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# “Singing on the Wing” as a Mechanism for Species Recognition in the Malarial Mosquito *Anopheles gambiae*

Cédric Pennetier, Ben Warren, R Dabire, Ian J Russell, Gabriella Gibson

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1 'Singing on the wing' as a mechanism for species recognition in the  
2 malarial mosquito *Anopheles gambiae*

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4 C. Pennetier<sup>1,2\*</sup>, B. Warren<sup>1\*</sup>, R. Dabire<sup>3</sup>, I.J. Russell<sup>1</sup> and G. Gibson<sup>1,2</sup>

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6<sup>1</sup>School of Life Sciences, University of Sussex, Falmer, Brighton, BN1 9QG, U.K.

7<sup>2</sup>Natural Resources Institute, University of Greenwich at Medway, Chatham

8Maritime, Kent, ME4 4TB, U.K.

9<sup>3</sup>Institut de Recherche en Science de la Santé/Centre Muraz, BP 390 Bobo-Dioulasso

1001, Burkina Faso

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13Running title: Species recognition in the malarial mosquito

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15Contact: Ian J. Russell, [i.j.russell@sussex.ac.uk](mailto:i.j.russell@sussex.ac.uk), Tel: + 44 (0)1273 678632

16Correspondence: [g.gibson@greenwich.ac.uk](mailto:g.gibson@greenwich.ac.uk), [i.j.russell@sussex.ac.uk](mailto:i.j.russell@sussex.ac.uk)

17Additional footnotes: \*These authors have contributed equally

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## 24Summary

25*Anopheles gambiae*, responsible for the majority of malaria deaths annually, is a  
26complex of seven known species and several chromosomal/molecular forms. The  
27complexity of malaria epidemiology and control is due, in part, to its remarkable  
28genetic plasticity, enabling its adaptation to a widening range of human-influenced  
29habitats, which leads to rapid ecological speciation as soon as reproductive isolation  
30mechanisms start to develop [1-6]. Although reproductive isolation is essential for  
31speciation, little is known about how it occurs in sympatric populations of its incipient  
32species[2]. We show that in such a population of the ‘M’ and ‘S’ molecular forms a  
33novel mechanism of sexual recognition (male-female pairs of mosquitoes match  
34flight-tones[7-9]), also confers the capability of mate recognition, an essential  
35precursor to assortative mating; frequency-matching occurs more consistently in  
36same-form pairs than in mixed-form pairs ( $P > 0.001$ ). We also show that the key to  
37frequency-matching is ‘difference tones’ produced in the nonlinear vibrations of the  
38antenna by the combined flight-tones of a pair of mosquitoes, and detected by the  
39auditory Johnston’s organ. Through altering their wing-beat frequencies to minimise  
40these difference tones, mosquitoes can match flight-tone harmonic-frequencies above  
41their auditory range. This is the first description of close-range mating interactions  
42between males and females of incipient *An. gambiae* species.

43

44

## 45Results and Discussion

46

47*Anopheles gambiae s.l.* has become a focus of research on the evolution of species  
48complexes to understand how populations diverge and become distinct species [4].  
49The essential mechanism leading to speciation is the evolution of reproductive  
50isolation between diverging populations. Within the *Anopheles gambiae* complex,  
51several degrees of reproductive isolation among its members can be observed in field  
52populations. On one hand, formally recognized species such as *An. gambiae s.s.* and  
53*An. arabiensis* have evolved strong reproductive isolation, although a permeable  
54species barrier still exists leading to a small degree of introgressive hybridization[1,  
555]. On the other hand, within *An. gambiae s.s.* cryptic incipient speciation has led to  
56the recognition of two molecular forms, named ‘M’ and ‘S’[6], that assortatively  
57mate[10] at different frequencies across different eco-geographical settings[3, 11].  
58The mechanisms responsible for reproductive isolation between M and S are not fully  
59understood and appear to vary across populations. In Mali, for example, unknown  
60behavioural cues used by the two forms to identify swarm sites have diverged and,  
61since they mate in segregated swarms, hybrids are rarely produced [12]. In Burkina  
62Faso, only 500 km away, M and S form mosquitoes can be found in the same swarm  
63[6, 10, 13, 14] and yet hybrids are also rare, indicating the potential existence of a  
64close-range barrier to interbreeding.

65       There are no published reports of close-range mate recognition in the *An.*  
66*gambiae* complex, and attempts to demonstrate mate recognition in the field with  
67volatile pheromones have not been successful (J.D. Charlwood, personal  
68communication, 2009). In this paper we report the first evidence of form-specific,  
69close-range (~ 2 cm) interactions between males and virgin females, characterized by

70continuously monitored audio-motor feedback between individual mosquitoes. This  
71behaviour, which provides the capability of mate recognition in mosquitoes, may  
72contribute to the observed assortative mating between M and S form mosquitoes  
73where they meet in mixed swarms.

74

#### 75Behavioural Interactions

76We recorded the flight tones and flight tone interactions produced by tethered wild  
77male and virgin female M and S form mosquitoes, individually and in same- and  
78mixed-form pairs under semi-natural conditions in Bobo Dioulasso, Burkina Faso  
79(Figure 1 inset). Individual male and female mosquitoes flew at mean fundamental  
80wing-beat frequencies (WBFs) similar to those reported previously[15], with males  
81flying at significantly higher WBFs (mean  $\pm$  SD; M males =  $704 \pm 25$  Hz,  $n=4$ ; S  
82males =  $682 \pm 27$  Hz,  $n=5$ ) than their conspecific females [M females =  $467 \pm 31$  Hz,  
83 $n=6$ ; S females =  $460 \pm 26$  Hz,  $n=5$ ;  $P < 1.0 \times 10^{-7}$ , Tukey's honest significant  
84difference (HSD), Experimental Procedures] for flight records of mean length = 8.7 s.  
85When male-female pairs of same-form and mixed-form were flown within auditory  
86range ( $\sim 2$  cm) of each other's flight tones, their flight behaviour altered significantly;  
87males and females of both molecular forms significantly increased their mean WBFs  
88(ANOVA;  $F=5.103$ ;  $df=1,101$ ;  $P = 0.026$ , for solo v. paired flight), with males  
89continuing to fly at significantly higher mean WBFs (M males =  $771 \pm 42$  Hz,  $n= 30$ ;  
90S males =  $715 \pm 55$  Hz,  $n = 14$ ) than their conspecific females (M females =  $489 \pm 33$   
91Hz,  $n = 24$ ; S females =  $475 \pm 28$  Hz,  $n = 20$ ;  $P < 1.0 \times 10^{-7}$  for both comparisons,  
92Tukey's HSD), irrespective of whether they were in same- or mixed-form pairs. All  
93types of mosquito also significantly increased the variability of their respective WBFs  
94(mean interquartile range, IQR) when flying in pairs ( $F = 20.137$ ;  $df =1, 101$ ;  $P = 1.9$

95X 10<sup>-5</sup>) from a mean value for males of 10 Hz for solo flights to 27 Hz for paired  
96flights, and for females from 5 Hz to 22 Hz, irrespective of the form they were paired  
97with.

98       The phenomenon of frequency-matching is, however, the most remarkable  
99feature of auditory interactions we observed in pairs of *An. gambiae* mosquitoes.  
100Frequency-matching is defined here as the maintenance of a relatively constant ratio  
101( $\pm 1\%$ ; Experimental Procedures) between the fundamental WBFs of two mosquitoes  
102through continuous audio-motor feedback interactions between them, as shown in  
103Figure 1. The closest audible frequency shared by females and males of both  
104molecular forms occurs at the 3<sup>rd</sup> harmonic of the female and the 2<sup>nd</sup> harmonic of the  
105male, given that the basic ratio between male and female WBFs is  $\sim 1.5$  and the range  
106of sensitivity of *An. gambiae* antennae is  $< 2,000$  Hz when they are flying  $\sim 2$  cm  
107apart (see below, Frequency Tuning). On the basis of our definition for frequency-  
108matching, 92% of matching sequences in our records occurred at the 3:2 harmonic  
109frequency, with matching frequencies that differed by  $< 22$  Hz (see discussion of  
110‘difference tones’ below).

111       Samples of male-female pairs of M and S form mosquitoes matching at a ratio  
112of 3:2 shown in Figure 1A & B illustrate our finding that the absolute mean matching  
113frequency is variable, unique to each interaction, and can change during a matching  
114sequence with one mosquito frequency-tracking the other. For example, in Figure 1A  
115& B the pairs of mosquitoes frequency-match for a few seconds at a time (light  
116coloured regions), reducing the variability in their respective WBFs when the ratio  
117between them is close to 3:2, but when they come back together after breaking apart,  
118the mean matching frequency has generally changed. Fine time-scale interactions are  
119shown in Figure 1C & D to illustrate the ability of mosquitoes to respond to changes

120in each other's WBF on a moment-to-moment basis with a brief (~50 – 60 ms) delay.  
121It is worth noting that both males and females actively respond to the other during  
122these interactions.

123 To accommodate this variability in behaviour between individual mosquitoes,  
124we developed a set of criteria for scoring the frequency-matching status of each  
125record, based on a minimum proportion of the record with matching and a minimum  
126duration of matching (frequency-match for > 20% of a record *and* for > 1s,  
127Experimental Procedures). Hence, based on the definition of frequency-matching,  
128the M-form pair in Figure 1 A matched for 25.5% (2.8 s) of the 11.0 s record, the S-  
129form pair in Figure 1B matched for 38.0% (4.9 s) of the 13.0 s record, the mixed-form  
130pair (S female-M male) in Figure 1E matched for only 5.4 % (0.7 s) of the 13.0 s  
131record, and the M female-S male pair in Figure 1F matched for 4.0% (0.5 s) of the  
13213.0 s record. Based on our set of scoring criteria, the pairs in A and B scored  
133'positive' and the pairs in E and F scored 'negative' for frequency-matching.

134 The results of this analysis show that frequency-matching occurred  
135significantly more often in same-form pairs (14 out of 24 pairs) than in mixed-form  
136pairs (2 out of 20 pairs) ( $\chi^2= 11.013$ ;  $df =1$ ;  $P=0.001$ ), thus demonstrating the  
137capability of M and S form mosquitoes to discriminate between 'same' and 'other'  
138form to a greater level of accuracy than any other adult phenotype assay described so  
139far [16].

140 Why does frequency-matching occur more often in same-form pairs? We  
141have evidence of physiological and behavioural factors that may potentiate sustained  
142frequency-matching in same-form pairs. The relative wing-beat frequencies of M and  
143S males and females at higher harmonics may constrain the range of possible WBF  
144ratios within mixed-form pairs. M-form pairs frequency-matched at significantly

145higher frequencies than S-form pairs (Fig 2A thick symbols, M-form =  $1510 \pm 78$  Hz  
146v. S-form  $1440 \pm 72$  Hz,  $F=9.347$ ;  $df= 1,10$ ;  $P=0.0121$ , Experimental Procedures).

147 Evidently there is a mechanism, or behavioural strategy yet to be identified,  
148that favours same-form frequency-matching. For example, having increased their  
149mean WBFs on hearing the sound of a nearby mosquito, if M-form males then  
150decrease and M-form females further increase their respective mean WBFs, they  
151would increase the likelihood of frequency-matching, whereas the reverse is true for  
152S-form mosquitoes (after the initial increase in WBFs, females decrease and males  
153further increase their respective WBFs to match). Were each type of mosquito to  
154respond always as if it were flying in a same-form pair, the chance of frequency-  
155matching in mixed-form pairs would be much reduced because the difference between  
156their respective 3:2 WBFs would increase.

157 Previous attempts to detect potential mate recognition characteristics in the  
158mean WBFs of *An. gambiae* species may have failed because WBFs were measured  
159only in solo flying mosquitoes. Our findings that mosquitoes increase the overall  
160frequency and variability of their wing beats when encountering others and the  
161potential importance of the relative WBFs of males and females at higher harmonic  
162ratios had not yet been appreciated[15, 17].

163 The interactive aspect of frequency-matching appears to be essential;  
164presentation of pure tones or pre-recorded mosquito flight tones to individual  
165tethered-flying *Anopheles* mosquitoes did not elicit frequency-matching in either  
166form. Analysis of factors controlling frequency-matching and subsequent mating  
167behaviour must be undertaken in free-flight experiments.

168 Frequency-matching may have evolved due to a selected advantage of mating  
169in free-flight; males are known to chase females by localising the source of their



170flight tone[17, 18], and frequency-matching at close-range would enable the relatively  
171small male to form a copula with the larger female in mid-flight by synchronising  
172with the potentially turbulent air stream generated by her wing beats[19, 20].

173 The findings presented here represent the first breakthrough in furthering our  
174understanding of mosquito mating interactions since Belton's analysis of male mate  
175localisation by sound > 35 years ago [18]. They are also the first documentation of  
176form-specific close-range interactions related to mating behaviour since Coluzzi first  
177put forward his theory of the evolution of reproductive isolation in diverging  
178populations [4, 21].

179

### 180**Frequency Tuning and Sensitivity of Mosquito Hearing**

181The physiological mechanism that controls frequency-matching is based on the  
182characteristics of one of the most sensitive hearing organs in the animal kingdom[7,  
18322-24]. Sounds are detected by the complex arrangement of sensillae (~15,000 in  
184males, ~7,500 in females) of the Johnston's organ (JO) in the pedicel of the antenna  
185(Figure 3A). The sensillae mechanoelectrically transduce and amplify the nanometre  
186displacements of the flagellum caused by the near-field component of sound[23, 24].  
187There is evidence for three species of mosquito, *Culex quinquefasciatus*[8], *Aedes*  
188*aegypti*[9] and now *An. gambiae*, that frequency-matching of flight tones occurs at  
189frequencies that are about three times higher than the fundamental WBF of females.  
190How do these high frequencies compare with the frequency bandwidth and tuning of  
191the flagellum and the JO? Male *An. gambiae* mosquitoes hydraulically extend and  
192collapse the fibrillae of their antennae[25] (Figure 3A, photo) on a diurnal cycle  
193linked to the swarming periods at dusk and dawn when mating occurs[17, 26]. These  
194mechanical changes in the antennae alter the response characteristics of the JO[27].

195Accordingly, we obtained antennal-mechanical and JO-receptor-potential frequency-  
196tuning-curves both during their diurnal phase of inactivity, when the fibrillae were  
197collapsed and at dusk when they were extended.

198 Mechanical threshold-tuning curves (0.2 nm criterion, noise floor 0.13 nm  
199R.M.S) measured with a laser-diode interferometer directed at the base of the  
200flagellum[28] from two male *An. gambiae* are shown in Figure 3B. With fibrillae  
201collapsed (solid symbols), the minima is at a frequency of  $235 \pm 14$  Hz and at a  
202particle velocity of  $4.1 \times 10^{-6} \pm 2.0 \times 10^{-7}$  ms<sup>-1</sup> (n = 7). With fibrillae extended (open  
203symbols), tuning shifts significantly upwards in frequency ( $P = 0.003$ ) to  $540 \pm 45$   
204Hz, but sensitivity is decreased to  $1.8 \times 10^{-5} \pm 5.7 \times 10^{-6}$  ms<sup>-1</sup> (n = 5), largely through  
205loss of the sensitive minima at ~ 200 Hz. Extension of the fibrillae is, therefore,  
206associated with an upwards shift in the most sensitive frequency of the antennae at the  
207expense of low-frequency mechanical sensitivity. Similar measurements from female  
208*An. gambiae* (red symbols, Figure 3B) did not reveal diurnal shifts in the sensitivity  
209and tuning of the flagellum (tuning frequency minima =  $209 \pm 33$  Hz; particle velocity  
210=  $1.4 \times 10^{-5} \pm 6.0 \times 10^{-6}$  ms<sup>-1</sup>, n = 5). The sensitivity and tuning of the female  
211flagellum, which was similar to that of the male's with collapsed fibrillae, had  
212noticeable and repeatable notches of sensitivity around the 1<sup>st</sup> and 2<sup>nd</sup> harmonics of the  
213male's flight tone (arrows, Figure 3B), similar to that reported for *Ae. aegypti* [22].  
214Accordingly, it can be observed from Figure 3B that the frequencies at which the  
215mosquitoes frequency-match are within the frequency range of the vibrations of the  
216flagellum (i.e., up to ~ 2,000 Hz at the particle velocity expected of mosquito wings  
217beating 2 cm away[20, B. Warren, unpublished]).

218 Voltage responses recorded from the JO are dominated by receptor currents  
219from the sensory cells (supplemental material S1) and henceforth, in this paper they

220will be referred to as compound phasic receptor potentials. The phasic receptor  
221potentials are twice the frequency ( $2f$ ) of the applied sound stimulus[29-31] and  
222preserve the temporal information necessary for frequency-matching[8]. Threshold  
223receptor-potential frequency-tuning curves (criterion = 1.4 times recording noise  
224floor,  $19.3 \mu\text{V R.M.S}$ ) are shown in Figure 3C. With fibrillae collapsed (solid  
225symbols), the minima frequency is  $200 \pm 15 \text{ Hz}$  (particle velocity =  $1.0 \times 10^{-6} \pm 9.1 \times$   
226 $10^{-7} \text{ ms}^{-1}$ ,  $n = 4$ ). With fibrillae extended (open symbols), tuning shifts upwards ( $300 \pm$   
227 $25 \text{ Hz}$ ) with increased sensitivity ( $1.5 \times 10^{-7} \pm 6.2 \times 10^{-8} \text{ ms}^{-1}$ ,  $n = 4$ ). In contrast to the  
228frequency range of the flagellum vibrations, the frequencies at which the mosquitoes  
229match their flight-tones is outside the bandwidth of the JO phasic receptor potentials  
230and thus outside the auditory range of *An. gambiae* mosquitoes. It has been reported  
231for *Ae. aegypti* [9] that the auditory range of the DC component of the JO receptor  
232potential extends far above that of the phasic response and encompasses the  
233frequency-matching range. We measured DC components of the receptor potential  
234and plotted DC frequency tuning curves (insets to Figure 3C). We also plotted DC  
235component tuning curves for *Cx. pipiens* mosquitoes (supplemental material S2). It is  
236clear from our findings that DC component frequency tuning curves are bounded by  
237the phasic receptor potential tuning and do not extend the auditory range of the JO.

238       Changes in the frequency tuning and sensitivity of the JO during extension of  
239fibrillae are complex and may not entirely be due to mechanical changes in the  
240flagellum. The electrical responses of the JO and mechanical responses of the  
241flagellum are metabolically vulnerable when the fibrillae are extended, and can  
242collapse within 5 min when disturbed by experimental procedures. It would be  
243interesting to discover if there is metabolic enhancement of the sensitivity of the JO  
244during the increased hydrostatic pressure that causes erection of the fibrillae.

245 We conclude that *An. gambiae* match their flight tones at frequencies that are  
246 outside the bandwidth of the JO's phasic responses to acoustic stimulation. It appears  
247 that the near-field auditory systems of *Anopheles* and *Culex* species[8] are similar.  
248 Each consists of a broadly-tuned, non-linear, detector (flagellum) that oscillates  
249 spontaneously at frequencies close to the female's WBF and can detect, through  
250 distortion, the higher harmonics of the flight tones. When pairs of tones (frequencies  
251  $f_1$  and  $f_2$ , or the mosquito's own flight tones and those of the other) are presented  
252 simultaneously, the flagellum generates distortion products, including one at the  
253 difference frequency ( $f_2 - f_1$ ), as can be seen in the amplitude spectra measured from  
254 the vibrations of the flagellum (Figure 4A,C,D). This difference tone is detected by  
255 the receptors of the JO even though the stimulus tones are beyond the frequency range  
256 of the JO and cannot be detected by it (Figure 4B,E,F). It is essential for the purpose  
257 of frequency-matching that difference tones can be generated at low frequencies by  
258 the flagellum and be detected by the JO. The JO can, for example, generate a strong  
259 difference tone at 12 Hz in response to pairs of tones at 1399 and 1411 Hz (Figure  
260 4C), which is within the frequency-matching range when in free flight and at stimulus  
261 levels equivalent to the flight tones mosquitoes produce when 10 mm apart[22]. We  
262 detected difference tones at 22 Hz in the receptor potentials (Figure 4E), which is  
263 similar to the magnitude of the differences in frequency between two mosquitoes  
264 when frequency-matching. Difference tones at lower frequencies were masked by  
265 low-frequency electrical noise that is generated in the JO. The basis of this noise was  
266 beyond the scope of this study, but a strong candidate is the pulsating antennal  
267 heart[32].

268 The detection of difference tones provides mosquitoes with a strategy for  
269 matching the harmonic components of their flight tones at frequencies they cannot

270hear (Figure 3B). By analogy with violinists who tune their instruments by detecting  
271beats, mosquitoes adjust their wing-beat frequencies to within a few Hz of each other  
272until the difference tones drop in frequency and disappear when the harmonics are  
273perfectly matched.

274

## 275**Conclusions**

276We report here the first quantifiable means of discriminating two molecular forms of  
277adult *An. gambiae* s.s. on the basis of an observed behaviour. This behaviour uses the  
278detection of difference tones as the basis of audio-motor interactions that occur  
279reliably between a male and a virgin female of the same form. The discovery of this  
280potential mate recognition mechanism constitutes the first evidence of a critically  
281necessary, albeit not sufficient, step in the process of assortative mating at close-  
282range, which is known to occur in this species complex. Our discovery of a mating-  
283related phenotype that is associated with genotype in the *An. gambiae* complex also  
284represents a breakthrough in research on how reproductive isolation can occur in  
285sympatric populations of incipient species.

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289

## 290**Experimental Procedures**

291

292Mosquito larvae were collected from breeding sites typical of the respective forms; M  
293form from rice paddies (VK7 village) and S form from rain-fed pools (Soumousso  
294village), and identified to form-level by PCR[33] at the end of experiments. Flight

295 tones were recorded with a particle velocity microphone[22] located within 2.0 cm of  
296 tethered mosquitoes[8] and equidistant between them when two mosquitoes were  
297 flown together (Figure 1). Factors known to affect wing-beat frequency[15] were  
298 controlled for[8]. Behavioural and biophysical experiments were conducted on 4 – 7-  
299 day old males and virgin females only during the 2 hours preceding dusk (period of  
300 inactivity) and the 2 hours following the onset of dusk (peak of maximum activity).

301       Methods for generating stimulus tones, recording flight tones from tethered  
302 flying mosquitoes, making and analysing mechanical measurements of the flagellum  
303 of the antenna with a self-mixing laser diode interferometer[28] and  
304 electrophysiological measurements from the JO, were as described previously[8].  
305 Measurements were made within half an hour of preparation because sensitivity,  
306 distortion products and spontaneous emissions usually deteriorated or disappeared  
307 after this period.

308       Fundamental WBFs were digitized from recordings[8] of duration = 4–18 s,  
309 (mean = 8.7 s), and analysed by three-way ANOVA (df= 1,101), followed by Tukey's  
310 honest significant difference (HSD) test for multiple comparisons of means to test  
311 effect of sex, form and solo vs paired flight on the mean WBF and associated  
312 interquartile ranges (IQR). Mean WBF data for frequency-matching same-form pairs  
313 were analysed using a linear model in R [32], which produced a significant difference  
314 between the weighted WBF means the M and S form pairs ( $F=9.347$ , d.f.= 1,10,  
315  $P=0.0121$ ). A Q-Q plot for the model of standardised residuals against theoretical  
316 quantiles showed a reasonable fit to the straight line, and a symmetrical distribution of  
317 points above and below the line.

318       'Frequency-matching' defined as a harmonic-based integer ratio between the  
319 fundamental WBFs of two mosquitoes  $\pm 1\%$  (i.e.,  $\pm 0.02$ ; since the range of values =

3200 - 2). 'Positive' score for frequency-matching ( $> 20\%$  *and*  $>1$  second) based  
321on analysis of the frequency distributions of the proportion and duration of records  
322that contained frequency-matching, which showed two overlapping curves for same-  
323and mixed-form data in proportion frequency-matching, with a clear breakpoint at  
324'20% of record matching'. To avoid false positives when scoring frequency-matching  
325due to multiple short bursts and crossing-over, a second criterion was added, that  
326required matching for  $> 1$  s, based on the frequency distribution of matching duration,  
327which showed that all but two records had matching sequences that lasted more than 1  
328s, and matching in these two records was mainly due to cross-over matching.

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433

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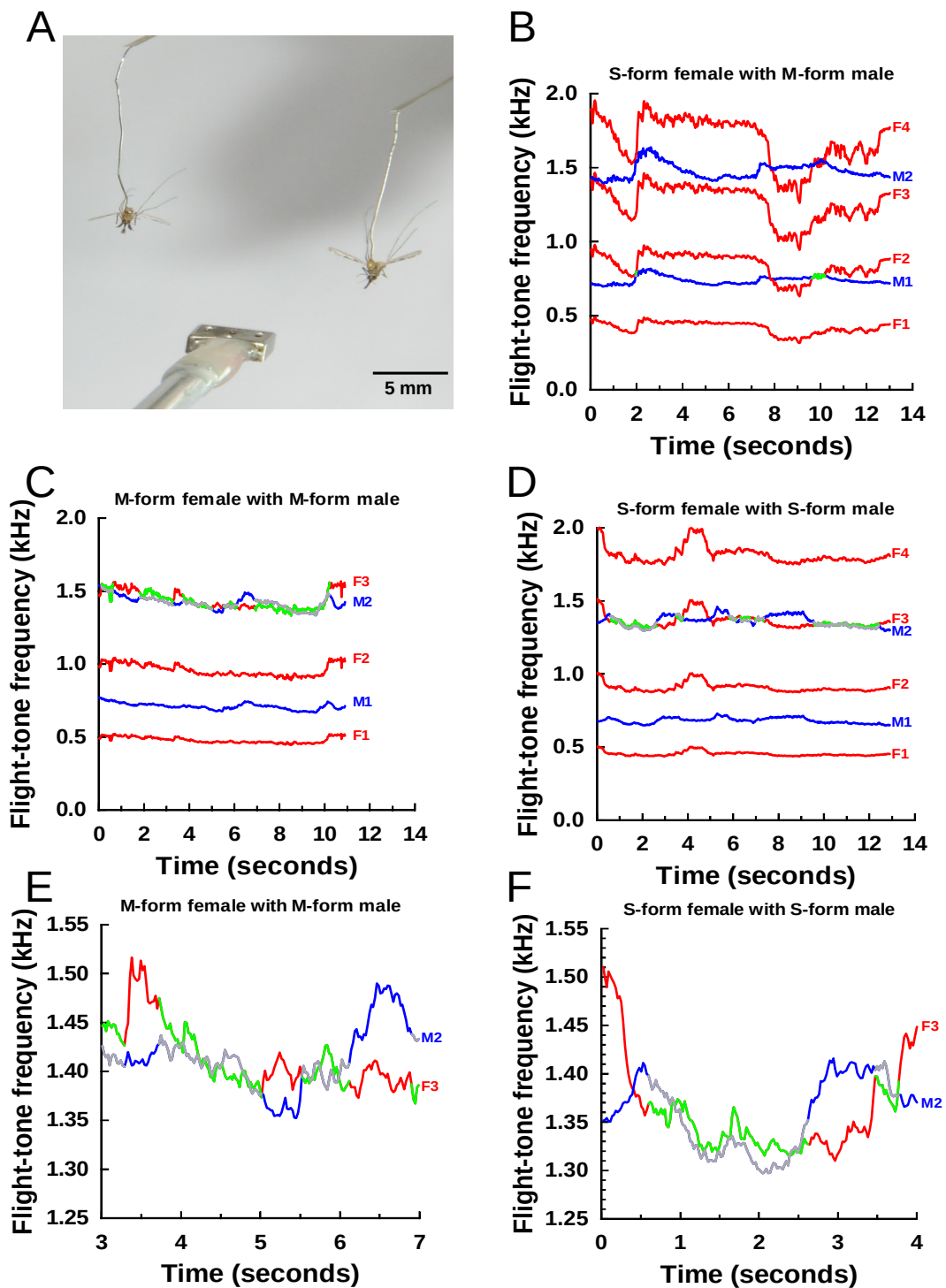
#### 443**Author information**

444Correspondence on behaviour should be addressed to G.G. ([g.gibson@gre.ac.uk](mailto:g.gibson@gre.ac.uk)) and  
445on neurophysiology should be addressed to I.J.R. ([i.j.russell@sussex.ac.uk](mailto:i.j.russell@sussex.ac.uk)).

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448 **Figure captions**



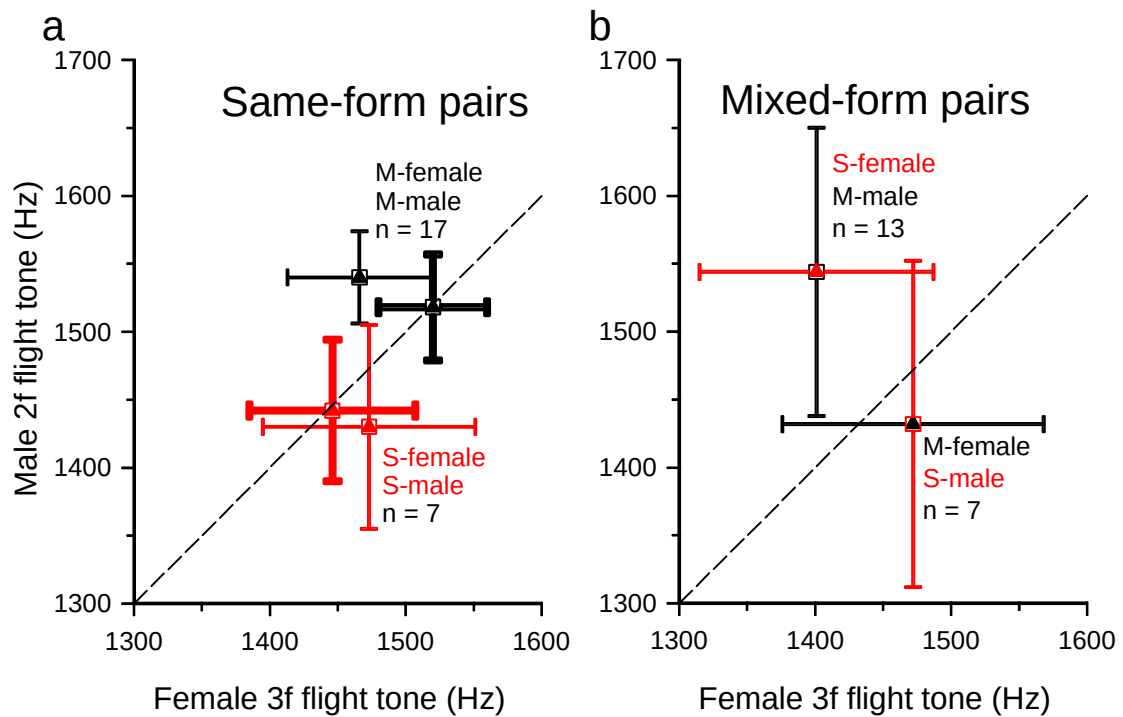
450 **Figure 1 Auditory interactions between tethered flying mosquitoes.**

451 Inset: Arrangement of particle velocity microphone and tethered mosquitoes during

452 sound recordings; A - F) Spectrograms (reconstructed from digitised fundamental

453frequencies) of flight tones with harmonics of males (blue) and females (red) and  
454periods of frequency matching (grey: male, green: female). A & B) Same-form pairs  
455of M-form and S-form mosquitoes, respectively, showing extended frequency-  
456matching (pale colours), when the female's 3<sup>rd</sup> and the male's 2<sup>nd</sup> harmonics converge,  
457at a ratio between their fundamental wing beat frequencies of 3:2 (i.e., 1.5, a  
458harmonic-based ratio); C & D) Expanded views of 4 s of the spectrograms of A and  
459B, respectively, showing periods of frequency-matching between the female's 3<sup>rd</sup> and  
460male's 2<sup>nd</sup> harmonics of their flight tones. E & F) Mixed-form pairs, S female & M  
461male and M male & S female, respectively, showing only transient periods of  
462frequency-matching between harmonics. The ratio between their fundamental wing-  
463beat frequencies does not stabilise at a harmonic-based value.

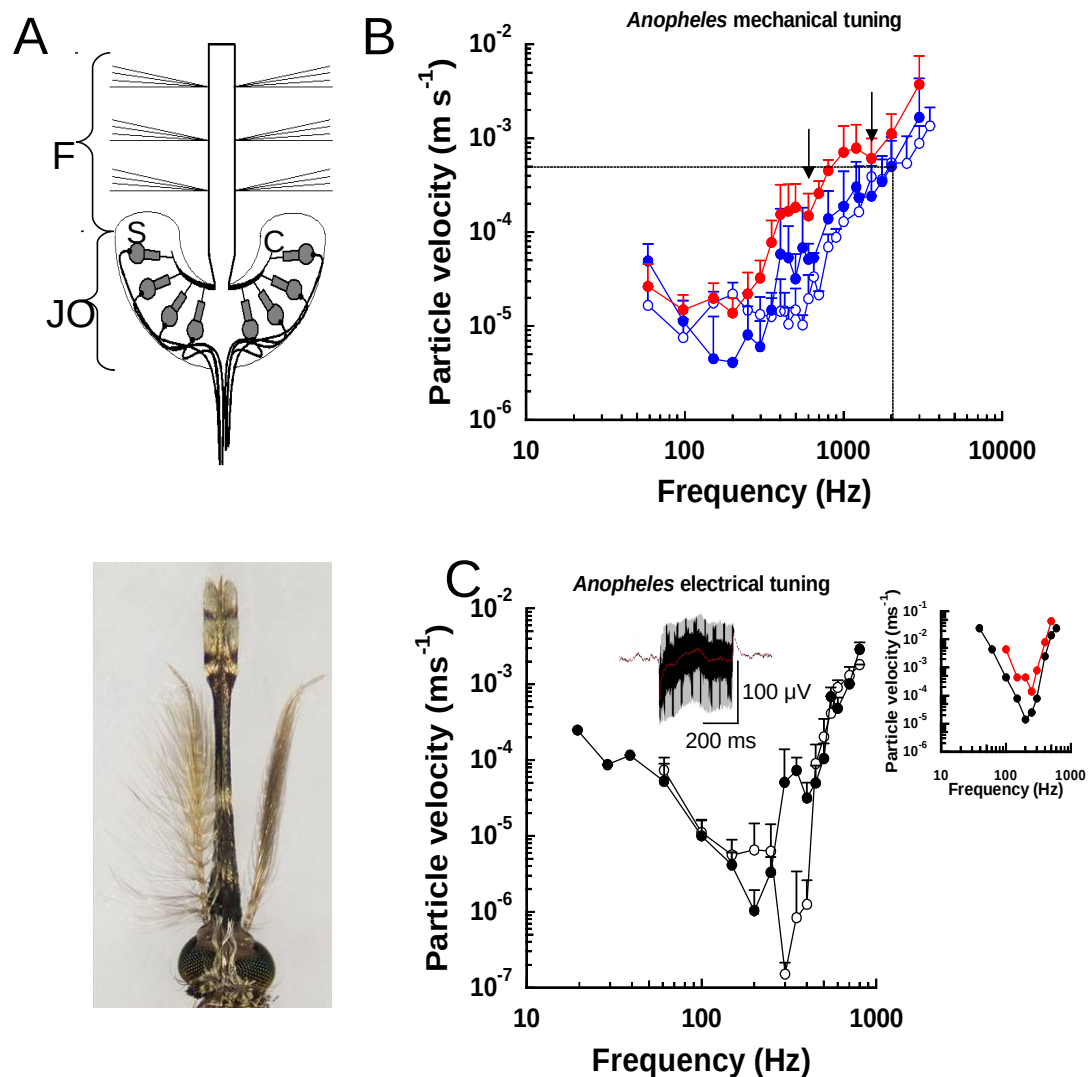
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465 **Figure 2 Comparison of the mean wing-beat frequencies of same-form and**  
 466 **mixed-form pairs of *Anopheles gambiae* M and S form mosquitoes at the 3:2**  
 467 **harmonic frequencies where most matching occurs.**

468 A) Same-form pairs: male (square) mean  $\pm$  standard deviation wing-beat frequency  
 469 plotted against female (triangle) mean wing beat frequency, for M form (black) and S  
 470 form (red) pairs during frequency-matching (thick lines) and non-matching (thin)  
 471 sequences. Dotted line denotes slope=1, i.e., perfect frequency-matching;

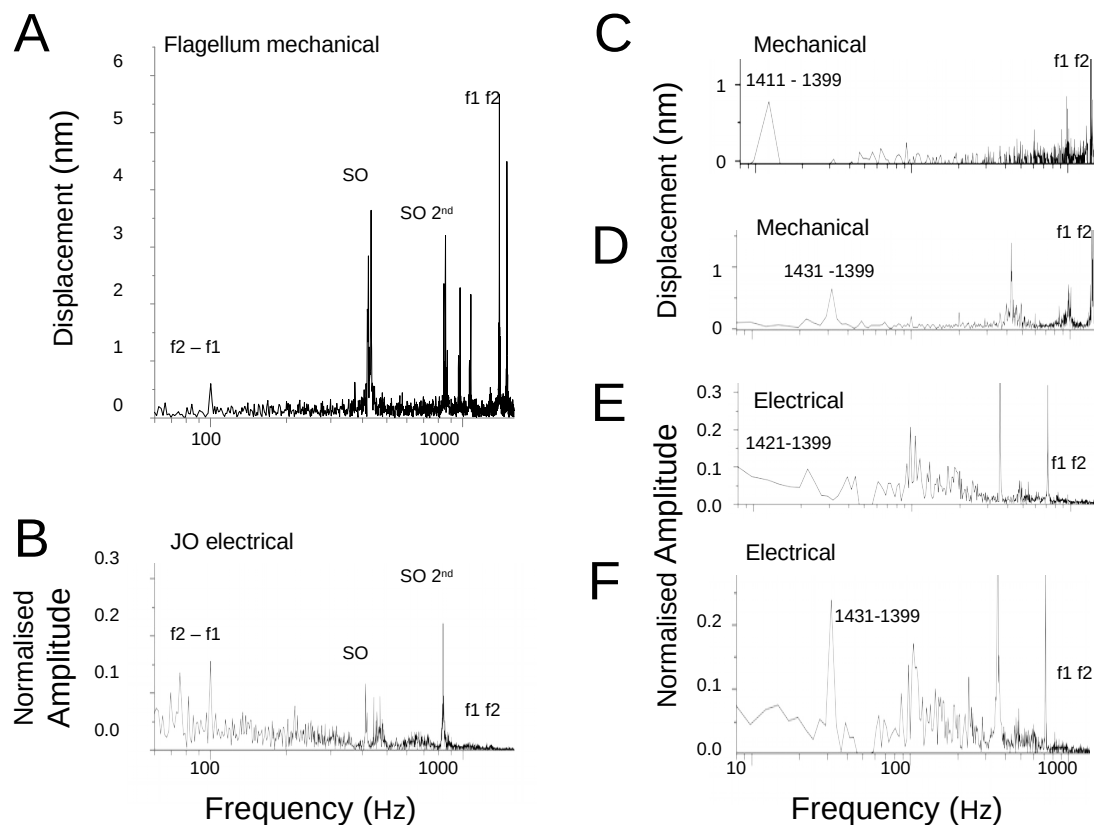
472 B) Mixed-form pairs: mean wing beat frequencies of non-matching S female & M  
 473 male pairs and M female & S male pairs.



475 **Figure 3 Mechanical and receptor-potential tuning-curves, from the flagellum**  
 476 **and Johnston's organ, respectively, of *Anopheles gambiae* (M form) mosquitoes.**  
 477 A) Schematic cross-section of mosquito antenna; flagellum (F) inserted into cup-  
 478 shaped pedicel that houses complex arrangement of cuticular processes (C) and  
 479 attached, mechanosensory scolopidia (S) of the Johnston's organ (JO)[31] and a  
 480 photomontage of male *An. gambiae* mosquito head; fibrillae extended (left, active  
 481 phase; dusk) and collapsed (right, inactive phase); B) Mechanical threshold  
 482 frequency tuning curve (mean and  $\pm$  standard deviation, vertical bars) measured from  
 483 base of flagellum in male mosquitoes (blue) with collapsed (solid) and extended (open

484symbols) fibrillae and female mosquitoes (red, solid). Arrows indicate sensitivity  
485peaks at 700 and 1400 Hz. Dotted lines indicate flight tone at highest frequency  
486mosquitoes are likely to encounter, and to which antennae can respond [23];  
487C) Main figure: Compound phasic (2f) receptor potential frequency tuning curves  
488(mean and  $\pm$  standard deviation, vertical bars) measured from JO of male mosquito  
489with collapsed (solid) and extended (open symbols) fibrillae; inset: receptor potential  
490(grey) with DC component (red) from a male with collapsed fibrillae in response to a  
491300 Hz tone, particle velocity  $0.0011 \text{ ms}^{-1}$ ; right: receptor potential tuning curves  
492derived from the 2f component (black) and DC component (red) of the receptor  
493potential.



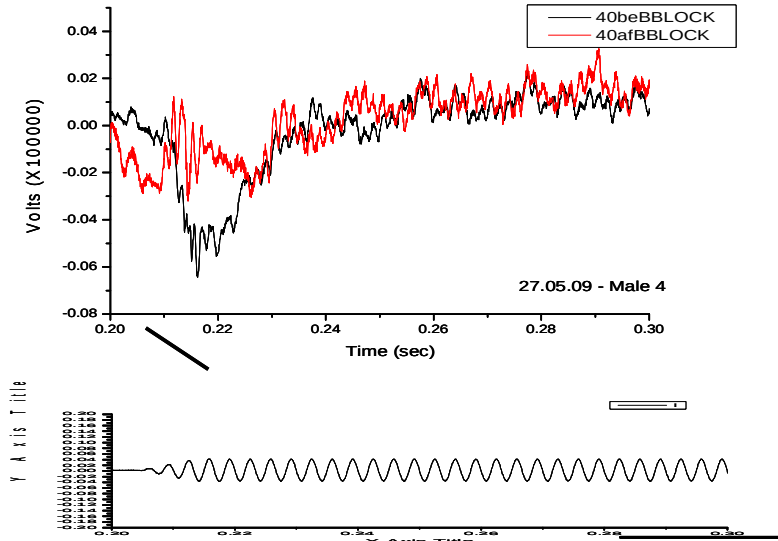


496 **Figure 4** Difference tones generated in the vibrations of the flagellum and  
 497 detected in the receptor potentials of the Johnston's organ of *Anopheles gambiae*  
 498 (M form) mosquitoes.

499 A) Recordings from male *An. gambiae* of amplitude spectra of flagellum vibrations  
 500 and B) JO compound receptor potentials (lower) in response to a pair of tones at 1399  
 501 Hz ( $f_1$ ) and 1499 Hz ( $f_2$ ), both at a particle velocity of  $0.0011 \text{ ms}^{-1}$ . C – F) Difference  
 502 tones in mechanical (flagellum) (C, D) and electrical (JO) (E, F) spectra in response to  
 503 tones at the frequencies indicated with particle velocities of  $0.005 \text{ ms}^{-1}$ . Responses to  
 504 the primary tones ( $f_1$  and  $f_2$ ) are seen in the mechanical but not in the electrical  
 505 responses. Difference tone ( $f_2 - f_1$ ) and spontaneous oscillation (SO) responses are  
 506 seen in both the mechanical and electrical spectra. The tones  $f_1$  and  $f_2$  also interact  
 507 with the SOs to produce further distortion products (A).

508**S1. The effect, on the compound JO potential of of an M-form male**  
 509***Anopheles gambiae*, of injecting TTX into the thoracic haemolymph**  
 510**sufficient to block the mosquito's observable motor activity.**  
 511

Time trace showing the electrical response of the JO to a 300.3 Hz tone at 5.58873E-4 m/s before and after diffusion of TTX into the mosquito body



40  $\mu\text{V}$

20 ms

512  
 513

514 Voltage responses recorded from JO of male M-type *Anopheles gambiae*

515 mosquito in response to a 300.3 Hz tone (particle velocity  $0.56 \text{ mms}^{-1}$ ) before

516 (black) and after (red) injecting  $1 \mu\text{M}$  TTX in insect saline (ref) into the thorax.

517 We assumed that the TTX had blocked neural activity when we no longer

518 observed spontaneous, reflexive, or mechanically evoked motor responses

519 from the palps, legs and wings. Note the phasic (2f) and DC (negative

520 baseline shift) components of the voltage response remains unchanged but

521 the initial negative peak (arrow) is greatly reduced following TTX injection. We

522 conclude that the voltage response recorded from the JO is dominated by the

523 compound receptor current of the sensory cells but the compound potential at

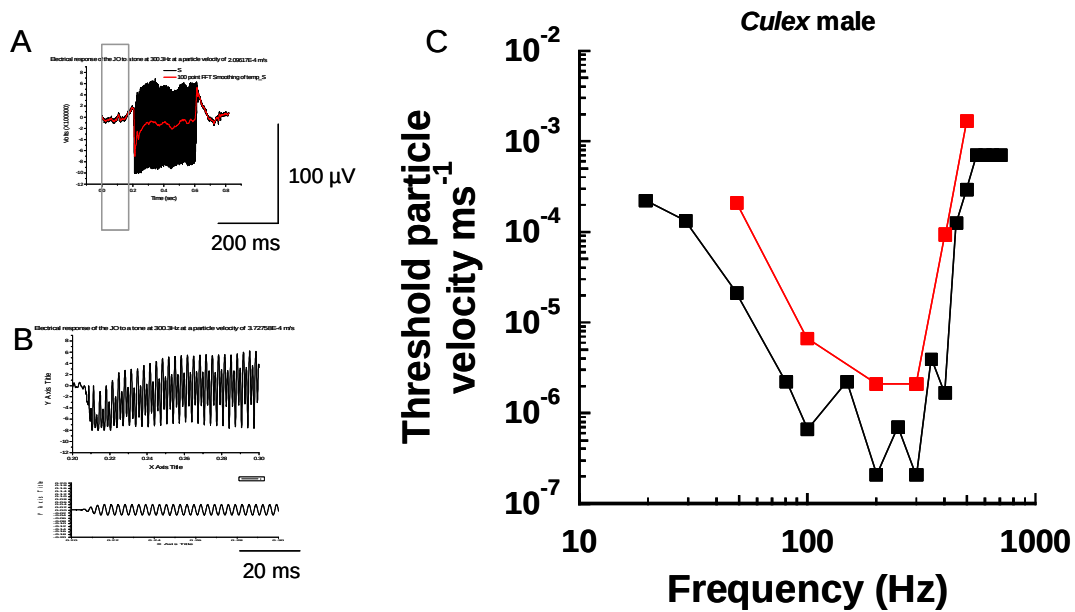
524 the onset of the voltage response to the tone is dominated by compound

525 neural responses. Extracellular potentials in response to tones recorded from

526 the JO are, therefore, similar to those recorded from the cochlea. Extracellular

529voltage responses recorded from the cochlea are dominated by receptor  
530currents from the outer hair cells and synchronised firing of nerve fibre  
531contribute to a compound action potential at the onset of the voltage  
532response.

533 **S2. DC and phasic voltage responses and frequency tuning curves**  
 534 recorded from the Johnston's organ of a male *Culex pipiens* mosquito  
 535



536  
 537

538 A, B Compound electrical responses (expanded view in B) recorded from the  
 539 JO in response to a 300 Hz tone (particle velocity,  $4.0 \mu\text{m s}^{-1}$ ). The red,  
 540 superimposed trace is the DC component of the voltage response. C)  
 541 Compound receptor potential (2f) (black) and DC receptor potential (red)  
 542 threshold tuning curves recorded from the JO of a male mosquito based on  
 543 the particle velocity necessary to produce a receptor potential 10 dB above  
 544 the recording noise floor.