

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

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HIGHLIGHTS

- Ablation of any one signal in *D. saltans* does not eliminate mating.
- Removal of the female midtarsi reduces mating occurrence.
- A novel courtship behavior described.
- Timing data used to discern stage in courtship affected by modality ablation.

1 **ABSTRACT**

2 Courtship signaling, necessary for the recognition of potential mates, is often complex
3 using many modalities with multiple components. *Drosophila* courtship is comprised of
4 chemical, tactile, visual and acoustic stimuli. Ablation of single sensory channels, either signal
5 production or reception, can determine the roles of individual modalities in overall reproductive
6 success. Adding measures of courtship timing, particularly courtship latency, the time for the
7 male to initiate courtship, and courtship duration, the time from courtship initiation until the
8 female accepts the male for copulation, allows us to identify the stage of courtship at which a
9 signal acts. This study focuses on *Drosophila saltans*, a member of the *saltans* species group.
10 Little is known about sexual behavior of species in this group, part of the *Sophophora* subgenus.
11 We find that the ablation of any one signal in *D. saltans* does not eliminate mating, thus
12 courtship is multimodal. In addition to examining the signals and signal reception common to
13 most *Drosophila* species, we also examine the role that the midtarsi play in courtship. The
14 removal of the female's midtarsi significantly reduces mating occurrence. Using timing data, we
15 discern that the absence of midtarsi in the female does not affect the male's ability to identify the
16 female as a potential mate, but the male may be unable to sufficiently stimulate the female to
17 copulation. Measuring courtship latency and courtship duration, as well as the occurrence of
18 courtship and copulation, allows us to determine if a signal plays a role in activating the male to
19 initiate courtship or stimulating the female to mate.

20

21 **KEYWORDS**

22 multimodal courtship, timing data, midtarsi, phenotypic manipulation, sensory channels

23 INTRODUCTION

24 Courtship behavior is comprised of one or more signals that ensure accurate recognition
25 and assessment of potential mates. A signal is any stimulus that, once emitted, benefits both the
26 signaler and receiver and has evolved under selection for the purpose of communicating
27 information (Maynard Smith & Harper, 2004). One role of courtship signaling is communication
28 of species identity (Ryan, 1990), which is necessary for choosing a compatible mate with whom
29 reproduction will result in fertile offspring (Andersson, 1994; Mendelson & Shaw, 2012).
30 Another role of courtship signaling is communication of mate quality. Recognition and sexual
31 selection signals can be understood under the same framework (i.e. unitary framework, Ryan &
32 Rand, 1993) and need not be examined using different experimental models. Examining the role
33 of signals in mate choice is necessary for understanding how species choose appropriate mates.

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

44 In most species of *Drosophila*, courtship communication occurs through chemical,
45 tactile, visual, and acoustic stimuli. For example, in *D. melanogaster*, courtship begins when the

46 male and the female come into contact, usually on a food source (Ewing, 1983). At this stage,
47 and throughout the entirety of courtship, visual signals may be important for either the male or
48 the female (Greenspan & Ferveur, 2000). Generally, the male initiates communication by
49 approaching the potential female mate and tapping her abdomen with his foretarsi (Spieth, 1974),
50 which contain chemoreceptors (Stocker, 1994). By “tasting” cuticular hydrocarbons (CHCs) that
51 function as pheromones (Ferveur, 1997), the male gains information about whether or not the
52 other individual is a female, if she is a conspecific, and if she has recently mated (Cobb & Jallon,
53 1990). In addition to perceiving CHCs through gustatory receptors on the foretarsi, the male may
54 also detect CHCs and other pheromones through olfactory receptors on the antennae (Stocker,
55 1994). At this point, the male may break off courtship if anti-aphrodisiac signals are received
56 (e.g. Cobb & Ferveur, 1996) or he may continue courtship and proceed by sending courtship
57 signals through other sensory channels. A male continuing courtship will vibrate his wing(s) to
58 create a species-specific courtship song (e.g. Liimatainen et al., 1992; Ritchie et al., 1999) that is
59 received by the female through her arista, the sound reception organ (Cook, 1973a, 1973b). The
60 male will closely follow the female, lick the tip of her abdomen with his proboscis (also
61 containing gustatory and olfactory receptors, Stocker, 1994) and periodically bend the tip of his
62 abdomen to meet hers to attempt to copulate. When the female is receptive, she will slow down
63 locomotion and spread her wings in order to allow the male to mount and copulate. Female
64 courtship signaling has been described to be limited to rejection signals with the exception of the
65 final acceptance signal, though female behaviors remain understudied (Dukas & Scott, 2015).

66 The importance of a particular sensory modality and associated signal(s) varies across the
67 *Drosophila* genus (Ewing, 1983; Spieth, 1974). For example, vision is necessary for male
68 reproductive success in *D. nebulosa* but not in *D. willistoni*, which are from the same species

69 group (Gleason et al., 2012). However, vision is necessary for males of both *D. subaquinaria* and
70 the closely related *D. recens* (Giglio & Dyer, 2013). Acoustic signals also vary in the role they
71 play in courtship success. Species-specific courtship song increases the rate at which mating
72 occurs in *D. melanogaster* and *D. simulans* (and to a lesser extent in *D. sechellia*) but is not
73 necessary for mating (Ritchie et al., 1999). Variation in courtship song contributes to
74 reproductive isolation in *D. melanogaster* and *D. lini* and their respective sibling species because
75 females use species-specific song components to discriminate against heterospecifics (Ritchie et
76 al., 1999; Wen et al., 2011). In contrast, in *D. montana* absence of courtship song inhibits mating
77 completely (Liimatainen et al., 1992).

78 Not only has the importance of different sensory modalities changed among species, but
79 also novel signals have evolved. One reason that vision is so important for *D. nebulosa* is that the
80 male produces an anal droplet that he fans towards the female; if he cannot orient in front of the
81 female, courtship fails (Gleason et al., 2012). This behavior has not been described for any other
82 species. Novelty is not reserved for the evolution of new signal modalities because existing
83 modalities may be modified with new signals. Courtship song seems to be particularly amenable
84 to these kinds of changes through the production of novel song types (e.g. Hawaiian *Drosophila*
85 song types, Hoikkala et al., 1994).

86 To understand the role of isolated signals in a multimodal courtship repertoire, one signal
87 must be ablated at a time and the subsequent effect on courtship and mating success examined
88 (e.g. Gleason et al., 2012; Hebets & Uetz, 1999; Liimatainen et al., 1992). Signal transmission
89 can be ablated by preventing the production of the signal or by obstructing the reception of the
90 signal. If a signal is essential to elicit courtship or copulation, the ablation will eliminate
91 courtship and/or copulation. Alternatively, single signals may not be necessary but may facilitate

92 courtship and copulation. This latter aspect may be missed by focusing solely on the *occurrence*
93 of courtship and/or copulation, as has been done in many studies (e.g. Benelli et al., 2012; Giglio
94 & Dyer, 2013; Gleason et al., 2012; Mayr, 1950; Narda, 1966; Robertson, 1983).

95 Measuring courtship latency (the time it takes courtship to start) and courtship duration
96 (time from the start of courtship start to the start of copulation) may help us to better understand
97 the stage of courtship at which a signal acts. An increase in courtship latency after a signal
98 ablation has different implications than an increase in courtship duration. Long courtship latency
99 means that the male is unable to detect a female signal efficiently or be sufficiently stimulated to
100 initiate courtship. In this case, either the male cannot receive a signal or the female cannot send a
101 signal with optimal efficacy. Long courtship duration can mean that the male is unable to
102 sufficiently stimulate the female to acceptance for copulation or that the male cannot receive the
103 female's acceptance signal. Using the information from both of these measurements allows us to
104 better understand the role a specific sensory modality plays in complex multimodal signaling.

105 In this study we focused on *D. saltans*, a member of the *saltans* species group. A
106 Neotropical clade comprised largely of sympatric species (de Campos Bicudo, 1973), the *saltans*
107 group is the most closely related group to the *willistoni* species. The mating behavior and sexual
108 signals of the other species groups in the *Sophophora* subgenus (the *melanogaster*, *obscura*, and
109 *willistoni* groups) have been studied extensively (reviewed in T. A. Markow & O'Grady, 2005)
110 but the *saltans* group has not received the same attention. Within the *saltans* group, species vary
111 greatly in their courtship song (Colyott, and Gleason unpublished), thus this group is a good
112 model to examine the importance of sensory modalities and use of sexual signals. Understanding
113 the sexual behavior of *D. saltans* (*sensu stricto*) will allow us to start filling in the gap of our

114 understanding of sexual behavior in the *Sophophora* subgenus and allow us to better examine
115 shifts in signals and their associated roles across the subgenus.

116 We examined the relative importance of individual sensory modalities in *D. saltans*. We
117 hypothesized that due to the multimodal nature of signaling in *D. saltans*, the ablation of a single
118 signal or its reception will not cause elimination of mating. We found this to be true for courtship
119 song and vision. Removing two sensory modalities, olfaction and hearing (olfaction cannot be
120 isolated because the hearing sensory organ is located at the tip of the olfactory sensory organ),
121 eliminates mating. In addition, through our experiments we discovered a new behavior involving
122 the midtarsi. Removal of the female's midtarsi significantly affected mating success. By
123 exploring timing data (courtship latency and courtship duration) we found evidence that the new
124 behavior may mediate an interaction between the male and female that significantly increases the
125 probability of mating.

126

127 **METHODS**

128 *Fly Culturing*

129 We maintained *Drosophila saltans* (*Drosophila* Species Stock Center stock number:
130 14045-0911.00) cultures 24 mm d x 94 mm h vials containing standard cornmeal-molasses
131 *Drosophila* food at 24°C with 12:12 light/dark cycle. The stock culture was maintained with 15-
132 30 flies of both sexes. Subcultures were standardized to generate the flies for our experiments.
133 Subcultures were started with ten potentially gravid females and one male. These flies were
134 removed after 2-3 weeks. Virgin experimental flies were collected under light CO₂ anesthesia
135 within 4 hours of eclosion. We housed virgins in single-sex groups of up to 10 individuals in
136 small food vials (16.5 mm d x 95 mm h) with cotton plugs.

137 *Behavioral Assays*

138 Individual virgins were removed from single-sex group vials at 7-9 days post eclosion
139 and were assigned to the manipulation treatments as described below. Post manipulation, flies
140 recovered for 24-48 hours before behavioral assays were performed. In each behavioral assay, a
141 single male and a female were aspirated into a new, small food vial. The cotton was pushed into
142 the vial to restrict the flies to approximately 1 cm³ space. A single trial consisted of observations
143 of all possible treatments simultaneously (control female with control male, manipulated female
144 with control male, control female with manipulated male and both sexes manipulated). The
145 observer watched the flies for an hour or until copulation was completed. The proportion of
146 males that courted and proportion of pairs that mated were calculated, as well as the courtship
147 latency and courtship duration for all pairs.

148 *vision*

149 To determine the general effect of light on mating success, pairs of virgin males and
150 females were placed in small food vials in a standard 12:12 light/dark (light treatment; N=87)
151 cycle or in a continuous dark (dark treatment; N=95) cycle for seven days. Females were
152 aspirated first into small vials and then randomly assigned a treatment. For the light treatment,
153 males were introduced into the vials assigned to a normal photoperiod (12:12 light/dark) in a lit,
154 24° C room. For the dark treatment, males were introduced into the vials under a red light and
155 kept in a 24° C, continuously dark incubator. Seven days later, all vials were scored for the
156 presence of larvae. Only vials with both parents alive at the end of the seven-day incubation were
157 used in analysis.

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

165 *wing removal*

166 To determine the effect of the production of song on mating success, wings (song
167 production organ) were removed (N= 15). Flies were anesthetized with light CO₂ and separated
168 into either a wing treatment (control) or wingless treatment. The wings were removed from the
169 wingless treatment individuals by severing the wing close to the body with a dissecting probe.

170 Individuals were group housed by treatment in single-sex groups of up to 10 individuals in new,
171 small food vials. Behavioral assays proceeded as described above.

172 *aristae removal*

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

179 *olfaction*

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

186 *gustation*

187 Gustation is inhibited by the removal of foretarsi, the location of chemoreceptors for
188 cuticular hydrocarbons (N= 13). Flies were anesthetized with light CO₂ and separated into
189 treatments with and without tarsi. Micro-dissection scissors were used to remove the five tarsal
190 segments of the foretarsi from both legs for treatment lacking foretarsi. Control individuals were
191 anesthetized for the same amount of time as required to perform the procedure. Behavioral
192 assays proceeded as described above.

193 *midtarsi*

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

198 *Data Analysis*

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

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

212 **RESULTS**

213 *Vision*

214 Single pairs held in constant darkness (N= 95) were less successful at producing progeny
215 than those held in a 12:12 hour light:dark cycle (N= 87; Figure 1; two tailed Fisher's exact test: P
216 < 0.0001). We concluded that mating is reduced in the dark because egg laying was not inhibited
217 by constant darkness; larvae were produced in vials in which mated females were transferred to
218 constant darkness (data not shown). The effect may be due to facilitation of male mating ability;
219 when males were blind (N=20) mating success was reduced but not eliminated (Figure 2a;
220 Fisher's Exact Test: $P = 0.0225$). However, when females were blind the reduction in mating
221 success was not significant (Figure 2a; Fisher's Exact Test: NS). When both sexes were blind,
222 there was a significant reduction of mating success (Figure 2a; Fisher's Exact Test: $P = 0.0095$)
223 presumably because the males were blind. The reduction in mating success was not caused by a
224 reduction in courtship occurrence (data not shown; Fisher's Exact Test: NS). Also, of the males
225 that courted, courtship latency and courtship duration were significantly longer when males were
226 blind (Table 1, Supplementary Figure 1). Courtship latency and courtship duration were not
227 affected when females were blind (Table 1, Supplementary Figure 1.).

228 *Wing Removal*

229 Males and females use their wings differently during courtship. Males vibrate their wings
230 to produce courtship song and wing vibration may produce visual as well as auditory cues.
231 Females spread their wings to signal receptivity, which serves as a visual signal of acceptance to
232 the males and allows the male to mount. Wing removal in both males and females (N=15)
233 significantly affected mating success. Wingless males had a significant decrease in mating
234 success (Figure 2b, Fisher's Exact Test: $P = 0.0025$). Removal of the female's wings also

235 significantly decreased mating success (Figure 2b, Fisher's Exact Test: $P = 0.05$). Furthermore,
236 when both sexes were wingless, mating success was significantly reduced (Figure 2b, Fisher's
237 Exact Test: $P < 0.001$). The reduction in mating success was not caused by a reduction in
238 courtship occurrence (data not shown, Fisher's Exact Test: NS). Courtship latency was not
239 affected by the absence of wings in either males or females (Table 1, Supplementary Figure 2)
240 indicating that courtship was initiated normally. However, of those that did court, courtship
241 duration was significantly longer when females and males were wingless (Table 1,
242 Supplementary Figure 2).

243 *Aristae Removal*

244 The arista are the auditory reception organs in *Drosophila* (Ferveur, 1997; Stocker,
245 1994) thus the removal of arista allowed us to isolate and examine the effect of hearing.
246 Aristaless females (N=16) had a significant decrease in mating success (Figure 2c, Fisher's
247 Exact Test: $P = 0.0025$). In contrast, aristaless males had no significant decrease in mating
248 success (Figure 2c, Fisher's Exact Test: NS). When both sexes had arista removed, mating
249 success was significantly reduced (Figure 2b, Fisher's Exact Test: $P = 0.0025$) likely because the
250 females were unable to hear. The reduction in mating success was not caused by a reduction in
251 courtship occurrence (data not shown, Fisher's Exact Test: NS). Courtship latency was not
252 affected by the removal of the arista in males or females (Table 1, Supplementary Figure 3).
253 Also, of those that did court, courtship duration was significantly longer (Table 1, Supplementary
254 Figure 3) when females lacked arista. Courtship duration was not affected when males lacked
255 arista (Table 1, Supplementary Figure 3).

256 *Olfaction*

257 The third antennae segment is an olfactory organ of *Drosophila* (Cook, 1973b). Because
258 antennae cannot be removed without removing the arista (thereby eliminating hearing), arista
259 were removed in the control treatment (N= 16). No males or females without antennae copulated
260 regardless of which sex was ablated; however, because the control individuals, which lacked
261 arista, mated at a very low rate (6%), sample sizes would need to be far larger than feasible to
262 detect specific effects on copulation caused specifically by lack of olfaction. Although we cannot
263 measure the effect of olfaction on copulation, unlike the previously examined senses, olfaction
264 has an effect on the initiation of courtship. Male lacking antennae had a significant reduction in
265 courtship occurrence (Figure 2d, Fisher's Exact Test: $P = 0.05$); the same was not true when the
266 female's antennae were removed (Figure 2d, Fisher's Exact Test: NS). Additionally, when both
267 sexes had antennae removed, courtship occurrence was significantly reduced (Figure 2d, Fisher's
268 Exact Test: $P = 0.05$) seemingly because the males were unable to smell. Latency of courtship
269 was not affected by the removal of the antennae in males or females (Table 1, Supplementary
270 Figure 4).

271 *Gustation*

272 The foretarsi, five distal segments of the leg, of *Drosophila* are lined with gustatory
273 receptors (Carlson, 1996). Males that had foretarsi removed (N=13) had a significant reduction
274 in courtship occurrence (data not shown, Fisher's Exact Test: $P = 0.0016$) and copulation
275 occurrence (Figure 2e, Fisher's Exact Test: $P = 0.0048$). Removal of the female's foretarsi did
276 not affect courtship occurrence (data not shown, Fisher's Exact Test: NS) or copulation
277 occurrence (Figure 2e, Fisher's Exact Test: NS). When both sexes had foretarsi removed,
278 courtship occurrence was significantly reduced (data not shown, Fisher's Exact Test: $P = 0.0149$)

279 as well as copulation occurrence (Figure 2e, Fisher's Exact Test: $P = 0.0472$) likely because the
280 males were unable to taste. Courtship latency and courtship duration were not affected when
281 males or females had foretarsi removed (Table 1, Supplementary Figure 5).

282 *Midtarsi*

283 Preliminary trials to examine the effect of gustation (foretarsal removal) on courtship and
284 copulation were done using the removal of midtarsi as a control (data not shown). We were
285 surprised by the effect of the midtarsi removal on behavior and thus performed separate
286 experiments to examine the effects of each set of legs separately. We almost missed the midtarsi
287 behavior because it is subtle and has not been considered in the assessment of courtship behavior
288 in *Drosophila*. Observations of courtship behavior of the control treatment of *D. saltans* progress
289 similarly as described in the introduction; after a brief interaction between the male and the
290 female (which includes the male tapping the female's abdomen with his foretarsi) the male
291 follows closely behind the female, alternating vibrating his wings and licking the tip of the
292 female's abdomen. Male attempts to mount at this point are often prevented by the female
293 kicking him with her hind legs, causing the male to resume following, singing, licking, and
294 circling. When the male is on the female's side with his head proximal to her abdomen, she may
295 extend her middle leg to touch his foretarsi with her midtarsi. This seems to be a signal initiated
296 by the female, but full ethogram analysis of *D. saltans* courtship behavior is needed to
297 demonstrate this conclusively. When the midtarsi of the female is ablated, the male approaches
298 her from the side and taps his foretarsi where her midtarsi would be if they were intact. The male
299 then continues courting the female, often even more aggressively, circling around her while he
300 vibrates his wings with intermittent abdomen licking and side-tapping.

301 Examining the role of midtarsi in courtship is unique to this study. Although many
302 studies have explored multimodal courtship in *Drosophila* species (reviewed in T. A. Markow &
303 O'Grady, 2005), recent studies have not examined the role of the midtarsi in courtship. Midtarsi
304 removal affected the sexes differently. When the midtarsi were removed from the males (N=15),
305 there was no significant effect on copulation occurrence (Figure 2f, Fisher's Exact Test: NS)
306 whereas when midtarsi were removed from the females, copulation occurrence was significantly
307 reduced (Figure 2f, Fisher's Exact Test: $P = 0.0209$). Also, when both sexes had midtarsi
308 removed, copulation occurrence was significantly reduced (Figure 2f, Fisher's Exact Test: $P =$
309 0.0209), likely because the females had no midtarsi. The removal of midtarsi in males or females
310 did not affect whether or not courtship occurs (data not shown, Fisher's Exact Test: NS).
311 Courtship latency was not affected when the midtarsi were removed from females (Figure 3,
312 Table 1), but courtship duration was significantly increased (Figure 3, Table 1). Courtship
313 latency and courtship duration were not affected when only males had midtarsi removed (Figure
314 3, Table 1).

315 **DISCUSSION**

316 *Courtship is Multimodal*

317 In each experiment we removed either signal production or signal reception in
318 *Drosophila saltans* courtship. The removal of any one signal or its reception did not completely
319 eliminate mating success or courtship (Table 2) indicating that *D. saltans* courtship is
320 multimodal with multiple sensory modalities influencing courtship success. Thus signals may be
321 redundant. The largest effect of the manipulations on mating success was on copulation: normal
322 production and reception of signals made copulation more likely. Every ablation had an effect
323 indicating that the previously described sensory modalities for *Drosophila* courtship
324 communication are all used by *D. saltans*.

325 Courtship occurrence was only altered by the ablation of the male antennae, which
326 involves the removal of both olfaction and audition. In all other cases courtship was initiated,
327 though it was delayed when the male was blind (Table 2) indicating that the male was unable to
328 receive stimulating signals from the female. Thus the female's initial signals to the male are
329 visual. This is reflected also in the reduction of mating success when pairs were kept in the dark,
330 though mating was not completely eliminated. Using the terms of Grossfield (1971), we can say
331 that *D. saltans* is a species for which mating is inhibited by darkness.

332 All signaling modalities tested affected *D. saltans* courtship success though none
333 eliminated mating, thus the species is similar to most other *Drosophila* relying on gustatory,
334 olfactory, tactile, acoustic and visual signals (e.g. Ewing, 1983; Giglio & Dyer, 2013; Gleason et
335 al., 2012; Spieth, 1974). Rarely is a single sensory modality necessary for courtship success
336 though there are species for which olfaction (e.g. *D. nebulosa*, Gleason et al., 2012), or vision
337 (e.g. multiple species, Grossfield, 1971) or courtship song (e.g. *D. pallidosa*, Doi et al., 2001) is

338 required for mating success. Mating success may be eliminated in the presence of a
339 heterospecific signal (e.g. *D. ananassae* females with *D. pallidosa* courtship song, Doi et al.,
340 2001). Our experiments were not designed to test species-specific signal recognition, but given
341 the multimodal nature of *D. saltans* courtship it is likely that discrimination against
342 heterospecifics involves assessing multiple signals and is not inhibited by single aberrant signals.

343 Our tests were no-choice experiments, which allowed us to measure effects on courtship
344 progression in individual pairs. Increased courtship duration implies that a male needed to work
345 harder to gain a female response, or that males failed to receive a signal from the female, leading
346 them to continue courting. Thus most signals as tested here may be reflecting mate quality
347 redundantly. If single signals were necessary for mate recognition, failure to receive them would
348 eliminate mating. This may be a possibility with olfactory signals, though we could not study
349 their reception without eliminating hearing as well, because copulation was completely
350 eliminated in the absence of olfaction. Currently nothing is known about pheromones produced
351 by *D. saltans*, or its relatives. In other species groups pheromones, particularly cuticular
352 hydrocarbons, which are largely gustatory signals, play a large role in reproductive isolation (e.g.
353 *melanogaster* group, Cobb & Jallon, 1990) or sexual selection by female choice (e.g. *D. serrata*,
354 Chenoweth & Blows, 2005).

355 Ablation of signals and their reception by physical manipulation is potentially damaging
356 to the fly in unanticipated ways, but lacking the genetic resources of *D. melanogaster*, we were
357 unable to use genetic ablation. Such mutations have been used to determine that the elimination
358 of a single modality in *D. melanogaster* does not prevent mating (T.A. Markow, 1987) though
359 elimination of both hearing and olfaction abolished mating success, implying a synergistic
360 interaction (Rybak et al., 2002). Given that our approach has similar effects, the use of physical

361 manipulations seems to be equivalent to that of genetic manipulations. Relying on genetic
362 manipulations, however, would have caused us to miss the midtarsi behavior.

363 *Different Effects on the Sexes*

364 The effects of a manipulation on the sexes were considered different when the ablation
365 of a body part in one sex did not alter mating success while the same ablation on the other sex
366 had a detrimental effect on mating (Table 2). This implies that males and females need to receive
367 different types of signals for courtship to progress, as has been seen in other species (Gleason et
368 al., 2012). In no cases did altering the female change the male's propensity to court her. Females
369 were always attractive to the male. Through our manipulations we were not able to alter female
370 pheromone production, though male failure to initiate as often when chemosensory reception
371 (through olfaction) was altered implies that female pheromones are instrumental in stimulating
372 male courtship.

373 Wing removal was the only ablation that had the same effect when removed from the
374 female as when removed from the male. The wing generates signals in different modalities for
375 each sex. For the male they are used to produce an acoustic signal. Lack of male wings is
376 paralleled by the reduction in mating when females cannot hear (lack of aristae). Lack of vision
377 for females does not affect mating success implying that male wings are not used for an
378 important visual signal.

379 However, lack of female wings was paralleled by the reduction in mating when males
380 could not see (lack of vision). In female courtship behavior wings are used in a visual signal of
381 acceptance; when males cannot see, mating success is reduced. In *D. melanogaster*, males need
382 vision to track the movement of females and to follow closely behind during courtship (reviewed
383 in Greenspan & Ferveur, 2000; Spieth, 1974). This may not be the case for *D. saltans*,

384 considering blind males attempted mounting often and failed because females had not spread
385 their wings (Odu, pers. obs.). However, when a female spread her wings, which allows easier
386 access for male mounting, the blind male often would not attempt to mount and therefore not
387 succeed in mating.

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

393 The differential effect of signaling modalities between males and females is probably a
394 ubiquitous characteristic of *Drosophila* courtship because each sex signals in different
395 modalities. For instance, although most *Drosophila* males produce an acoustic signal (reviewed
396 in T. A. Markow & O'Grady, 2005), most females do not produce acoustic signals, with a few
397 exceptions (e.g. Donegan & Ewing, 1980). For *D. melanogaster* males, lack of aristae does not
398 affect a male's ability to produce normal courtship song (Burnet et al., 1977), thus inability to
399 hear affects courtship more when females are deficient than when males are deficient. In
400 competition experiments using genetic mutants, visually defective females are as successful as
401 wild-type females, although visually defective males are never successful when competing with
402 wild-type males (T.A. Markow, 1987), likely because males need to be able to follow females,
403 whereas females are not similarly restricted. Similarly, a *D. nebulosa* male requires vision to
404 position himself in front of a female to fan a pheromone towards her but she does not need vision
405 to mate (Gleason et al., 2012). He does not need to be able to smell the pheromone, though the
406 female will not mate if she cannot smell (Gleason et al., 2012).

407 *Midtarsi: a Potential Tactile Signal?*

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

417 The observed interactions between the female and male centered around the female
418 midtarsi and are potentially part of a two-way conversation between the male and female. All
419 other signals involved in courtship are one-way signals from one individual that causes a change
420 in behavior in the other individual. Because we have observed the females reaching out with
421 their legs toward the males, the midtarsi may convey a tactile signal to the male that is an active
422 encouragement signal preceding the wing spreading posture. Or the female may need a tactile
423 response from the male to progress to acceptance. Regardless of the nature of the
424 communication, in the absence of the female midtarsal signal, males may continue courtship
425 more aggressively not knowing to proceed to the next stage thereby delaying copulation.

426 Although observations of the role of midtarsi in courtship have not been noted in other
427 well-studied *Drosophila* species, this behavior is probably not restricted to *D. saltans* as similar
428 behaviors were noted in the distantly related *D. malerkotliana* (*melanogaster* group). A *D.*
429 *malerkotliana* male uses his foretarsi to tap the midtarsi of the other individuals; following the

430 midtarsi tap, courtship progresses if the female is a conspecific or breaks off if she is a
431 heterospecific or a male (Narda, 1966). This behavior may be analogous to the initial foretarsi
432 tap of other species where the male uses his foretarsi to tap the body of the female. For a *D.*
433 *malerkotliana* male, absence of foretarsi does not prevent the progression of courtship but the
434 male fails to distinguish male and female targets as well as heterospecific and conspecific
435 females (Narda, 1966). A male that taps a female that lacks midtarsi proceeds with courtship in
436 the same way that he continues if he lacks foretarsi.

437 Given that the midtarsi-associated behavior of *D. malerkotliana* results in the interruption
438 of courtship when the target individual is the wrong species or sex, whereas in *D. saltans*
439 midtarsi are associated with the continuation of courtship of the opposite sex, these are probably
440 behaviors with different messages. For most *Drosophila* species, although females have control
441 over accepting males, few female signals have been described, although females may set the
442 dynamics of courtship interactions (Dukas & Scott, 2015). This behavior should be examined in
443 other species because it may have gone undetected.

444 Given that the midtarsi-associated behavior of *D. malerkotliana* results in the interruption
445 of courtship when the target individual is the wrong species or sex, whereas in *D. saltans*
446 midtarsi are associated with the continuation of courtship of the opposite sex, these are probably
447 behaviors with different messages. Few discrete female signals have been described for
448 *Drosophila* species, though the most common one is wing spreading by the females, a visual
449 signal indicating receptivity required before males will mount females (reviewed in T. A.
450 Markow & O'Grady, 2005). The midtarsi signal by females of *D. saltans* may be an additional
451 signal to encourage courtship, though not of final acceptance, which is the wing spreading signal.
452 This may be a further mechanism through which females can control the dynamics of courtship,

453 as has been suggested for *D. melanogaster* (Dukas & Scott, 2015). The midtarsi behavior should
454 be examined in additional species because it may be present but not yet detected.

455 *Timing Data*

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

468 *Conclusions*

469 We found that the removal of no single modality eliminated courtship or copulation
470 indicating that *D. saltans* courtship is multimodal. We also described a courtship behavior that
471 should be considered in future studies of *Drosophila* courtship signaling. Lastly, we suggest that
472 future studies should consider measuring courtship latency and courtship duration as well as the
473 occurrence of both to understand the role that courtship signals play in the progression of
474 courtship.

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569

570 **FIGURES**

571 Table 1: Summary of ANOVA results for timing data

Ablation	Courtship latency				Courtship duration			
	Female effect		Male effect		Female effect		Male effect	
	F-value	P-value	F-value	P-value	F-value	P-value	F-value	P-value
Eyes	F _{1,58} = 1.83	0.182	F _{1,58} = 7.95	0.007^a	F _{1,58} = 1.88	0.176	F _{1,58} = 7.09	0.010
Wings	F _{1,56} = 2.15	0.148	F _{1,56} = 3.01	0.088	F _{1,56} = 6.45	0.014	F _{1,56} = 30.7	8.32e-07
Aristae	F _{1,56} = 0.15	0.701	F _{1,56} = 2.10	0.153	F _{1,56} = 33.0	3.92e-07	F _{1,56} = 0.70	0.408
Antennae ^b	F _{1,41} = 0.57	0.454	F _{1,41} = 0.14	0.709				
Foretarsi	F _{1,34} = 0.32	0.573	F _{1,34} = 1.46	0.235	F _{1,34} = 0.00	0.983	F _{1,34} = 2.62	0.116
Midtarsi	F _{1,55} = 0.53	0.469	F _{1,55} = 3.49	0.067	F _{1,55} = 16.8	0.000	F _{1,55} = 0.02	0.889

572

573 ^aSignificant P-values are bold. ^bFor the antennae, the proportion of the control individuals that
574 courted and copulated was too low for comparisons to be made with ablated individuals.

575

576 Table 2: Summary of results

Ablation	Courtship occurrence ^a		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 ^b	NS	↓			NS	NS		
Foretarsi	NS	NS	NS	↓	NS	NS	NS	NS
Midtarsi	NS	NS	↓	NS	NS	NS	↑	NS

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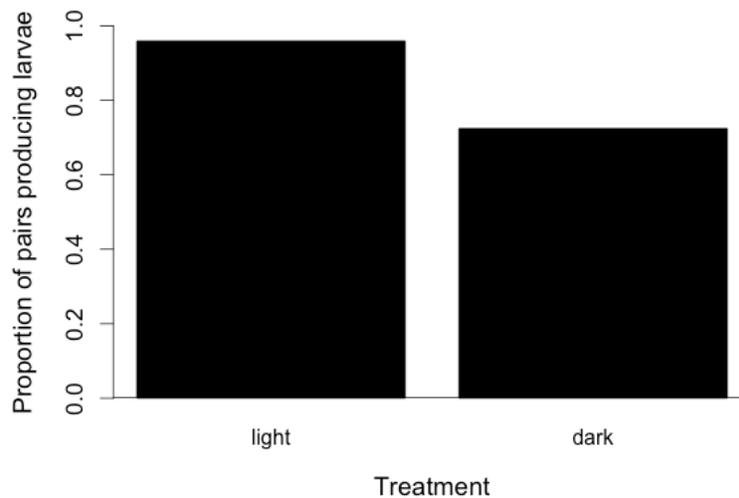
578 ^aAbbreviations: NS: no significant change; ↓: significant decrease; ↑: significant increase.

579 ^bFor the antennae, the proportion of the control individuals that courted and copulated was so
580 low that comparisons could not be made with ablated individuals.

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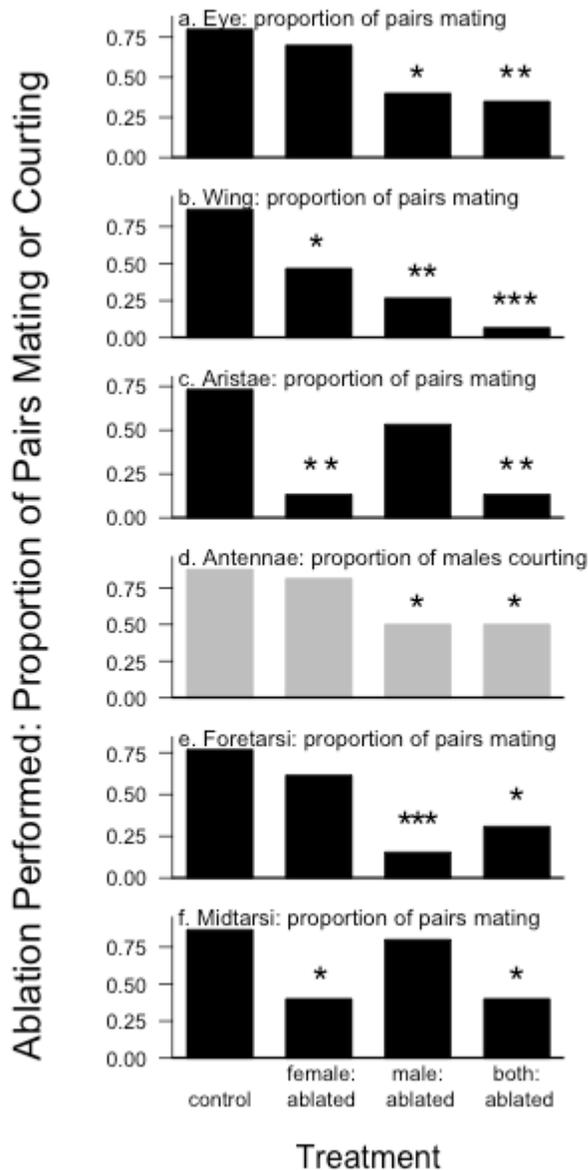
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584

585 **Figure 1. Effect of light on mating.** Mating pairs were left in the light (N=87; 12 hr light: 12 hr
586 dark) or in the dark (N=95; continuous darkness). After seven days, the vials were scored for
587 presence of larvae. Pairs in continuous darkness produced offspring significantly less often than
588 pairs in a normal light: dark cycle (two-tailed Fisher's Exact Test: $P < 0.001$).

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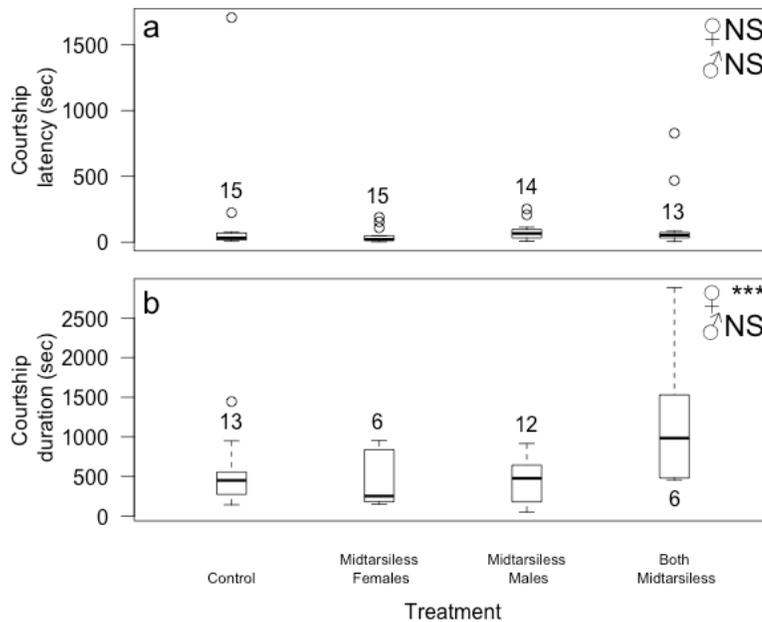
590

591 **Figure 2. Proportion of pairs mating or courting for ablation experiments.** Mating trials
 592 were conducted with control pairs, ablated females, ablated males, and both sexes ablated. Pairs
 593 were observed for an hour or until copulation ended and the proportion that courted and
 594 copulated were recorded. A Fisher's Exact Test was used to compare each treatment to control.
 595 Significance level of test is indicated on bar plots ($P < 0.05$ *, $P < 0.01$ **, and $P < 0.005$ ***)

596 a. Male blindness inhibits copulation (N=20 per treatment). b. Absence of wings inhibits

597 copulation (N=15 per treatment). c. Female inability to hear inhibits copulation (N=15 per

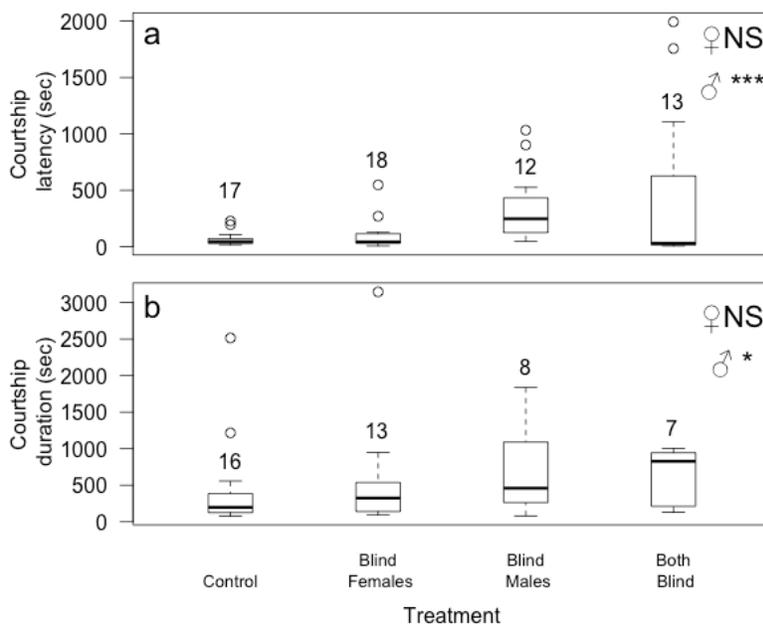
598 treatment). d. Male inability to smell inhibits courtship (N=16 per treatment). e. Male inability to
 599 taste inhibits courtship and copulation (N=13 for each treatment). f. Female inability to side-tap
 600 male inhibits copulation (N=15 for each treatment).
 601



602
 603 **Figure 3. Effects of midtarsi on courtship latency and courtship duration.** Mating trials were
 604 conducted with control pairs, midtarsi ablated females, midtarsi ablated males, and both sexes
 605 midtarsi ablated. Pairs were observed for an hour or until copulation ended and the time it took
 606 males to court (a. courtship latency), and courting males to copulate (b. courtship duration) was
 607 recorded. An ANOVA was used to test for an effect of male treatment and female treatment on
 608 log transformed timing data. The effect of female treatment and male treatment are in the upper
 609 right corner of plot (NS: not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$). For ANOVA
 610 analyses data for pairs that did not copulate were scored as described in the methods. To
 611 construct the boxplots this data was left out for better visualization of the recorded data.
 612

613 **SUPPLEMENTARY FIGURES**

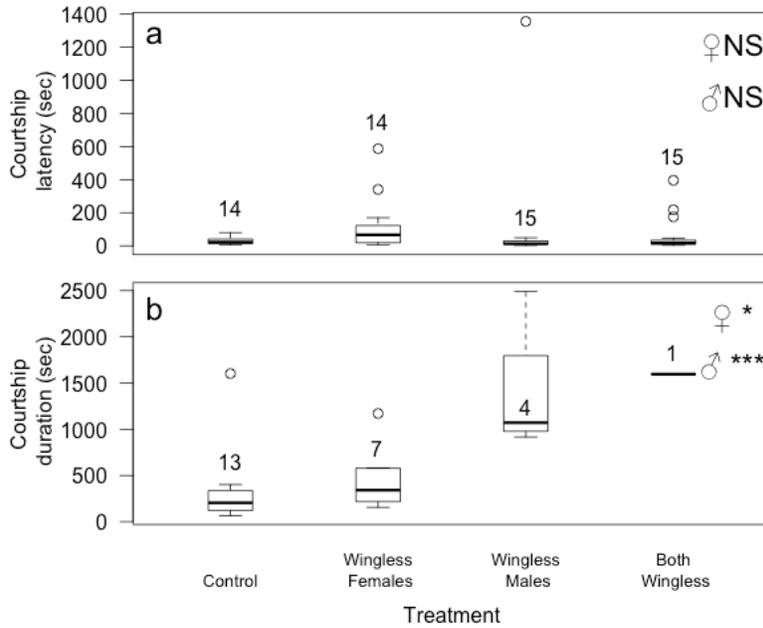
614 For the following figures (1-5) mating trials were conducted with control pairs, ablated females,
615 ablated males, and both sexes ablated. Pairs were observed for an hour or until copulation ended
616 and the time it took males to court (a. courtship latency), and courting males to copulate (b.
617 courtship duration) were recorded. An ANOVA was used to test for an effect of male treatment
618 and female treatment on log transformed data. The number of courting (a. courtship latency) and
619 copulating pairs (b. courtship duration) for each treatment appears in the figures. The effect of
620 female treatment and male treatment are in the upper right corner of plot (NS: not significant; *
621 $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$; ND: not enough data to perform analysis). For ANOVA
622 analyses data for pairs that did not copulate were scored as described in the methods. To
623 construct the boxplots this data was left out for better visualization of the recorded data.
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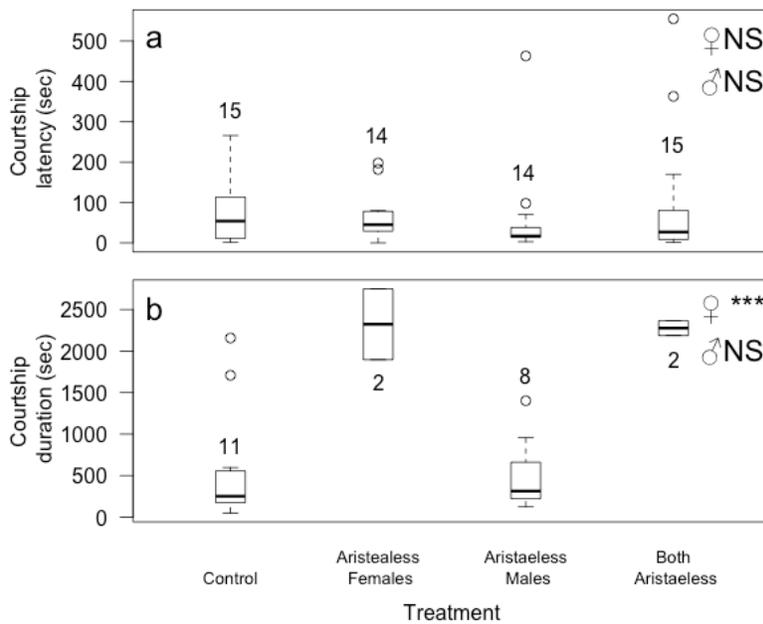
626 **Supplementary Figure 1. Effects of vision on courtship latency and courtship duration.**

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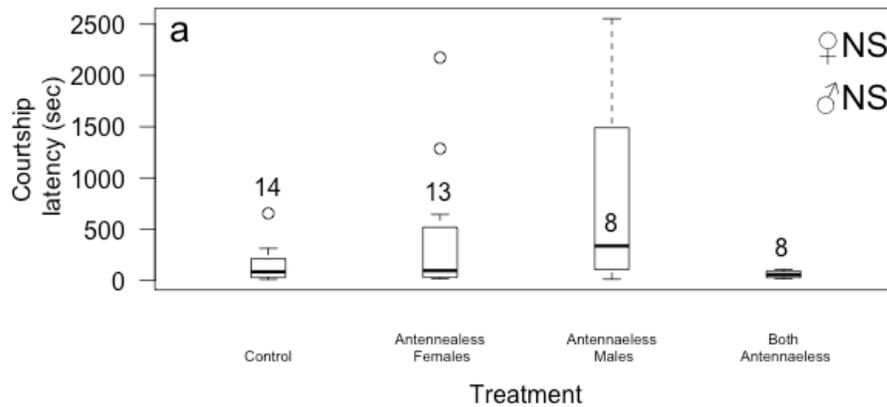
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629 **Supplementary Figure 2. Effects of wings on courtship latency and courtship duration.**



630

631 **Supplementary Figure 3. Effects of hearing on courtship latency and courtship duration.**

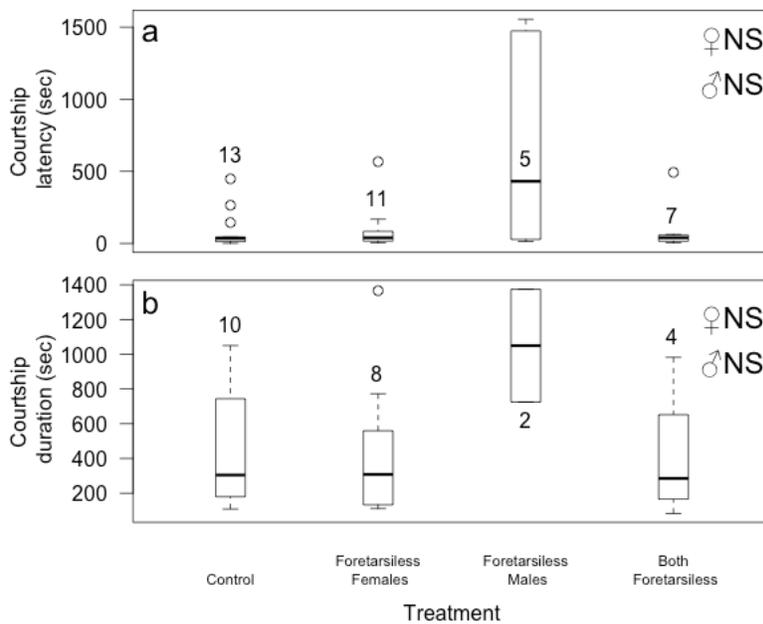


632

633 **Supplementary Figure 4. Effects of antenna on courtship latency and courtship duration.**

634 Only one control (lacking aristae) pair mated so we were unable to assess the effect on courtship
 635 duration.

636



637

638 **Supplementary Figure 5. Effects of foretarsi on courtship latency and courtship duration.**

639

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