

Author accepted manuscript of

Gleason, Jennifer M, Amanda A. Pierce, Anne L. Vezeau and Stephanie F. Goodman. 2012.
Different sensory modalities are required for successful courtship in two species of the
Drosophila willistoni group. *Animal Behaviour* 83:217-227

<http://dx.doi.org/10.1016/j.anbehav.2011.10.029>

Different sensory modalities are required for successful courtship in two species of the
Drosophila willistoni group

Jennifer M. Gleason^{a*}, Amanda A. Pierce^{b,1}, Anne L. Vezeau^{b,2} and Stephanie F. Goodman^{b,3}

^aDepartment of Ecology and Evolutionary Biology, University of Kansas, Lawrence, USA

^bUndergraduate Biology, University of Kansas, Lawrence, USA

*Corresponding author:

Jennifer M. Gleason
Department of Ecology and Evolutionary Biology
1200 Sunnyside Ave.
University of Kansas
Lawrence, KS 66045,
Telephone: +1-785-864-5858
FAX: +1-785-864-5860
jgleason@ku.edu

¹Current address
Graduate Division of Biological and Biomedical Sciences
Emory University
Atlanta, Georgia
USA

²Current address:
School of Medicine
University of Kansas
Kansas City, KS
USA

³Current address:
College of Education
Kansas State University
Manhattan, KS
USA

Word count: 6,908

During courtship, specific signals are transmitted and received between the male and the female. To understand the evolution and function of courtship behaviours, these signals must be tested individually to determine their relative effects. *Drosophila* species have a variety of courtship patterns that use different sensory modalities, including chemosensory, tactile, visual and auditory cues, and thus provide an opportunity for exploring different sensory modalities. In most of the *Drosophila willistoni* group species, males produce an auditory cue (courtship song) through wing vibration. One species, *D. nebulosa*, does not produce an auditory signal in courtship although both sexes perform wing-lifting movements and males fan an anal droplet towards the female. We compared the sensory modalities important for mating success in *D. nebulosa* with *D. willistoni* through a series of phenotypic manipulation experiments in which individuals had disrupted transmission or reception of signals. In *D. nebulosa*, females have to be able to smell, presumably to detect the anal droplet, for successful mating to occur, whereas for males of the same species, vision is essential. In contrast, no single sensory modality is absolutely necessary for *D. willistoni* mating success, although hearing greatly increases mating success for females and courtship song greatly increases mating success for males. Thus these species are divergent in the sensory modalities necessary for courtship success indicating that courtship behaviour may change rapidly within species groups.

Key words: anal droplets; antennal removal; chemosensory cues; courtship song; *Drosophila nebulosa*; *Drosophila willistoni*; mate choice; phenotypic manipulation; visual cues; wing displays

Courtship consists of specific signals conveyed and perceived by a male and female prior to mating. Such signals may provide information about the quality of the individual and/or the identity of the species (Hebets & Papaj 2005). Courtship communication can use multiple sensory channels, or modalities, such as chemosensory, auditory, visual and tactile cues that need to be understood in conjunction (reviewed in Hebets & Papaj 2005; Partan & Marler 2005). These cues can convey multiple messages, be redundant, interact synergistically, or one signal may dominate other signals (Candolin 2003; Hebets 2008; Hebets & Papaj 2005). Untangling these signals is important for understanding the evolution and functions of courtship behaviour.

Display signals can be examined in isolation through ablation or masking of single signal components to study the role of different signalling modalities and their effect on courtship success (Gibson & Uetz 2008; Hebets & Uetz 1999; Rundus et al. 2010; Uetz & Roberts 2002). If a signal is important in mate choice, then receptivity and mating success are predicted to be reduced in the absence of a signal. However, the display signal is only half of the courtship interaction: reception is equally important. Thus another way to test signal efficacy is to remove reception through masking or ablation. By phenotypically manipulating each or both sexes involved, the sufficiency or necessity of signals may be tested.

Members of the genus *Drosophila* display a variety of courtship behaviours using many sensory modalities (reviewed in Markow & O'Grady 2005). Males of the majority of *Drosophila* species vibrate their wings to produce a courtship song that plays an integral part in species differentiation and mate choice (e.g. Bennet-Clark et al. 1974; Ritchie et al. 1999; Tomaru et al. 1995) and song parameter values may reflect genetic quality (Hoikkala et al. 1998). In the most well known *Drosophila* species, *D. melanogaster*, singing increases male mating success, but is not absolutely necessary (Kyriacou & Hall 1982; Ritchie et al. 1999; Tayln & Dowse 2005). Additional signals and signal reception are involved in male mating

success. The loss of a single sensory input does not completely impede courtship and mating ability in *D. melanogaster* (reviewed in Tompkins 1984). However, signals can have a synergistic effect: males that are able to express both auditory and olfactory signals are more successful than males that produce only one of these cues (Rybak et al. 2002).

The relative roles of different signal modalities on courtship success have not been examined in many *Drosophila* species. The acoustic modality, courtship song, has received the most attention, particularly with respect to its evolution in *Drosophila* species groups (e.g. Alonso-Pimentel et al. 1995; Blyth et al. 2008; Costa & Sene 2002; Cowling & Burnet 1981; Hoikkala et al. 1994). In studies of the evolution of courtship songs of the *Drosophila willistoni* sibling species, males of the outgroup species, *D. nebulosa*, were found not to produce an acoustic signal despite actively using their wings throughout courtship (Gleason & Ritchie 1998; Ritchie & Gleason 1995). *Drosophila nebulosa* is a member of the *willistoni* species group, although not a sibling species, and appears to be unique in this group by not having an acoustic signal. We thus set out to explore the sensory modalities that are important to *D. nebulosa* courtship and compare them to those that are important to *D. willistoni*.

Courtship in *D. nebulosa* was described by Spieth (1947) and is composed of both male and female behaviours. Although males do not produce an acoustic signal, both males and females spread, lift and drop their wings both in the presence and absence of other individuals. This behaviour has only been recorded in one other species, *D. bocainensis*, another *D. willistoni* group species. The wings of *D. nebulosa* appear distinctly more grey to the investigators than the wings of *D. melanogaster* and members of the *D. willistoni* group, suggesting that wings may be involved in visual signals conveyed by both the male and female during wing lifting as speculated by Spieth (1947). In courtship, the male approaches the side of the female, uppercuts by striking the female with his legs just under her wings,

taps her and then circles to the front. The tapping behaviour allows the male to assess the female's cuticular pheromones through gustatory receptors in his feet. Standing perpendicularly to the female, the male bends his abdomen laterally toward the female and extrudes a liquid droplet from the anus. At the same time, he silently fans the wing on the side closest to the female. After this posture, he runs to the rear of the female and attempts copulation. If the female accepts the male, she spreads her wings in an acceptance display and copulation commences. If not, the male repeats the posture in front of the female, always bending his abdomen toward the female (Spieth 1947). The female, with the exception of the acceptance display, is primarily passive though she may walk away from the male until she accepts him.

The mating behaviour of *D. willistoni* is similar to that of *D. nebulosa* but the male does not produce an anal droplet. Instead, the male extends one wing that he vibrates to produce a species-specific courtship song (Ritchie & Gleason 1995). The female signals receptivity with wing spreading (Spieth 1952). The courtship songs of the other *D. willistoni* sibling species are like that of *D. willistoni* although there are major differences in both the temporal and structural parameters of courtship songs and the song may be under sexual selection in this group (Gleason & Ritchie 1998; Ritchie & Gleason 1995).

We determined the modalities of courtship signals important for mating success for both *Drosophila nebulosa* and *D. willistoni*. Specifically we tested the roles of vision, antennal olfaction, and hearing, all of which were eliminated through phenotypic ablation experiments. In addition, we tested the role of wings in signal transmission. In *D. nebulosa*, wings may play a role in pheromone transmission through the fanning of the anal droplet or in a visual display. In contrast, wing removal in *D. willistoni* affects the male's ability to produce the acoustic signal. Our examination of sensory modalities in these two species differ significantly from what has been done in *D. melanogaster*: in those studies ablation

was performed using mutations (Markow 1987; Rybak et al. 2002; Tompkins 1984). Here we physically alter the flies to ablate vision, hearing and olfaction as well as to block acoustic and visual signal transmission.

MATERIALS AND METHODS

Culturing and Virgin Collection

Cultures of both *D. nebulosa* (14030-0761.00) and *D. willistoni* (14030-0811.24) were obtained from the UC San Diego Drosophila Stock Center. All experiments and culturing were conducted at 25°C with flies raised on standard cornmeal molasses fly food on a 12:12 light: dark cycle. For all experiments, male and female virgins were isolated within 1-3 hours of eclosion under a light CO₂ anaesthesia. Single sex groups of 10-20 individuals were kept in 25 x 95 mm vials until randomly assigned to treatment groups and manipulated before use in experiments at 7 to 10 days old. Except as noted, all mating pairs within an experiment consisted of one virgin male and one virgin female placed in a 15 x 95 mm mating vial containing standard *Drosophila* cornmeal-molasses media.

Treatments of each species were approximately the same, however the method of scoring mating success differed as described below. Most of the *D. nebulosa* observations were completed before the *D. willistoni* observations were started and thus informed the change in protocol. In particular, the *D. nebulosa* trials indicated that direct observation of mating trials could potentially be more informative. Descriptions of the methods are presented by species.

Drosophila nebulosa

Vision tests: presence and absence of light

The necessity of visual cues during courtship was tested by comparing pairs of flies incubated with a normal photoperiod to pairs incubated in constant darkness. For the pairs with a normal photoperiod, a male and a female *D. nebulosa* were aspirated into a mating vial

and then placed in an incubator at 25°C with a 12:12 light:dark cycle. For the pairs in constant darkness, a female was aspirated into a mating vial first under normal light. Under red light, a male was then introduced into the vial and then placed in constant darkness. Mating pairs were incubated for one week at which time vials were checked for the presence and absence of larvae and for whether or not the parents were still alive. Any vials in which both parents were not alive were discarded from analysis. The presence of larvae indicated that the pair had successfully copulated.

To determine if incubation in darkness had an effect on female egg laying or egg development, *D. nebulosa* females that successfully mated and laid eggs in the light:dark cycle above were moved onto new food and placed in constant darkness for a week. At that time, the vials were checked for whether or not the females were still alive and for the presence of larvae. Only vials with live females were included in analysis.

Vision tests by sex

To determine the separate role of vision in *D. nebulosa* courtship for males and females, mating tests were performed with flies with and without their eyes covered by paint. Sexually mature virgins were aspirated into a shortened micropipette tip that allowed the fly's head to protrude. The compound eyes of blinded flies were covered using a Sharpie metallic silver oil-based opaque paint marker (Sanford LP, Oakbrook, IL). To control for the effect of immobilization and the marker, all control flies were immobilized and a dot of marker paint was placed on their heads between the compound eyes but not covering the ocelli. After 24 hours, one virgin male and one virgin female were aspirated into each mating vial. Each trial consisted of one pair each in one of four treatments: female-only blind, male-only blind, both blind, and a control in which both sexes could see. A total of 10 trials were conducted for mating periods of 2 and 24 hours, each. Upon completion of the mating period, the flies were separated using CO₂ anaesthesia and the males were discarded. The females were submerged

in potassium buffered saline and their reproductive tracts were extracted with forceps by pulling on the ovipositor. A Leica DM5000B compound microscope was used to identify whether a successful copulation had occurred by observing the presence or absence of sperm within the seminal receptacle and spermathecae. The presence of sperm indicated a successful mating had taken place. The total number of trials reported in the results varied because some individuals died in the course of the experiments.

Single side male vision tests

Given that males were incapable of mating when blind (see Results), a further test was done of male *D. nebulosa* mating ability. Males were blinded in one eye only, using the same technique as above, with half of the males being blinded on the right and half on the left. Males were also blinded in both eyes. Control males were immobilized in the same manner as the blinded males. Male and female pairs were placed into a copulatron (Drapeau & Long 2000; Ruedi & Hughes 2008), a device that allows simultaneous observation of multiple pairs of flies. The copulatron has multiple circular mating chambers (2.5 cm diameter x 3 cm deep) with a semicircular (6 mm diameter) side compartment in which food is placed. Males were introduced into the lower half of the chamber under CO₂ anesthesia after eye painting and one day before observations. A plastic sheet was placed above the males and then the females were placed in the upper chamber under CO₂ anesthesia. The following day, the plastic sheet was removed and each pair was observed for 15 minutes. The observer counted the number of times the male bent to the left and to the right during anal droplet fanning. Bouts of fanning were counted separately if the male stopped fanning between abdominal bending movements or if the male moved around the female.

Wing Removal Tests

To determine whether the presence and absence of wings influences reproductive behaviour in *D. nebulosa*, mating tests were performed with wingless and winged flies.

Sexually mature virgin flies were lightly anesthetized using CO₂ and half of all flies to be used had their wings removed by severing the wings with a dissecting probe as close to the body as possible. After anesthesia, flies were placed in fresh food vials to recover and were grouped by sex and treatment. After 24 hours, one virgin male and one virgin female were aspirated into a mating vial. Each trial consisted of one pair each in one of four treatments: female-only wing removal, male-only wing removal, both male and female without wings, and a control with intact wings for both sexes. A total of 20 and 22 trials were conducted for mating periods of 2 and 24 hours, respectively. After the mating period, the female reproductive tract was dissected and the presence or absence of sperm was scored by the same method used in the vision tests. Total number of trials reported for each treatment varied because some individuals died before testing.

Antennae Removal Tests

To test the role of olfaction in *D. nebulosa* courtship, parts of the antennae were removed. Removal of antennae does not deprive the fly completely of all of its ability to smell because olfactory sensilla are also located on the maxillary and labial palps (reviewed in de Bruyne & Baker 2008), however, the majority of the olfactory receptor neurons are in the antennae (reviewed in Hallem et al. 2006). The antennal olfactory neurons respond strongly to fly odors (van der Goes van Naters & Carlson 2007) and thus may be important for courtship. Olfaction is only part of chemoreception in flies; gustation also plays a role in mating in *D. melanogaster*, primarily through the detection of cuticular hydrocarbons (reviewed in Watanabe et al. 2011) via gustatory receptors that are widely distributed in the fly (reviewed in Montell 2009; Vosshall & Stocker 2007). Thus, our manipulations here only sampled a portion of chemosensory detection, but one that is relevant to volatile compounds.

The antennae are also the organs of hearing in *Drosophila* in that vibrations are transmitted through the arista (reviewed in Tauber & Eberl 2003). Thus removal of

antennae may influence more than olfactory reception. *Drosophila nebulosa* males, despite performing wing movement during courtship, do not produce courtship song or any other detectable sounds with standard acoustic recording equipment (Gleason & Ritchie 1998; Ritchie & Gleason 1995). Thus, in *D. nebulosa*, lack of hearing should not impact behaviour.

The third and second antennal segments, along with the arista, were removed by pinching with forceps. As in the vision tests, sexually mature virgins were aspirated into a shortened micropipette tip that allowed the fly's head to protrude. To control for the effect of immobilization, all flies in the trials were subjected to the same restraint. The antennae of the control flies were tugged with forceps but were not removed. Immobilization and antennal ablation occurred one day prior to mating trials. After manipulations, flies were placed in fresh food vials to recover and were grouped by sex and treatment.

Each of the four treatments was conducted within a trial. The treatments for mating pairs included female-only antennal removal, male-only antennal removal, both male and female antennal removal and a control in which both male and female had intact antennae. One male and one female were aspirated into a mating vial. Trials were conducted for 2 hours and 24 hours. Upon completion of the mating period, the female reproductive tract was dissected and the presence or absence of sperm was scored by the same method used in the previous tests. The presence of sperm indicated a successful mating had taken place. A total of 11 trials were conducted for each mating period.

Drosophila willistoni

Vision tests: presence and absence of light

Tests for the effects of visual cues on the courtship of *D. willistoni* were performed as in *D. nebulosa* with one group of pairs of flies placed on a normal 12:12 hour light:dark cycle and the other group in constant darkness. After one week, the vials with both parents still alive were scored for the presence of larvae to determine if mating occurred.

Vision tests by sex

In order to selectively remove sight, *D. willistoni* flies were individually blinded in the same manner as *D. nebulosa*, with a couple exceptions. We used a gold Sharpie metallic extra fine point oil-based opaque paint marker rather than the silver (Sanford LP, Oakbrook, IL). Control flies underwent the same procedure, but the ommatidia on the right eye were covered. This was to account for any unanticipated effects the paint marker may have on the fly that may alter courtship and mating ability. After manipulation, each fly was placed alone in a 15 x 95 mm vial with food and allowed to recover for 24 hours. Test couples were then placed in 15 x 95 mm vials with food. The males were aspirated into the vials first, followed by the females. Males were removed from the test vials after 24 hours. Females were removed two days following male removal to allow females time to lay eggs, if mating occurred. Test vials were scored one week after the initial introduction of the test couple for the presence of larvae. In each trial, four combinations of male and female were tested in equal numbers: blinded male with control female, control male with blinded female, both male and female blinded and both male and female controls.

Wing removal

Because courtship song is produced by wing vibration, the importance of the wings for *D. willistoni* mating success was examined by comparing winged and wingless flies. Wing removal and treatment of control flies was the same as with *D. nebulosa*. After wing removal, the flies recovered for 24 hours in individual vials before observation using the copulatron. Males were aspirated into the bottom of each mating chamber of the copulatron and separated from the top half of the mating chamber by a sheet of plastic. Females were then aspirated into the top half. After a 30-minute acclimation period, the plastic sheet was removed, starting the mating trial. Pairs were observed for one hour and the presence or absence of mating was scored. In each trial, four combinations of male and female were

tested in equal numbers: wingless male with control female, control male with wingless female, both male and female wingless and both male and female controls.

Removal of aristae (hearing ablation) and antennae (hearing and olfaction ablation)

The arista, a feathery structure attached to the 3rd antennal segment, is primarily responsible for auditory reception and oscillates in response to auditory stimulation. Oscillation is accompanied by movement in the 3rd antennal segment, which then transmits the rotation to the 2nd antennal segment (Eberl 1999; Gopfert & Robert 2002). While the 3rd antennal segment plays a role in auditory reception, it is the location of the majority of olfactory reception in *Drosophila* (Carlson 1996). Thus, antennal removal to disrupt olfaction also eliminates hearing.

In the first set of experiments, aristae were removed. A fly was aspirated into a micropipette tip as in the blinding experiment. Both aristae were removed by pinching with forceps. Control flies were also restrained, but aristae were not removed, although they were manipulated with forceps. Flies were allowed to recover for 24 hours in individual vials before testing in the copulatron as in the wing tests. In each trial, four combinations of male and female were tested in equal numbers: aristaeless male with control female, control male with aristaeless female, both male and female aristaeless and both male and female controls.

The procedure for antennal removal was the same as for the aristae, but instead the 2nd and 3rd antennal segments were pinched off with forceps. Flies were allowed to recover for 24 hours before testing in the copulatron as in the wing tests. In each trial, four combinations of male and female were tested in equal numbers: antennaeless male with control female, control male with antennaeless female, both male and female antennaeless and both male and female controls.

RESULTS

Drosophila nebulosa

Vision tests: presence and absence of light

For *D. nebulosa*, light had a statistically significant effect on mating success: pairs kept in the dark (N=86) never mated whereas 91.3% of those on 12:12 light: dark cycle (N=80) mated (two tailed Fisher's exact test: $P < 0.0001$). The deficit was not because females do not lay eggs in the dark. Of the 15 females that had mated under a normal light: dark cycle and survived for another week, all females laid eggs that hatched into larvae when kept in continuous darkness.

Vision tests by sex

Male and female *D. nebulosa* differed significantly in their ability to mate when they were blinded (Fig. 1). When blind, males were completely unsuccessful at mating as compared to controls (two tailed Fisher's exact test: $P=0.002$, 2 hr; $P < 0.0001$, 24 hr) whereas blind females did equally well as controls (two tailed Fisher's exact test: $P=1.0$, 2 hr; $P=1.0$, 24 hr). When both sexes were blind, no matings were observed (two tailed Fisher's exact test: $P=0.007$, 2 hr; $P < 0.0001$, 24 hr), but this is caused presumably by the males being blind.

Single side male vision tests

To determine if blinding of males affected male courtship ability directly, further tests were conducted on only the males in which they were blinded in both eyes or one eye with results compared to males that were not blinded. Blinding of any sort reduced the probability of courtship being observed in the 15 minute observation period of the trials. In 31 trials with males blinded in both eyes, none performed courtship, whereas courtship was observed in 11 of 30 control trials (two tailed Fisher's exact test: $P=0.0001$). When the males were completely blind, although they walked around the mating arena, they did not engage the females. Females were observed on two occasions to walk up to males and tap them, a

behaviour not previously described in other species. Females often performed wing waving displays in front of the males.

Blinding in a single eye also reduced courtship as compared to the control. When the left eye was covered, courtship was observed in 12 of 58 trials (comparison to control, two tailed Fisher's exact test: $P=0.0001$) and for the right eye in 13 of 45 trials (comparison to control, two tailed Fisher's exact test: $P=0.004$). When neither eye was blinded, males fanned on each side equally (Fig. 2). When blinded in the left eye, a significantly great number of fanning events occurred on the right as compared to the control (two tailed Fisher's exact test: $P=0.002$). Similarly for the right eye, a significantly great number of fanning events occurred on the left as compared to the control (two tailed Fisher's exact test: $P<0.001$).

Wing Removal Tests

The role of wings in courtship behaviour was more complex than antennae (Fig. 3). For the males, male mating success was reduced compared to the controls at 2 hours but not 24 hours (two tailed Fisher's exact test: $P=0.004$, 2hr; $P=0.324$, 24 hr). When the female lacked wings but the male did not, there was not a statistically significant effect compared to the controls at either 2 hours or 24 hours (two tailed Fisher's exact test: $P=0.200$, 2 hr; $P=0.373$, 24 hr). When both males and females were wingless, fewer matings occurred as compared to the controls at 2 hours but not 24 hours (two tailed Fisher's exact test: $P=0.010$, 2 hr; $P=0.069$, 24 hr), presumably because of the effect of the males alone.

Antennal removal tests

Removal of antennae had a clear effect on females but not males (Fig. 4), preventing females from successfully mating as compared to the controls (two tailed Fisher's exact test: $P=0.002$, 2 hr; $P=0.011$, 24 hr). Removal of male antennae did not significantly change their behaviour from that of the controls (two tailed Fisher's exact test: $P=1$, 2 hr; $P=1$, 24 hr).

The amount of time a pair was housed did not change the results: females cannot overcome a deficiency in antennal olfaction as there was never a mating when the female lacked antennae. When neither sex had antennae, no mating was observed but this presumably is caused by the females lacking antennae.

Drosophila willistoni

Vision tests

Unlike *D. nebulosa*, continuous darkness slightly, but not statistically significantly reduced mating success of *D. willistoni* with 9 of 30 trials failing to produce larvae whereas only 7 of 33 pairs failed to produce larvae with a normal light:dark cycle (two tailed Fisher's exact test: $P=0.56$). To examine this effect further, we separately blinded each sex and both together to compare with the normally sighted control. In none of the treatments was a significant effect found in comparison to the control (two-tailed Fisher's exact test: $P=0.2021$ females blind; $P=0.1065$, males blind; $P=0.0915$, both blind), though there was a non-significant trend toward decreased mating success with each blind treatment (Fig. 5). A partitioned Chi square test showed a statistically significant overall effect of treatment ($X^2_1=4.57$, $P<0.03$).

Wing removal tests

Male *D. willistoni* vibrate their wings to produce a species-specific courtship song (Gleason & Ritchie 1998; Ritchie & Gleason 1995). When wings were removed (Fig. 6), the proportion of pairs mating decreased for wingless females, but this was not a statistically significant effect as compared to the control (two-tailed Fisher's exact test: $P=0.3406$). Removal of the male's wings had a significant effect in both the male wingless treatment (two-tailed Fisher's exact test: $P=0.0095$) and when both sexes were wingless (two-tailed Fisher's exact test: $P=0.0031$). The results imply that the male's ability to produce auditory cues plays an integral role in the courtship and mating success of *D. willistoni*.

Removal of aristae

The aristae are primarily responsible for auditory reception in the antennae of *Drosophila*. By only removing aristae, we were able to isolate the effect of lack of hearing (Fig. 7). When aristae were removed from the female, the proportion of successful matings decreased as compared to the control (two-tailed Fisher's exact test: $P=0.0079$). Males lacking aristae were not less successful as compared to the controls (two-tailed Fisher's exact test: $P=0.5160$). Surprisingly, removal of aristae from both males and females did not decrease mating success (two-tailed Fisher's exact test: $P=0.2049$). The separate effects on females and males implies that females need to be able to receive the auditory cues produced by the males but males do not need to be able to hear themselves sing. Given that female mating success decreased without aristae, absence of aristae in both sexes was expected to lower mating success.

Removal of antennae

The third segment of the antennae is the main olfactory receptors for *Drosophila*. Removal of antennal segments confounds olfaction and reception because removal of these segments also removes the aristae. As with the aristae, removal of the antennae for the female substantially decreases mating success (Fig. 8) as compared to the control (two-tailed Fisher's exact test: $P=0.0011$) and there was not a significant effect for the males (two-tailed Fisher's exact test: $P=0.0991$). However, unlike removal of just the aristae for both sexes, removal of the antennae with aristae for both sexes eliminated mating success entirely (two-tailed Fisher's exact test: $P=0.0003$). Although not directly comparable, because the trials were not conducted at the same time, there was a significant decrease for both sexes lacking antenna as compared to both sexes lacking aristae (two-tailed Fisher's exact test: $P=0.0094$). There was no difference for the success of the controls in these two tests (two-tailed Fisher's exact test: $P=1$).

DISCUSSION

In our experiments we blocked the reception of signals (blinding, removal of arista, and removal of antennae with arista) and blocked the generation of a signal (removal of wings). Males of the two species, *D. nebulosa* and *D. willistoni*, are clearly more successful when they can produce a signal and when the female of their species can perceive that same signal, and those signals differ between species. For *D. nebulosa*, if the female cannot smell the anal droplet produced, she will not mate. For *D. willistoni*, if the female cannot hear the courtship song of the male, she is less likely to mate, though mating may occur.

In interpreting these results, we need to keep in mind that all of these tests were done in a laboratory with no choice tests. In nature, many individuals of both sexes may be present in an aggregation allowing for differential assessment of individuals. In addition, in nature individuals have the ability to leave the mating arena and effectively refuse a mate, an option not afforded here and apparent in the difference between 2 hour and 24 hour tests with *D. nebulosa*. In the absence of an increase in mating success at 24 hours, it is clear that vision is essential for *D. nebulosa* males and antennal olfaction for *D. nebulosa* females.

Differences in female receptivity among individuals may also have an effect on the results, but all experimental conditions were performed at the same time with individuals randomly assigned to treatment groups to minimize inter-individual effects. Thus, our experiments have been successful at determining what sensory modalities are necessary for mating success, but the sensory modalities that are used in assessing relative choice among individuals need to be explored in more detail, ideally through choice tests.

The role of vision

Grossfield (1971) classified *Drosophila* species as light independent (Class I: mate equally well in the dark as in the light), light facilitated (Class II: significant reduction in matings in the dark, but matings still occur) or light dependent (Class III: do not mate in the

dark). Thus, these species may be classed as Class III (*D. nebulosa*) and Class I (*D. willistoni*). Vision thus has different effects on the mating success of each species.

In addition to allowing visual cues, light is a Zeitgeber signal for setting the timing of circadian rhythm (reviewed in Peschel & Helfrich-Förster 2011). Such signals can be important of the timing of courtship events because differences in timing of activity may result in the reproductive isolation of species (Tauber et al. 2003). However, there is no reason to expect that the circadian rhythms of these flies would be disrupted in a way that would change their propensity to mate: flies kept in continuous darkness keep mating activity rhythms and locomotor activity rhythms in complete darkness (Konopka & Benzer 1971; Sakai & Ishida 2001). In *D. simulans*, a species that is light facilitated (Class II), maintenance of flies in complete darkness changes mating activity rhythms, but light is still the primary cue that affects mating behavior (Sakai & Ishida 2001). For the species studied here, continuous maintenance of *D. willistoni* in the dark did not significantly affect mating behavior. For *D. nebulosa*, if mating only depended upon rhythm, then 66-75 % of the pairs kept entirely in the dark should have mated as this is the proportion of pairs mating in two hours as controls in all of the other experiments. The pairs were placed in an incubator during the middle of the light cycle, at a similar time to when all the other trials were conducted, and thus should have maintained their mating activity rhythm at least for the first of the seven days. Instead, none of the pairs kept in the dark mated; therefore, the major effect on these flies is the absence of light and not a change to mating rhythms.

As far as we are aware, the separate roles of vision in each sex have not previously been tested in any other *Drosophila* species. Blinded male *D. nebulosa* do not mate successfully whereas blind females were as successful as sighted females. Males need sight to follow the females and do not initiate courtship if they cannot see. This was particularly evident by the males predominantly fanning on the side on which he could see the female.

The failure is not because the males are not receiving a visual wing signal produced by the females; removal of the female's wings did not decrease mating success.

In contrast, *D. willistoni*, did not have a sex difference in mating success when flies were blinded instead of placed in the dark, although there was an overall effect of treatment. The discrepancy here may imply that light provides an environmental cue, perhaps detectable through the ocelli, for mating but that visual acuity is not necessary for successful courtship. From these results, we cannot predict what the role of vision might be in the other *D. willistoni* sibling species because visual effects may be dependent upon the ability of the male to track the female. If males are able to use other cues, as suggested by *D. willistoni*, then vision may facilitate mating but not be necessary.

The role of wings

Because of extensive wing lifting by both male and female *D. nebulosa*, the distinct shading of the wings, and male fanning while performing abdominal bending with release of the anal droplet (Spieth 1947), we hypothesized that wings could be important for both visual and olfactory cues. The absence of wings on the females did not significantly affect mating success, implying that males are not responding to visual wing cues from the females when females perform wing lifting, nor are wings necessary for signalling receptivity.

The absence of wings in male *D. nebulosa*, however, had a short-term effect that disappeared after 24 hours. Together with the fact that females are as receptive when blind as when they can see, this implies that females do not need to receive visual signals (at least at the short distances tested here) and that wings are used by the *D. nebulosa* male to transmit anal droplet pheromones with greater efficiency. The lack of evidence for a visual wing signal was surprising because female *D. nebulosa* wing lifting has been shown to deter courtship attempts of heterospecific males of the *D. willistoni* group (Spieth 1949). Wing lifting behaviour possibly may be a long distance signal that aids in aggregation: our

experiments in small mating chambers and vials do not allow us to test this possibility.

Knowledge of the ecological habits of this fly would help to determine if wings may play a role in visual communication.

In contrast, wings played a greater role in the courtship success of *D. willistoni* males: if they are not able to generate their species-specific courtship song, males are less successful than with their song. The role of song varies among *Drosophila* species with wingless males of some species failing to mate, as in *D. montana* (Liimatainen et al. 1992) and *D. buskii* (Bixler et al. 1992), where as in other species wingless males have a decreased mating success, as in *D. parabiopectinata* (Crossley & Bennet-Clark 1993) and both *D. melanogaster* and *D. simulans* (Ritchie et al. 1999). In one case, song has been shown to have an inhibitory effect on interspecific mating: *D. ananassae* females mate more readily with wingless *D. pallidosa* males than with winged ones (though intraspecific mating of *D. pallidosa* males decreases in the absence of wings; Doi et al 2001). Thus *D. willistoni* is like other species for which an acoustic signal facilitates mating, but is not necessary for mating. Loss of acoustic signal, either through mute (wingless) or deaf (aristaless) females, increases heterospecific matings for *D. sechellia* and *D. auraria* species (Tomaru & Oguma 2000; Tomaru et al. 1998; Tomaru et al. 2000), further emphasizing the role of wings in acoustic signals.

The role of hearing

The role of hearing was not specifically tested in *D. nebulosa* because of a lack of an acoustic signal. For *D. willistoni*, when females lacked aristaes, the number of pairs mating was significantly reduced. This is the reciprocal of the effect of males without wings: lack of an acoustic signal reduced mating success. Thus, the acoustic signal of *D. willistoni* is not necessary for mating success, but substantially increases mating, which may be similar for the rest of the *D. willistoni* sibling species, but this remains to be tested. Absence of an effect of aristaes removal in males implies that males do not need to hear their own courtship song to

generate it effectively. This has been demonstrated in *D. melanogaster* in that males that lack aristae produce normal courtship song (Burnet et al. 1977).

The role of olfaction

We were unable to test the role of olfaction through the antennae alone in either species because the elimination of olfaction through antennal segment removal necessarily removes audition at the same time. With a lack of acoustic signal in *D. nebulosa*, the removal of the antennae is presumed to be an effect on olfaction. A female *D. nebulosa* must receive chemical signals from the male, as evidenced by the complete absence of mating when the female does not have antennae. Males do not need to be able to sense chemical cues through their antennae as their courtship success was not affected in the absence of antennae. Thus, the anal droplet is probably transmitting an important olfactory signal to the female. Anal droplets are rare among *Drosophila* but have been observed in both sexes. The single instance in females is an observation that in 50% of copulating pairs, *D. melanogaster* females produce a tiny droplet at the tip of the ovipositor that effectively increases male sexual activity (Lasbleiz et al. 2006); this particular behaviour has not been observed in any other studies of *D. melanogaster*, though the majority have focused on the more conspicuous male behaviours and not the female behaviours. Clearly this behaviour needs to be investigated more closely and may rely on observations with video rather than direct observation.

More common than female droplets are male anal droplets. In the Hawaiian picture-winged *Drosophila*, the male does not fan the droplet but instead curls his abdomens up over his head toward the females head. In this position, the male pulsates his abdomen while producing a droplet (Spieth 1978) with the droplet invariably preceding mating (Tompkins et al. 1993). Because *D. nebulosa* and Hawaiian species are distantly related, anal droplets are likely convergent olfactory signals and may exist in other species, although they have not

been documented. *D. subobscura* males also produce droplets but theirs are regurgitated and fed to females as a nuptial gift (Immonen et al. 2009). The anal droplets of *D. nebulosa* are unlikely to be nuptial gifts because the females do not come into direct contact with the droplets.

The effect of removal of antennae has not been studied in many species. Antennal removal reduces mating success in *D. melanogaster* and *D. pseudoobscura* (Mayr 1950) though, at least for *D. melanogaster*, the effect goes away after 24 hours, providing further evidence that courtship is multimodal and males may compensate for missing signals. In comparison with Mayr's results, we cannot say definitively that *D. nebulosa* females are incapable of mating without antennae because our sample sizes are small. However, comparing our 24-hour data with Mayr's 24-hour data for wild type *D. melanogaster* females without antennae (16 matings, 5 failures), we see a significant reduction in mating for *D. nebulosa* without antennae (two tailed Fisher's exact test: $P=0.0001$).

For *D. willistoni*, our studies were not conducted in a way that we could determine the relative effects of hearing and antennal olfaction, though the reduction in mating success of females without antennae versus females without aristae suggests that antennal olfaction may play a role in female mating success. As with the other experiments in *D. willistoni*, elimination of a sensory modality did not eliminate mating success. Mating success was higher when both the male and the female lacked aristae than when just females lacked aristae, but eliminated when both lacked antennae, further suggesting an interaction in antennal olfaction in both sexes. The effect of antennal olfaction needs to be examined in other species in the group. In addition, combining ablation of antennal olfaction with other sensory modalities may give insights into whether antennal olfaction is redundant with other signals, for example vision, particularly with respect to the male's ability to follow the female throughout courtship.

Future studies should be performed in determining the cause of the discrepancy between the mating success of the treatment group involving both sexes altered and females altered in the two antennal experiments of *D. willistoni* (Figs. 7 and 8). A more appropriate control for the antennal removal experiments is probably not intact antennae, but antennae lacking arista to allow an estimation of the decrease in mating due entirely to olfaction ablation. In addition, a method to disrupt antennal olfactory reception while maintaining auditory reception would more directly determine the role of antennal olfaction in courtship. In addition, other further experiments in *D. willistoni* should examine the interaction of vision and acoustic signals or antennal olfaction through observation of flies under dim red light, which effectively blinds the flies. These experiments will be informative for determining potential redundancies of signals.

Evolutionary implications

Given the vast diversity of the genus *Drosophila*, with nearly 1500 described species, studies of the sensory modalities involved in successful courtship, particularly with respect to the different senses required by males and females separately, may give great insight into the evolution of courtship. Variation in ability to mate in the dark, female wing acceptance postures, song types and cuticular hydrocarbons (potential pheromones) have been noted across the genus (reviewed in Markow & O'Grady 2005), but systematic examination of the senses used by each species, and by each sex within a species, is needed to understand how sensory modality changes may be affecting reproductive isolation in closely related species.

D. nebulosa males and females are using separate signalling modalities/ sensory channels during courtship. Unlike other species in which signals may be redundant or composite (reviewed in Partan & Marler 2005) each sex is using a separate, unimodal signal channel and that sense has a dominant role. Elimination of vision, through keeping cultures in the dark, prevents mating in many species (reviewed in Markow & O'Grady 2005) but

elimination of other senses, at least one at a time, does not completely disrupt courtship. For example, in *D. melanogaster*, the loss of vision and olfaction does not abolish mating (Gailey et al. 1986; Tompkins et al. 1980) but the loss of olfaction, vision and tactile cues does eliminate courtship (Gailey et al. 1986). Similarly for *D. malerkotliana*, various amputations (wings, antennae, legs) in both sexes reduce mating success but mating success is not completely abolished until two amputations are performed, one on each sex (Narda 1966). Thus, whereas most *Drosophila* studies demonstrate the use of multiple cues in courtship, we show here that different, single cues are important for each sex.

Through this study, it appears that, although *D. willistoni* is more closely related to *D. nebulosa* than to *D. melanogaster*, *D. willistoni* uses sensory inputs during courtship that are more similar to those of *D. melanogaster*. In *D. nebulosa*, the lack of one sensory input is enough to prevent mating. However in *D. melanogaster* and *D. willistoni*, the lack of one sensory signal may diminish mating success, but it does not completely prevent it. This suggests that multiple signaling modalities are used in the courtship of *D. melanogaster* and *D. willistoni*. Perhaps the cause of *D. nebulosa* mating being disrupted by the removal of a single sensory input lies in the absence of courtship song. This lack of a song already removes a possible auditory signal, thus making *D. nebulosa* more dependent on other sensory inputs. Thus the important courtship signals for mating success change over evolutionary time. By comparing the *D. willistoni* findings with those of *D. nebulosa*, it is apparent that there is evolution in the importance of various courtship signals in *Drosophila* species. The relative importance of courtship signals greatly differ among these closely related species, suggesting that the rate of divergence is rapid. Because courtship may contribute to reproduction isolation, this rapid divergence may contribute to the speciation process.

Additional studies investigating multimodal signaling in the courtship and mating success of other members of the *willistoni* group are needed to understand how these signals are evolving. Two other species, *D. tropicalis* and *D. insularis* have structurally, but not temporally, similar courtship songs whereas the songs of *D. equinoxialis* and *D. paulistorum* are structurally different from each other and from the other species (Gleason & Ritchie 1998; Ritchie & Gleason 1995). The song of *D. equinoxialis* has probably been under extensive sexual selection given that the divergence in the song of this species greatly exceeds that of the others (Gleason & Ritchie 1998). Testing the importance of the acoustic signal to mating within each of these species may reveal more about how the songs have evolved and how signal modalities are evolving in general.

ACKNOWLEDGMENTS

Pauly Cartwright allowed us to use her compound microscope. John Carlson, Stuart Macdonald and Robert Ward provided advice on dissections and Terry Gleason on statistics. Mike Ritchie, Eileen Hebets, Leanna Birge, Jeff Cole, Jenny Hackett and the KU EEB Genetics Group commented on an early manuscript. This work was supported NSF grant IBN-0347419 to JMG. AAP, ALV and SFG were supported by separate Gould Undergraduate Research Awards in Entomology from the EEB Department at KU. AAP was also supported by the KU Initiative to Maximize Student Development (IMSD; NIH 5R25GM062232).

REFERENCES

Alonso-Pimentel, H., Spangler, H. G. & Heed, W. B. 1995. Courtship sounds and behaviour of the two saguaro-breeding *Drosophila* and their relatives. *Anim. Behav.*, 50, 1031-1039.

- Bennet-Clark, H. C., Dow, M., Ewing, A. W., Manning, A. & von Schilcher, F.** 1974. Courtship stimuli in *Drosophila melanogaster*. *Behav. Genet.*, 6, 93-95.
- Bixler, A., Jenkins, J. B., Tompkins, L. & McRobert, S. P.** 1992. Identification of acoustic stimuli that mediate sexual behavior in *Drosophila busckii* (Diptera: Drosophilidae). *J. Insect. Behav.*, 5, 469-478.
- Blyth, J. E., Lachaise, D. & Ritchie, M. G.** 2008. Divergence in multiple courtship song traits between *Drosophila santomea* and *D. yakuba*. *Ethology*, 114, 728-736.
- Burnet, B., Eastwood, L. & Connolly, K.** 1977. The courtship song of male *Drosophila* lacking arista. *Anim. Behav.*, 25, 460-464.
- Candolin, U.** 2003. The use of multiple cues in mate choice. *Biol. Rev.*, 78, 575-595.
- Carlson, J. R.** 1996. Olfaction in *Drosophila*: from odor to behavior. *Trends in Genetics*, 12, 175-180.
- Costa, C. T. A. & Sene, F. M.** 2002. Characterization of courtship sounds of species of the subgroup *fasciola* (Diptera, Drosophilidae, *Drosophila repleta* group): Interspecific and interpopulational analyses. *Braz. J. Biol.*, 62, 573-583.
- Cowling, D. E. & Burnet, B.** 1981. Courtship songs and genetic control of their acoustic characteristics in sibling species of the *Drosophila melanogaster* subgroup. *Anim. Behav.*, 29, 924-935.
- Crossley, S. A. & Bennet-Clark, H. C.** 1993. The response of *Drosophila parabiopectinata* to simulated courtship songs. *Anim. Behav.*, 45, 559-570.
- de Bruyne, M. & Baker, T. C.** 2008. Odor detection in insects: volatile codes. *J. Chem. Ecol.*, 34, 882-897.
- Drapeau, M. D. & Long, A. D.** 2000. The Copulatron, a multi-chamber apparatus for observing *Drosophila* courtship behaviors. *Dros. Inf. Serv.*, 83, 194-196.

- Eberl, D. F.** 1999. Feeling the vibes: chordotonal mechanisms in insect hearing. *Curr. Opin. Neurobiol.*, 9, 389-393.
- Gailey, D. A., Lacaillade, R. C. & Hall, J. C.** 1986. Chemosensory elements of courtship in normal and mutant, olfaction-deficient *Drosophila melanogaster*. *Behav. Genet.*, 16, 375-405.
- Gibson, J. S. & Uetz, G. W.** 2008. Seismic communication and mate choice in wolf spiders: components of male seismic signals and mating success. *Anim. Behav.*, 75, 1253-1262.
- Gleason, J. M. & Ritchie, M. G.** 1998. Evolution of courtship song and reproductive isolation in the *Drosophila willistoni* species complex; do sexual signals diverge the most quickly? *Evolution*, 52, 1493-1500.
- Gopfert, M. C. & Robert, D.** 2002. The mechanical basis of *Drosophila* audition. *The Journal of Experimental Biology*, 205, 1199-1208.
- Grossfield, J.** 1971. Geographic distribution and light-dependent behavior in *Drosophila*. *Proc. Natl. Acad. Sci. USA*, 68, 2669-2673.
- Hallem, E. A., Dahanukar, A. & Carlson, J. R.** 2006. Insect odor and taste receptors. *Ann. Rev. Ent.*, 51, 113-135.
- Hebets, E. A.** 2008. Seismic signal dominance in the multimodal courtship display of the wolf spider *Schizocosa stridulans* Stratton 1991. *Behav. Ecol.*, 19, 1250-1257.
- Hebets, E. A. & Uetz, G. W.** 1999. Female responses to isolated signals from multimodal male courtship displays in the wolf spider genus *Schizocosa* (Araneae: Lycosidae). *Anim. Behav.*, 57, 865-872.
- Hebets, E. A. & Papaj, D. R.** 2005. Complex signal function: developing a framework of testable hypotheses. *Behav. Ecol. Sociobiol.*, 57, 197-214.

- Hoikkala, A., Kaneshiro, K. Y. & Hoy, R. R.** 1994. Courtship songs of the picture-winged *Drosophila planitibia* subgroup species. *Anim. Behav.*, 47, 1363-1374.
- Hoikkala, A., Aspi, J. & Suvanto, L.** 1998. Male courtship song frequency as an indicator of male genetic quality in an insect species, *Drosophila montana*. *Proc. R. Soc. London, Ser. B*, 265, 503-508.
- Immonen, E., Hoikkala, A., Kazem, A. J. N. & Ritchie, M. G.** 2009. When are vomiting males attractive? Sexual selection on condition-dependent nuptial feeding in *Drosophila subobscura*. *Behav. Ecol.*, 20, 289-295.
- Konopka, R. J. & Benzer, S.** 1971. Clock mutants of *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA*, 68, 2112-2116.
- Kyriacou, C. P. & Hall, J. C.** 1982. The function of courtship song rhythms in *Drosophila*. *Anim. Behav.*, 30, 794-801.
- Lasbleiz, C., Ferveur, J.-F. & Everaerts, C.** 2006. Courtship behaviour of *Drosophila melanogaster* revisited. *Anim. Behav.*, 72, 1001-1012.
- Liimatainen, J., Hoikkala, A., Aspi, J. & Welbergen, P.** 1992. Courtship in *Drosophila montana*: the effects of male auditory signals on the behaviour of flies. *Anim. Behav.*, 43, 35-48.
- Markow, T. A.** 1987. Behavioral and sensory basis of courtship success in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA*, 84, 6200-6204.
- Markow, T. A. & O'Grady, P. M.** 2005. Evolutionary genetics of reproductive behavior in *Drosophila*: Connecting the dots. *Ann. Rev. Genet.*, 39, 263-291.
- Mayr, E.** 1950. The role of antennae in the mating behavior of female *Drosophila*. *Evolution*, 4, 149-154.
- Montell, C.** 2009. A taste of the *Drosophila* gustatory receptors. *Curr. Opin. Neurobiol.*, 19, 345-353.

- Narda, R. D.** 1966. Analysis of the stimuli involved in courtship and mating in *D. malerkotliana* (Sophophora, drosophila). *Anim. Behav.*, 14, 378-383.
- Partan, S. R. & Marler, P.** 2005. Issues in the classification of multimodal communication signals. *Am. Nat.*, 166, 231-235.
- Peschel, N. & Helfrich-Förster, C.** 2011. Setting the clock- by nature: Circadian rhythm in the fruitfly *Drosophila melanogaster*. *FEBS Lett.*, 585, 1435-1442.
- Ritchie, M. G. & Gleason, J. M.** 1995. Rapid evolution of courtship song pattern in *Drosophila willistoni* sibling species. *J. Evol. Biol.*, 8, 463-479.
- Ritchie, M. G., Halsey, E. J. & Gleason, J. M.** 1999. *Drosophila* song as a species-specific mating signal and the behavioural importance of Kyriacou & Hall cycles in *D. melanogaster* song. *Anim. Behav.*, 58, 649-657.
- Ruedi, E. A. & Hughes, K. A.** 2008. Natural genetic variation in complex mating behaviors of male *Drosophila melanogaster*. *Behav. Genet.*, 38, 424-436.
- Rundus, A. S., Santer, R. D. & Hebets, E. A.** 2010. Multimodal courtship efficacy of *Schizocosa retrorsa* wolf spiders: implications of an additional signal modality. *Behav. Ecol.*, 21, 701-707.
- Rybak, F., Sureau, G. & Aubin, T.** 2002. Functional coupling of acoustic and chemical signals in the courtship behaviour of the male *Drosophila melanogaster*. *Proc. R. Soc. London, Ser. B*, 269, 695-701.
- Sakai, T. & Ishida, N.** 2001. Circadian rhythms of female mating activity governed by clock genes in *Drosophila*. *Proc. Natl. Acad. Sci. USA*, 98, 9221-9225.
- Spieth, H. T.** 1947. Sexual behavior and isolation in *Drosophila* I. The mating behavior of species of the *willistoni* group. *Evolution*, 1, 17-31.
- Spieth, H. T.** 1949. Sexual behavior and isolation in *Drosophila*. II. The interspecific mating behavior of species of the *willistoni* group. *Evolution*, 3, 67-81.

- Spieth, H. T.** 1952. Mating behavior within the genus *Drosophila* (Diptera). *Bull. Amer. Mus. Nat. His.*, 99, 395-474.
- Spieth, H. T.** 1978. Courtship patterns and evolution of the *Drosophila adiantola* and *planitibia* species subgroups. *Evolution*, 32, 435-451.
- Tauber, E. & Eberl, D. F.** 2003. Acoustic communication in *Drosophila*. *Behav. Proc.*, 64, 197-210.
- Tauber, E., Roe, H., Costa, R., Hennessy, J. M. & Kyriacou, P.** 2003. Temporal mating isolation driven by a behavioral gene in *Drosophila*. *Current Biology*, 13, 140-145.
- Tayln, B. C. & Dowse, H. B.** 2005. The role of courtship song in sexual selection and species recognition by female *Drosophila melanogaster*. *Anim. Behav.*, 68, 1165-1180.
- Tomaru, M. & Oguma, Y.** 2000. Mate choice in *Drosophila melanogaster* and *D. sechellia*: Criteria and their variation depending on courtship song. *Anim. Behav.*, 60, 797-804.
- Tomaru, M., Matsubayashi, H. & Oguma, Y.** 1995. Heterospecific inter-pulse intervals of courtship song elicit female rejection in *Drosophila biauraria*. *Anim. Behav.*, 50, 905-914.
- Tomaru, M., Matsubayashi, H. & Oguma, Y.** 1998. Effects of courtship song in interspecific crosses among the species of the *Drosophila auraria* complex (Diptera: Drosophilidae). *J. Insect. Behav.*, 11, 383-398.
- Tomaru, M., Doi, M., Higuchi, H. & Oguma, Y.** 2000. Courtship song recognition in the *Drosophila melanogaster* complex: heterospecific songs make females receptive in *D. melanogaster*, but not in *D. sechellia*. *Evolution*, 54, 1286-1294.
- Tompkins, L.** 1984. Genetic analysis of sex appeal in *Drosophila*. *Behav. Genet.*, 14, 411-440.

- Tompkins, L., Hall, J. C. & Hall, L. M.** 1980. Courtship-stimulating volatile compounds from normal and mutant *Drosophila*. *J. Insect Phys.*, 26, 689-697.
- Tompkins, L., McRobert, S. P. & Kaneshiro, K. Y.** 1993. Chemical communication in Hawaiian *Drosophila*. *Evolution*, 47, 1407-1419.
- Uetz, G. W. & Roberts, J. A.** 2002. Multisensory cues and multimodal communication in spiders: Insights from video/audio playback studies. *Brain Behav. Evol.*, 59, 222-230.
- van der Goes van Naters, W. & Carlson, J. R.** 2007. Receptors and neurons for fly odors in *Drosophila*. *Current Biology*, 17, 606-612.
- Vosshall, L. B. & Stocker, R. F.** 2007. Molecular architecture of smell and taste in *Drosophila*. *Annual Review of Neuroscience*, 30, 505-533.
- Watanabe, K., Toba, G., Koganezawa, M. & Yamamoto, D.** 2011. Gr39a, a highly diversified gustatory receptor in *Drosophila*, has a role in sexual behavior. *Behav. Genet.*, 41, 746-753.

FIGURE LEGENDS

Figure 1. Vision tests in *D. nebulosa*. Mating trials were conducted with control test couples, blinded females, blinded males, and both sexes blinded. The time course of the experiment was 2 or 24 hours. The proportion of pairs in each category in which sperm was found in the female reproductive tract was used to estimate the proportion of successful matings. Sample sizes are given above each bar. A two-tailed Fisher's exact test was used to compare each treatment to the control (*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, absence of asterisks indicates lack of statistical significance).

Figure 2. Single side vision tests in *D. nebulosa*. Males were blinded in either the right or left eye and compared to the controls males (no blinding). The proportion of fanning to the left or right was calculated for the males that courted in the 15 minute observation period (sample sizes are given with each treatment). Males fanned more often on side with unimpaired vision. Sample sizes are given above each pairs of bars. A two-tailed Fisher's exact test was used to compare each treatment to the control (**: $P < 0.01$, ***: $P < 0.001$, absence of asterisks indicates lack of statistical significance).

Figure 3. Wing tests in *D. nebulosa*. Mating trials were conducted with control test pairs, wingless females, wingless males, and both sexes wingless. The time course of the experiment was 2 or 24 hours. The proportion of pairs in each category in which sperm was found in the female reproductive tract was used to estimate the proportion of successful matings. Sample sizes are given above each bar. A two-tailed Fisher's exact test was used to compare each treatment to the control (*: $P < 0.05$, **: $P < 0.01$, absence of asterisks indicates lack of statistical significance).

Figure 4. Antennal removal tests in *D. nebulosa*. Mating trials were conducted with control test pairs, females without antennae, males without antennae and both sexes without antennae. The time course of the experiment was 2 or 24 hours. The proportion of pairs in each category in which sperm was found in the female reproductive tract was used to estimate the proportion of successful matings. Sample sizes are given above each bar. A two-tailed Fisher's exact test was used to compare each treatment to the control (*: $P < 0.05$, **: $P < 0.01$, absence of asterisks indicates lack of statistical significance).

Figure 5. Vision tests in *D. willistoni*. Mating trials were conducted with control test couples, blinded females, blinded males, and both sexes blinded. Test couples were paired for 24 hours at which time the male was removed. Females were removed 2 days following the males. One week after pairing, the presence of larvae in the vial was scored to indicate a successful mating. The lack of sight does not significantly decrease the proportion of pairs mating successfully in any of the experimental treatments as compared to the control (two-tailed Fisher's exact test; $P > 0.05$ in all cases). Sample sizes are given above each bar.

Figure 6. Wing tests in *D. willistoni*. Mating trials were conducted with control test pairs, wingless females, wingless males, and both sexes wingless. The time course of the experiment was one hour and observation was used to determine successful matings. A two-tailed Fisher's exact test was used to compare each treatment to the control (*: $P < 0.01$, absence of asterisks indicates lack of statistical significance). Sample sizes are given above each bar.

Figure 7. Aristae removal tests in *D. willistoni*. Mating trials were conducted with control test couples, females without aristae, males without aristae, and both sexes without aristae.

The time course of the experiment was one hour and observation was used to determine successful matings. A two-tailed Fisher's exact test was used to compare each treatment to the control (**: $P < 0.001$, absence of asterisks indicates lack of statistical significance). Sample sizes are given above each bar.

Figure 8. Antennal removal tests in *D. willistoni*. Mating trials were conducted with control test couples, females without antennae, males without antennae, and both sexes without antennae. The time course of the experiment was one hour and observation was used to determine successful matings. A two-tailed Fisher's exact test was used to compare each treatment to the control (*: $P < 0.01$, **: $P < 0.001$, absence of asterisks indicates lack of statistical significance). Sample sizes are given above each bar.

Figure 1

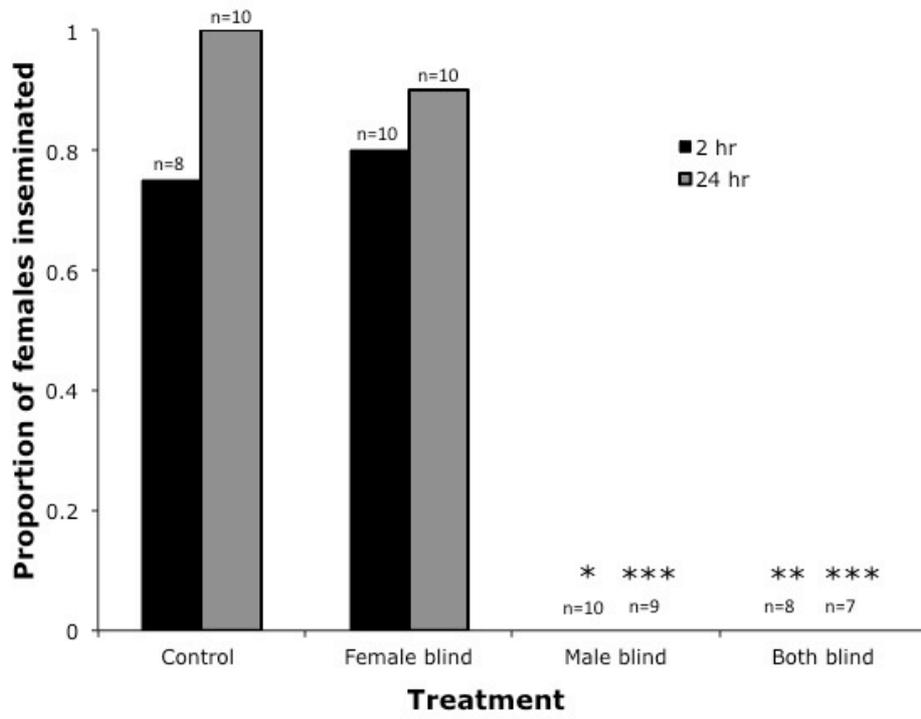


Figure 2

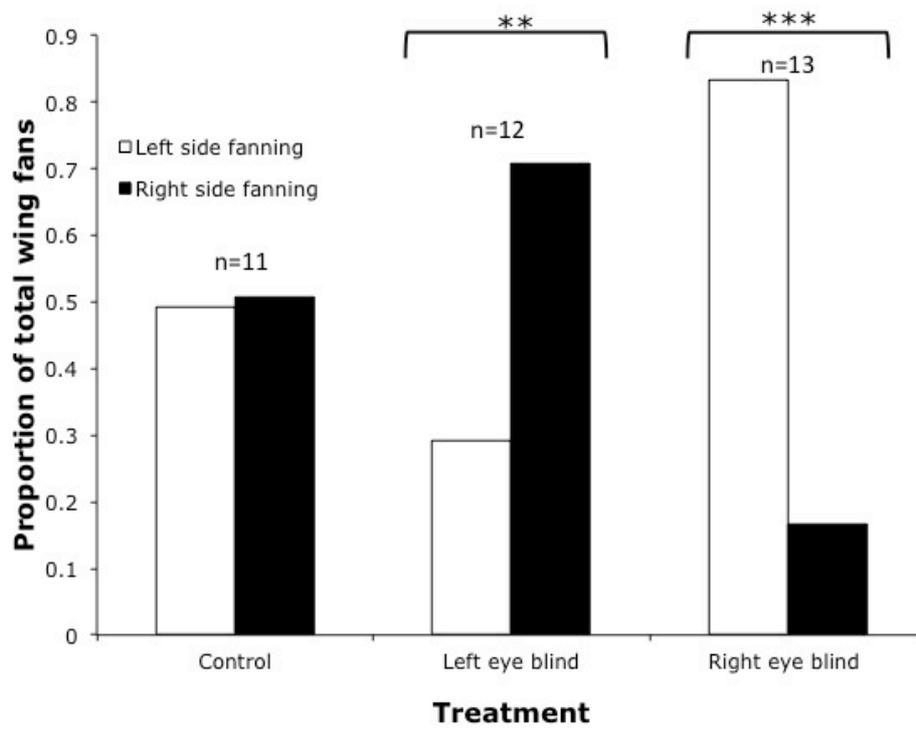


Figure 3

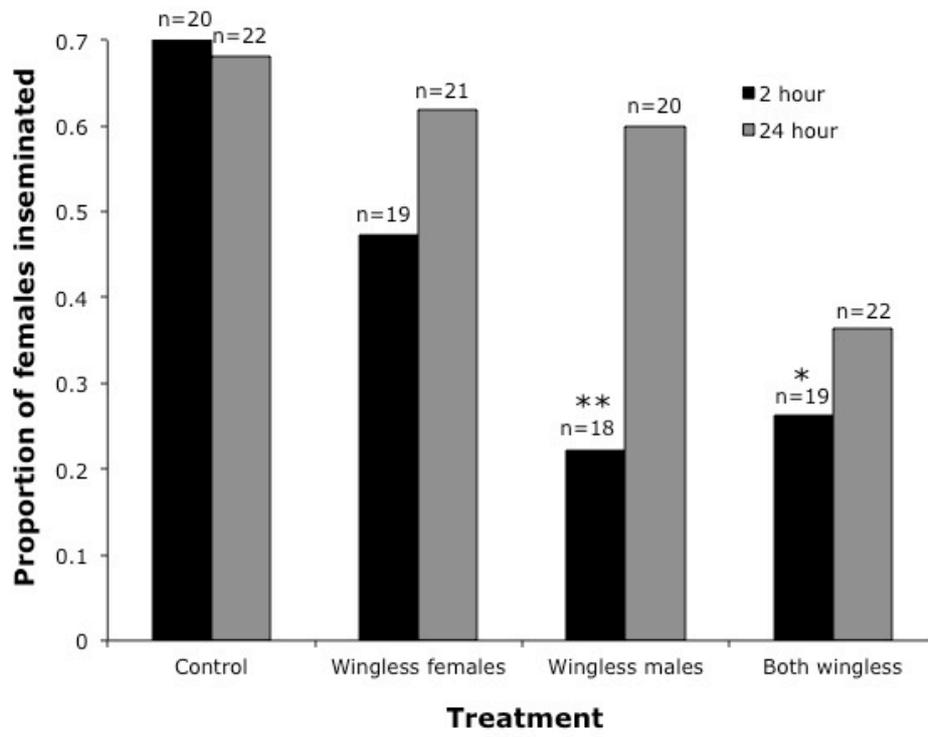


Figure 4

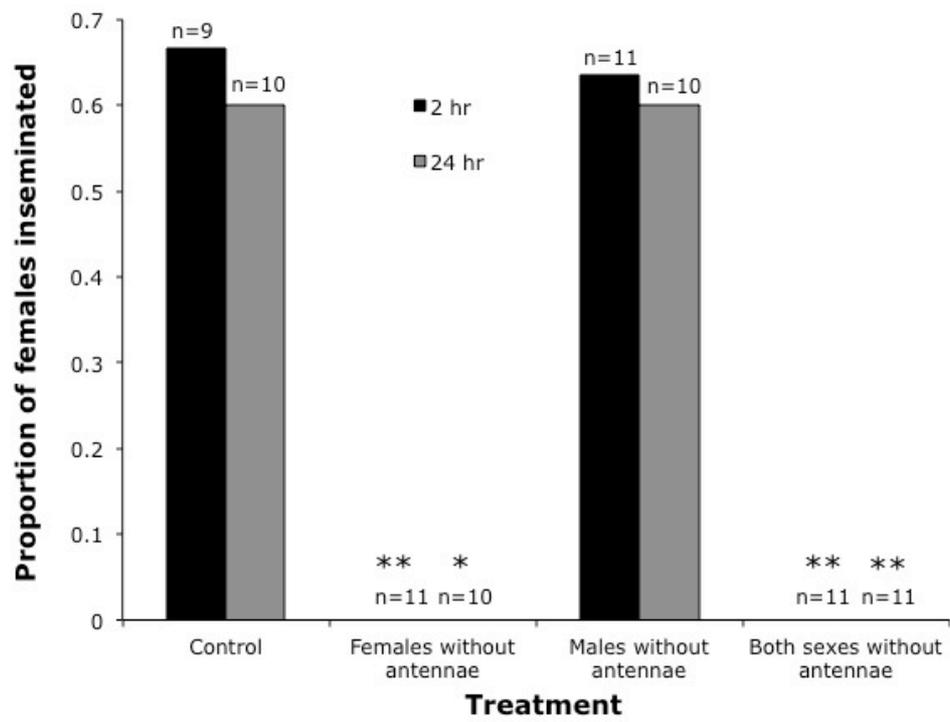


Figure 5

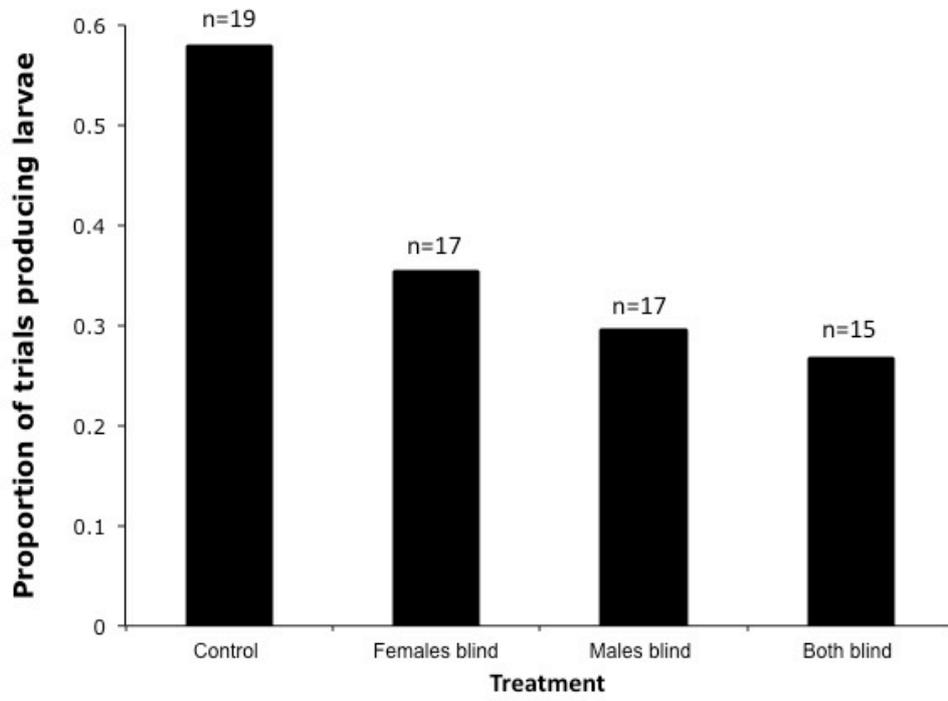


Figure 6

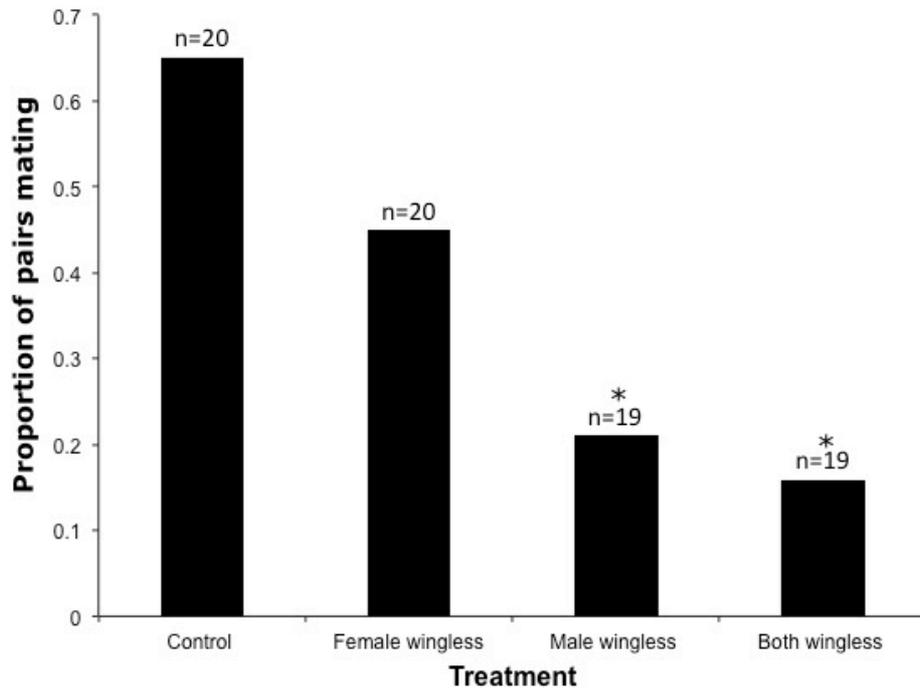


Figure 7

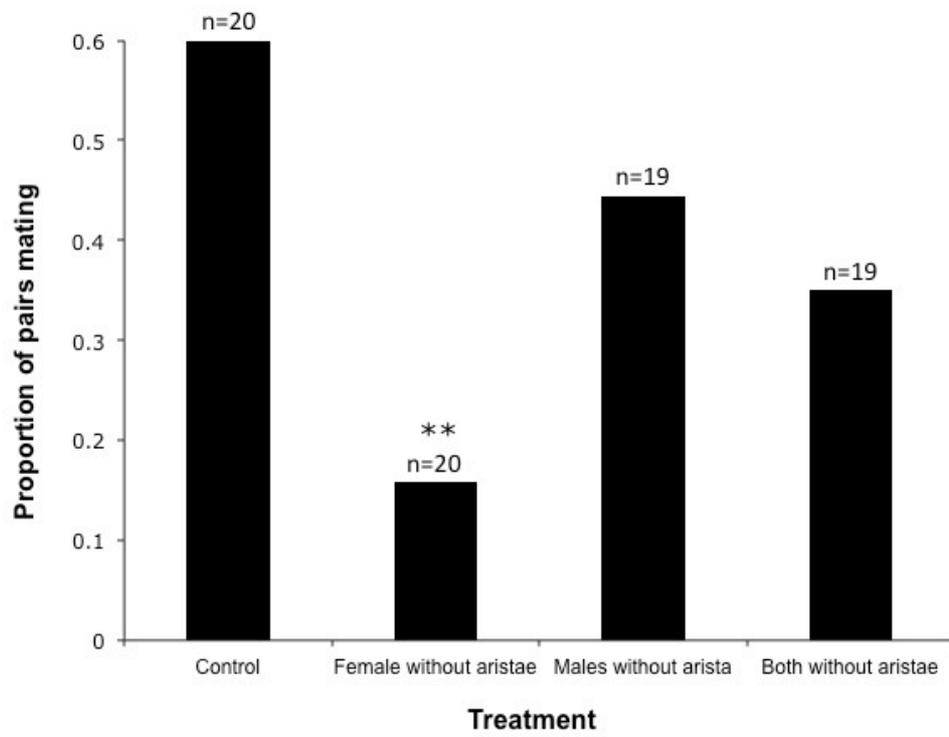


Figure 8

