson's correlation test was done to examine the relationship between the mean carrier frequency of beeps and adjusted mean IPI of songs of advanced cross generation flies (F6 – F8). The correlation was done on the deviations from the generation mean to control for differences between generations. All mean IPI measurements that did not have a measurement for temperature during recording and had less than 75 IPIs measurements were removed prior to analysis. All songs that did not contain beeps, and therefore no carrier frequency measurement, were removed prior to analysis. Mean carrier frequency was negatively correlated with adjusted mean IPI (t_{301}= -7.09, R= -0.38, P< 0.001, N= 303). The gray shading represents the 95% confidence interval around the regression line. As IPI of pulse song increased (pulse rate slowed), the beeps of the song had a lower carrier frequency.

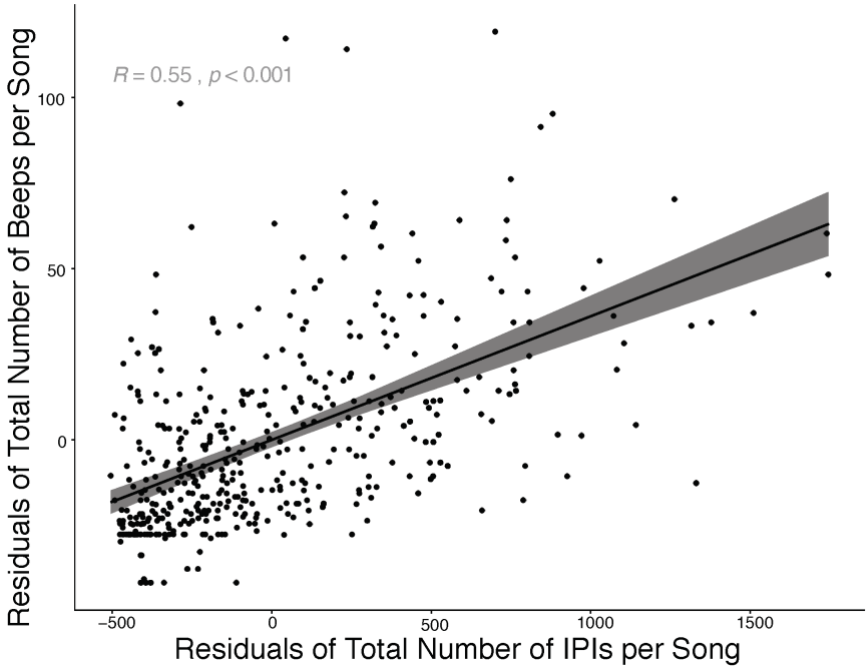


Figure 3.6. Correlation analysis of total IPIs and total number of beeps in a song. A Pearson's correlation test was done to examine the relationship between total number of beeps and total number of IPIs per song for advanced cross generation flies (F5– F8). The correlation was performed on the deviations from the generation mean to control for differences between generations. All recordings that included at least one IPI or at least one beep were included in the analysis. Total number of IPIs was positively correlated with total number of beeps in a song (t_{436}= 13.654, R= 0.547, P< 0.001, N= 438). The gray shading represents the 95% confidence interval around the regression line. As the total number of beeps in song increased, the total number of IPIs in song increased.

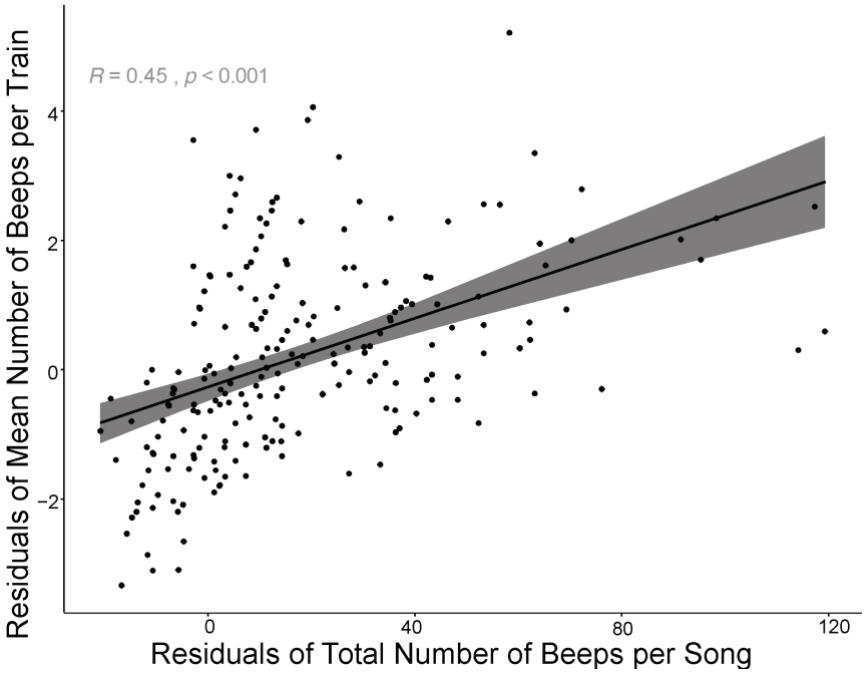


Figure 3.7. Correlation analysis of mean beeps per train and total number of beeps in a song. A Pearson's correlation test was done to examine the relationship between mean number of beeps per train and total number of beeps per song for advanced cross generation flies ($F_5 - F_8$). The correlation was performed on the deviations from the generation mean to control for differences between generations. Songs that included more that 3 trains of beeps were included in the analysis. Mean beeps per train was positively correlated with total beeps in a song (t_{240}= 7.805, R= 0.450 , *P*< 0.001, N= 242). The gray shading represents the 95% confidence interval around the regression line. As total number of beeps in song increased, the average number of beeps per train increased.

Table 3.1. Song summary statistics.

	Total number of IPI per song† mean ± SD N	Adjusted IPI (ms) ‡ mean ± SD N	Mean number of beeps per train ¶ mean ± SD N	Beep carrier frequency (Hz) ¶ mean ± SD N
Overall	505.10± 430.00 463	50.43± 3.04 361	5.58± 1.92 406	433.8± 33.34 403††
1-7	602.86± 751.06 7	48.21± 1.57 7	6.37± 2.09 7	438.4± 62.59 7
5-11	486.57± 338.94 7	52.97± 3.38 7	5.48± 2.50 7	441.6± 18.72 7
$F_{1(5\text{-}11)}$	843.67± 629.66 6	52.21± 5.35 6	4.89± 1.35 6	432.4± 24.41 6
$F_{1(1\text{-}7)}$	908.29± 608.84 7	50.11± 3.36 7	6.35± 1.96 7	440.9± 27.17 7
$F_{5\text{-}8}$ pooled	495.02± 415.40 436	50.40± 2.97 334	5.58± 1.91 379	433.5± 33.18 376
F_5	461.17± 397.41 30	NA§	5.91± 2.03 24	442.2± 53.71 24
F_6	522.83± 405.29 60	49.06± 2.06 27	4.81± 1.38 50	436.4± 35.50 50
F_7	495.81± 421.23 282	50.48± 3.01 252	5.54± 1.96 246	433.5± 31.60 246
F_8	481.33± 414.80 64	50.67± 3.03 55	6.20± 1.81 59	426.9± 25.35 56††

†Includes all songs in which flies produced pulses. ‡Songs with less than 75 IPI measurements or that did not have a measurement for temperature during recording were not included. IPI was adjusted to the common temperature of 25º C. §All generation 5 flies were discarded prior to calculating adjusted IPI because they were recorded without a mean temperature. ¶Includes only songs that produced beeps. ††We were unable to measure carrier frequency for three songs that had beeps due to there not being enough data to produce a fast Fourier transform with a Hanning window of 2048 (i.e., data was analyzed in windows that contained 2048 data points).

Table 3.2. Correlation analyses of song traits.

$t_{d.f.}$ † *P*-value / R_{XY}‡ SD_X SD_Y	Adjusted Mean IPI¶	Total Beeps	Total IPIs	Mean Beeps per Train§	Mean Carrier Freq. of Beeps
Adjusted Mean IPI¶		t_{332}= -0.93 0.36¶	t_{332}= -1.46 0.15¶	t_{199}= -0.03 0.97¶§	**t_{301}= -7.09** **< 0.001¶**
Total Beeps	-0.05 2.95 28.73		**t_{436}= 13.65** **< 0.001**††	**t_{240}= 7.81** **< 0.001**§	t_{374}= 1.20 0.23
Total IPIs	-0.08 2.95 410.25	**0.55** 27.45 415.54		t_{240}= -0.03 0.97§	t_{374}= 1.24 0.22††
Mean Beeps per Train§	-0.00 3.01 1.55	**0.45** 25.50 1.51	-0.03 436.67 1.51		t_{238}= 0.89 0.38§
Mean Carrier Freq. of Beeps	**-0.38** 2.95 30.81	0.06 27.45 32.99	0.06 415.54 32.99	0.06 1.51 31.81	

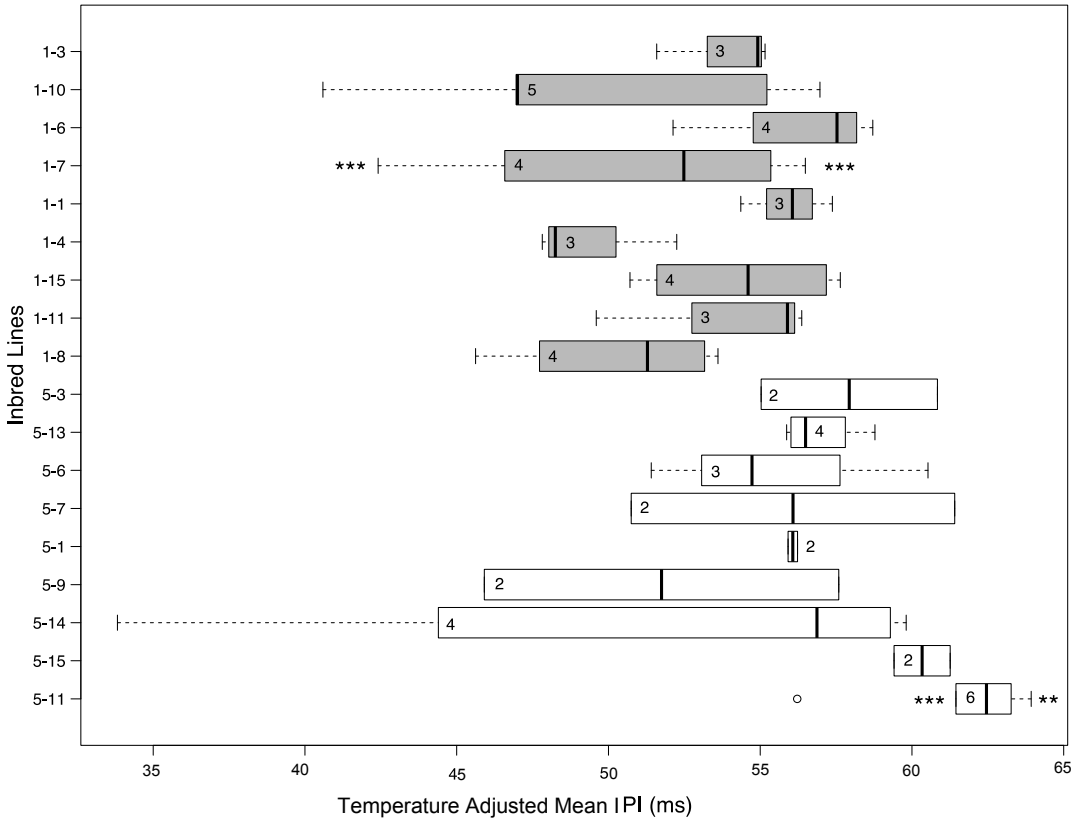
†A Pearson's correlation test was done to compare each song characteristic with each other song characteristic. *t*-values with degrees of freedom and *P*-values for each test are in the top diagonal of the table. Significant *P*-values (α value of 0.01 was used as is common when performing all by all correlation analyses) are in bold. ‡Coefficient of correlation, R values and standard deviations for X (character on horizontal axis of table) and Y (character on vertical axis of table) are in the bottom diagonal of the table. R values associated with significant *P*-values are in bold. ¶Data were processed to exclude songs that did not have a measurement for temperature during recording and/or had less than 75 IPI measurements. §Data were processed to exclude songs that had less than 3 beep trains. ††All recordings that did not have any beeps or any IPIs were excluded.

Table 3.3. Factor loadings, eigenvalues, and proportions of total variance explained by the first two axes extracted by PCA of residuals of five song measurements.

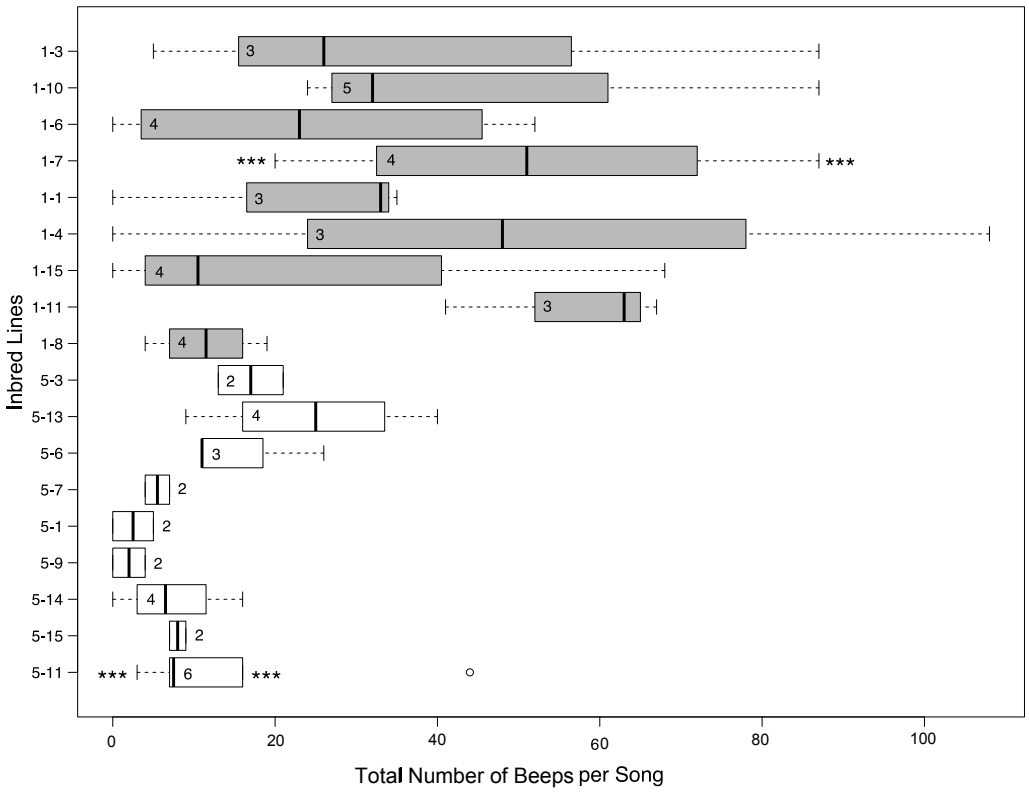
	PC1	PC2
Eigenvalue†	1.257	1.171
Proportion of variance explained	0.316	0.274
Adjusted Mean IPI	0.004	**-0.698**
Total Beeps	**0.706**‡	0.017
Total IPIs	**0.440**	-0.029
Mean Beeps per Train	**0.549**	0.108
Mean Carrier Freq. of Beeps	-0.079	**0.707**

†Data were processed to exclude songs that did not have a measurement for temperature during recording, had less than 75 IPI measurements, had less than 3 beep trains (N=199). ‡Factor loadings with an absolute correlation greater than 0.4 are shown in bold.

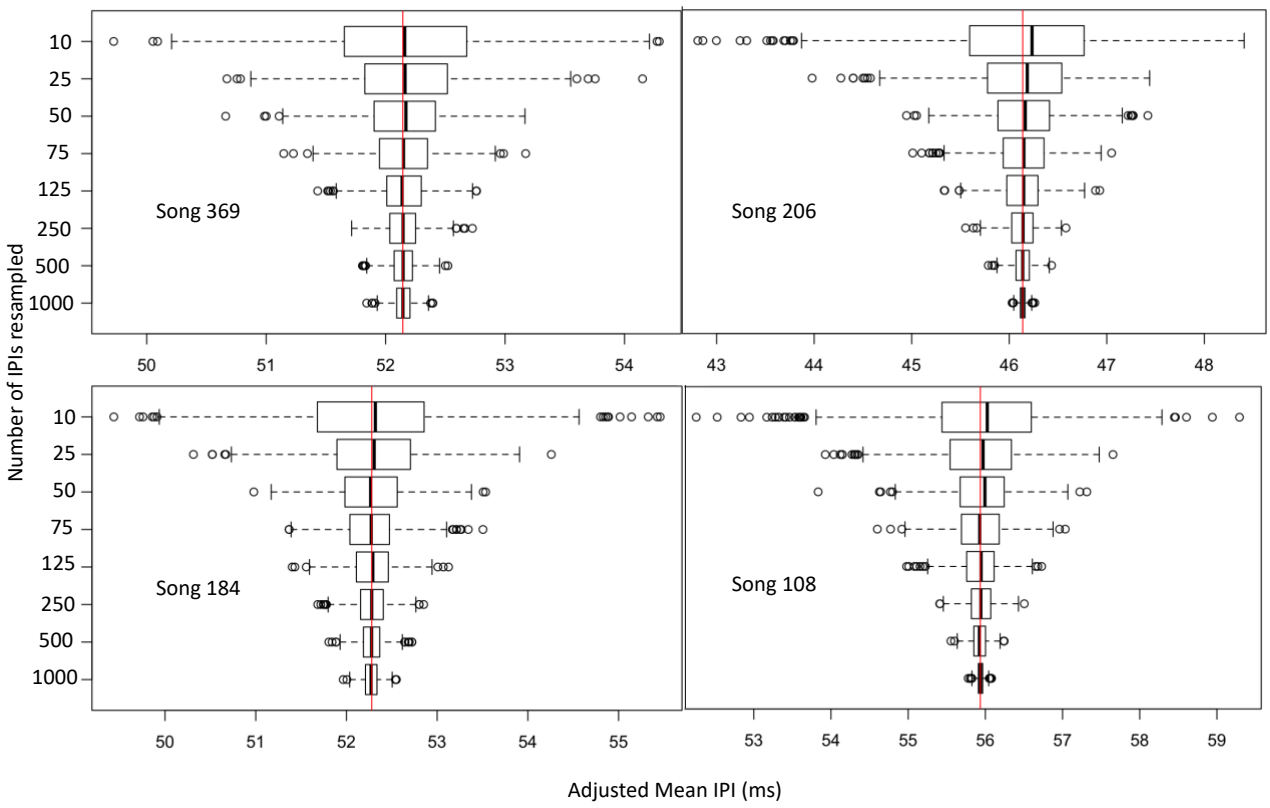
Supplementary Figures



Supplementary Figure 3.1. Temperature adjusted mean IPI for inbred lines of *D. sturtevanti* strain one and five. Boxplots were used to present the data associated with each line; bold line in box is median, box is interquartile range, whiskers are minimum and maximum, and open circles are outliers. The number of songs analyzed for each line is presented in the box associated with the group. Inbred lines marked with asterisks, one-seven (short IPI) and five-eleven (long IPI) were chosen and crossed to produce the advanced intercross flies recorded.



Supplementary Figure 3.2. Total number of beeps per song for inbred lines of *D. sturtevanti* strain one and five. Boxplots were used to present the data associated with each line; bold line in box is median, box is interquartile range, whiskers are minimum and maximum, and open circles are outliers. The number of songs analyzed for each line is presented in the box associated with the group. Inbred lines marked with asterisks, one-seven (high beeps) and five-eleven (low beeps) were chosen and crossed to produce the advanced intercross flies recorded.



Supplementary Figure 3.3. Subset of songs used to assess number of IPI measurements needed to produce an accurate mean IPI measurement. A custom bootstrap technique was used to assess the 20 songs with highest number (range?) of IPI measurements. A number of IPI measurements (10, 25, 50, 75, 125, 250, 500, 1000) were resampled 1000 times with replacement and the mean IPI was calculated after each resampling. Confidence interval plots of the means calculated in each resampling group (10, 25, 50, 75, 125, 250, 500, 1000) were lined up against the mean using all data (red line) to choose a minimum number of IPIs for subsequent analysis. A minimum of 75 IPI measurements was chosen. All songs that had less than 75 IPIs measurements were removed (N= 47), leaving 418 songs.

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