

1 **Masking of an auditory behaviour reveals how male mosquitoes use distortion to detect**
2 **females**

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10 **Keywords**

11 Insect hearing; Johnston's organ; *Culex quinquefasciatus*; Distortion products; Frequency
12 tuning; Acoustic behaviour; Phonotaxis; Rapid frequency modulation behaviour.

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14

15 **Abstract**

16 The mating behaviour of many mosquito species is mediated essentially by sound: males
17 follow and mate with a female in mid-flight by detecting and tracking the whine of her flight-
18 tones. The recent discovery of a stereotypical male behaviour (Rapid Frequency Modulation,
19 RFM), initiated in response to the detection of the female's flight-tones, has provided a
20 means of investigating these auditory mechanisms while the mosquitoes are free-flying.
21 Mosquitoes hear with their antennae, which vibrate to near-field acoustic excitation. The
22 antennae generate nonlinear vibrations (distortion products, DPs) at frequencies that are equal
23 to the difference between the two simultaneously presented tones, e.g. the male and female
24 flight-tones, which are detected by mechanoreceptors in the auditory (Johnston's) organ (JO)
25 at the base of the antenna. Recent studies showed the male mosquito's JO is tuned not to the

26 female flight-tone, but to the frequency difference between the male and female flight-tones.
27 To test this hypothesis males were presented simultaneously with a female flight-tone and a
28 masking tone, which should suppress the male's acoustic RFM response to sound. The free-
29 flight behavioural and *in vivo* electrophysiological experiments reported here revealed that
30 acoustic masking suppresses the RFM response to the female's flight-tones by attenuating the
31 DPs generated in the nonlinear vibration of the antennae. These findings provide direct
32 evidence in support of the hypothesis that male mosquitoes detect females when both are in
33 flight through difference tones generated in the vibrations of their antennae due to interaction
34 between their own flight-tones and those of a female.

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36

37 **Introduction**

38 Acoustic masking has been used as a tool to measure frequency selectivity of the auditory
39 system at all levels from the receptor to behaviour in mammals, including humans [1, 2].
40 More recently it has been used to study the sensory and neural mechanisms of acoustic
41 detection in insects with tympanic hearing, such as in crickets or *Ormia* flies [3-8]. Here we
42 use tone-on-tone acoustic masking to investigate the hearing mechanisms of the mosquito
43 *Culex quinquefasciatus*, an insect with antennal hearing. We used simultaneous acoustic
44 masking of a stereotypical, acoustically-driven behaviour – Rapid Frequency Modulation
45 (RFM) – [9, 10] and of the electrical responses of the auditory Johnston's organ (JO) [9, 11-
46 14], to explore what it is that male mosquitoes listen to when they detect the flight-tones of
47 females and if their behaviour is determined by the nonlinear properties and frequency
48 responses of the JO.

49 Mosquitoes detect the near-field component of sound (air particle displacement)
50 through vibrations of the antennae [15, 16]. Antennal vibrations are detected by and

51 transduced into electrical signals by several thousand mechanosensory scolopidia, which
52 compose the JO housed in the pedicel at the base of each of the antennae [17]. The mechanics
53 of the antenna are nonlinear; it behaves as a rod that becomes stiffer with increasing
54 displacement [11]. When the antenna is vibrated by two tones, it generates a strong distortion
55 product (vibration) at a frequency that is the arithmetic difference of these two tones,
56 including those that mimic the wing beat frequency (WBF) of male and female mosquitoes
57 [11-13]. Largely because of this nonlinearity, the male JO is tuned overall to detect the
58 difference in frequency between the male's own WBF and that of the female [9, 11-14]. The
59 bandwidths of the sensory receptors of the JO set upper frequency limits on the phasic
60 electrical responses that can be recorded from the JO [9, 11-16]. Male and female WBFs are
61 above the frequency bandwidth of the JO. It is therefore hypothesized that male mosquitoes
62 must fly to detect and locate females through listening to difference tones [9, 11-14].

63 It has long been established that male mosquitoes are attracted by female flight-tones
64 [18-25]. In the case of *C. quinquefasciatus*, males aggregate over visual markers, forming
65 swarms at dawn and dusk [26, 27]. Unmated females approach established swarms,
66 whereupon flying males detect the female flight-tones and a mating chase ensues [17, 26, 27].
67 Male mosquitoes are also attracted to artificial sound sources emitting pure tones that
68 simulate the flight-tones of a conspecific female mosquito [9, 10, 22-25]. Recently, we
69 reported that free-flying male mosquitoes in two different taxonomic sub-families, *C.*
70 *quinquefasciatus* [9] and *Anopheles gambiae s.l.* [10], exhibit the stereotypical RMF
71 behaviour in response to the fundamental frequency of female flight-tones. The RFM
72 behaviour is defined by its spectrographic characteristics and by the flight path of the flying
73 male; it consists of a steep increase in the male's WBF concomitant with fast phonotactic
74 flight towards the female (or artificial sound source), followed by rapid modulation of their
75 WBF when in the immediate vicinity of a female or the female-like sound source [9]. RFM is

76 performed only when the male mosquito has detected, located and reached (within ~5 cm) the
77 sound source and represents an acoustic measure of a mating attempt by the male [9, 10].
78 Thus, this pre-copulatory behaviour provides an acoustic assay with which to monitor the
79 conditions under which a male responds to a sound source emitting a female flight-tone.

80 The outcome of the free-flight behavioural and *in vivo* electrophysiological
81 experiments reported here indicate that acoustic masking is caused through suppression of the
82 DPs generated in the non-linear vibration of the antennae. Our findings provide direct
83 behavioural evidence in support of the hypothesis that male mosquitoes detect females when
84 both are in flight by detecting difference tones generated in the vibrations of their antennae
85 through the interaction between their own flight-tones and those of a female.

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87

88 **Materials and Methods**

89 *Mosquitoes*

90 Colonies of *Culex quinquefasciatus* Say (Muheza strain) were reared in controlled-
91 environment chambers; 70-75% rH, 26±2°C and 12 h light: 12 h dark cycles. Larvae were
92 reared on cat food pellets (Purina® PetCare, Gatwick, UK) and adults were provided with
93 10 % sugar solution *ad libitum*. Larval density was ~70 l⁻¹ of water. Experiments were done
94 with adult male mosquitoes between 4-14 days post-emergence and during the first 3 h of the
95 scotophase, when mating behaviour occurs under natural conditions.

96

97 *Behavioural experiments*

98 The set-up used to record the acoustic behaviour of free-flying mosquitoes consisted of a 30
99 cm sided metal-framed cube covered by white cotton gauze (the flight arena) placed on a
100 vibration-damped table (Newport®, Irvine, CA, USA) inside a sound attenuated booth (IAC

101 Ltd, Winchester, UK). Acoustic stimulation consisted of two different pure-tones – a probe
102 tone and a masking tone – delivered simultaneously to the flight arena from two different
103 sound sources. The probe tone was delivered to the centre of the arena through a calibrated
104 sound source consisting of a 0.5 cm diameter plastic probe tip, damped with acoustic foam,
105 connected via a 1 cm diameter polythene tube to an adapted Audio Techniques[®] ATH
106 A700AX speaker. The masking tone was delivered from a large calibrated Beyerdynamics[®]
107 DT 770 speaker placed on a side wall of the arena at a distance of 15 cm from the probe
108 speaker. In Experiment 1 the sounds produced by the two speakers and the male mosquitoes
109 inside the arena were recorded using a calibrated [15] particle velocity microphone (Knowles
110 NR-3158, Itasca IL, USA) located ~2 cm from the probe speaker tip and a pressure
111 microphone (Knowles 23132, Itasca IL, USA) mounted at the focal point of an 18” parabolic
112 reflector (Edmunds) and located on one side of the cage. Because some males approached the
113 probe speaker and others approached the masking speaker, the set-up was then altered. Given
114 that our set-up was limited to 2-channel sound acquisition, in Experiment 2 the parabolic
115 microphone was substituted with a second calibrated particle velocity microphone placed ~2
116 cm in front of the centre of the masking speaker. The acquired signals were amplified x100
117 with a purpose built 2-channel preamplifier and digitized with a Fireface[®] UC sound card
118 (sampling rate: 192 kHz). Digital sound outputs were recorded and analyzed using
119 Spectrogram 16 (Visualization Software, LLC).

120 Probe and masking tones were produced using the sine wave function of Test Tone
121 Generator 4.4 (EsserAudio[®], 2011) and cosine windowed to avoid acoustic transients. All
122 tone presentations lasted for 10 s and the interval between presentations was ~5 s. Three
123 different probe tone frequencies were used, all within the stimulus range for eliciting an RFM
124 response in males [9]: 340 Hz, which is within the 10 dB bandwidth of the JO; 400 Hz,
125 within the best frequency of the male’s behavioural audiogram, and; 450 Hz, within the

126 higher frequency range of free-flying females [9]. The particle velocity of the probe tones
127 was set, using a calibrated particle velocity microphone, to be $5.7 \times 10^{-5} \text{ ms}^{-1}$ at a reference
128 distance of 2 cm, which is similar to the sound intensity produced by tethered-flying females
129 at the same distance [9]. The frequencies of the masking pure tones varied according to the
130 experiments and probe tones used (Supplemental Tables 1 and 2), ranging between 100-1000
131 Hz. The masking sound source was set to deliver a particle velocity of $\sim 8 \times 10^{-5} \text{ ms}^{-1}$ at a
132 reference distance of 2 cm.

133 One to four male mosquitoes were placed inside the flight arena and after ~ 10 min
134 period of adaptation, the mosquitoes started to fly spontaneously, whereupon sound recording
135 and stimuli presentation were initiated. Experiments consisted of the simultaneous
136 presentation of a probe tone and a masking tone. Control treatments consisted of stimulation
137 with the probe tone alone (i.e. without a masking tone present) to confirm that males
138 consistently exhibited the RFM response when in close proximity of a sound source emitting
139 a female-like tone [9]. Males were stimulated successively with different tone pairs
140 (presented pseudo-randomly to avoid repetition) until they stopped flying.

141 The RFM acoustic response was used to indicate whether or not a male mosquito
142 detected the presence of a female-like tone. Masking experiments were designed to quantify
143 the occurrence of RFM response in the presence of two simultaneous tones. Simultaneous
144 presentation of two tones (the probe tone and the masking tone) stimulated males to express
145 one of three responses: 1) males flew toward the probe speaker and initiated RFM within ~ 5
146 cm of the probe speaker, 2) males showed no conspicuous response to either speaker, or 3)
147 males flew toward the masking speaker and initiated RFM towards the masking speaker
148 (Figure S1). The spectrograms of all acoustic outputs were monitored in real time and
149 recorded for later analysis. An RFM response was observed towards a particular speaker
150 when, upon acoustic stimulation, the spectrogram showed the stereotypical frequency

151 modulation of a male's WBF [9]. Moreover, the sound level during RFM was observed to be
152 20-30 dB higher than that during normal flight in the vicinity of the speaker it directs the
153 behaviour towards (Figure S1). Occasionally (<5% of the records), the presentation of a tone
154 pair elicited an RFM response to both speakers; in this situation, we registered the response
155 towards the first speaker to which the male directed his response. For each probe/masking
156 tone pair, the proportion of RFM response was calculated by dividing the number of observed
157 RFM responses by the number of tone pair presentations. Two behavioural experiments were
158 conducted; in Experiment 1, due to the microphone arrangement, only the RFM responses
159 towards the probe speaker were quantified, whereas in Experiment 2, both speakers were
160 monitored. In Experiment 1, each probe/masking tone combination was tested 26 times using
161 a total of 36 male mosquitoes, while in Experiment 2, each pair tone was tested 32 times
162 using a total of 47 males.

163 The proportion of RFM responses to the probe tone alone provided the extrinsic null
164 hypothesis used in G-tests for goodness-of-fit to test the effect of the experimental
165 probe/masking tone pairings on the proportion of response [28]. G-tests for independence
166 were used to compare different probe tones, while comparisons within the same probe tone
167 were performed using G-tests for goodness-of-fit.

168

169 *Electrophysiology*

170 Mosquitoes were immobilized by cold narcosis and fixed with beeswax to a small brass
171 block. The pedicel, head and legs were fixed using superglue. Female- and male-like pure
172 tones were delivered through separate speakers to the preparation from a pair of modified
173 DT48 speakers, each coupled to a 7 mm plastic tube. The tip of each tube was positioned 1
174 cm from the mosquito on opposite sides of the head. Phasic, compound receptor potentials
175 were measured from the JO with tungsten electrodes (5-7 M Ω , 1 μ m tip, Microprobes.com).

176 USA, part # WE30032.OH3) that were advanced with a Märzhäuser[®] PM10 manipulator so
177 that the tip of the electrode just penetrated the wall of the pedicel. In this location, voltage
178 responses from the JO are dominated by phasic compound receptor potentials from the local
179 population of sensory cells [12], but still demonstrate a response twice the frequency of the
180 acoustic stimulus [17]. Signals from the electrodes were amplified (x10000) and low pass
181 filtered (5 kHz) using custom built differential pre-amplifier. Probe tones of 82 ms duration
182 with 8 ms rise/fall time were delivered via a 5 kHz low pass filter and calibrated against a
183 known 94 dB SPL microphone (Bruel & Kjaer 4230) [15, 16]. Voltage control signals for the
184 sound system were generated and voltage signals from the electrodes were digitized at 250
185 kHz via a Data translation 3010 D/A A/D card using programs written in Matlab. The
186 magnitude and phase of the phasic voltage signals were stored for further analysis. All
187 measurements were made on a Newport[®] isolation table inside an IAC sound attenuated
188 booth. Temperature control was provided by placing the preparation in a chamber machined
189 in a Peltier controlled heat-sink [9, 29]. All recordings were made within 30 min of
190 preparation.

191 Pairs of pure tones simulating the approximate fundamental flight-tones and particle
192 velocities of male ($f_1 = 700$ Hz, 4×10^{-4} ms⁻¹) and female ($f_2 = 400$ Hz, 1×10^{-5} ms⁻¹)
193 mosquitoes were delivered to the antennae of 4 sensitive males mounted in the recording
194 setup. These parameters were chosen to simulate the likely stimuli received by the antennae
195 of a free-flying male mosquito in the close presence of a female [9, 11]. The electrical
196 responses of the JO were recorded from just below the cuticle of the pedicel, adjacent to the
197 scolopidia, which generate graded, non-spiking potentials [12]. Pair tone acoustic stimulation
198 resulted in electrical responses of the JO at the tone frequencies and at the resulting DP (300
199 Hz). Masking pure tones were generated by a Philips[®] PM5193 function generator and
200 delivered simultaneously with f_1 and f_2 tones through a Beyerdynamics[®] DT 770 speaker

201 positioned 7.5 cm in front of the preparation. Masking tones ranging between 125-450 Hz
202 were delivered successively at increasingly particle velocity levels and their 10dB and 15dB
203 suppression effects on the DP compound electrical potential generated in response the f1 and
204 f2 tones were recorded.

205

206

207 **Results**

208 *Acoustic masking of male mosquito RFM*

209 In Experiment 1, we tested the effect of masking tones on the proportion of RFM responses
210 that were directed towards the probe speaker by free-flying male mosquitoes. Probe tone-only
211 elicited an RFM response towards the probe speaker in > 80% of the presentations (Figure
212 1A-C, dashed horizontal lines; probe 340 Hz: 81%; 400 Hz: 85%; 450 Hz: 88%). The
213 proportion of mosquitoes that gave an RFM response was similar for all three probe tones (G-
214 test of independence: $G=0.596$; d.f.=2; $P=0.742$).

215 Pure tone acoustic masking, regardless of the probe frequency, caused significant
216 suppression of the RFM response (when compared to probe-only presentations) for masking
217 frequencies between 300-550 Hz (G-test goodness-of-fit; probe: 340 Hz, $G \geq 5.16$, $p \leq 0.023$;
218 probe: 400 Hz, $G \geq 3.87$, $p \leq 0.049$; probe: 450Hz, $G \geq 4.60$, $p \leq 0.032$) (Figure 1A-C, closed
219 circles). Outside this range, the response proportion was similar to the probe-only stimulation
220 (Figure 1A-C, open circles). The masking tones that caused maximum suppression of the
221 RFM response fell within the same narrow frequency range (390-420 Hz), independently of
222 the probe tone frequency (Figure 1, Supplemental Table 1).

223 Results shown in Figure 1 reveal that the proportion of RFM response in male
224 mosquitoes can be reduced significantly or totally suppressed when a second pure tone is
225 delivered simultaneously with the initial probe tone. Two possible processes can be

226 considered for the observed behavioural masking: *i*) interference, in which the presence of a
227 masking tone impairs the mosquito's ability to detect, locate and/or express RFM response to
228 the probe tone; or *ii*) competition, in which the frequency of the masking tone is more
229 attractive to the male than the frequency of the probe tone, resulting in an increased
230 probability of RFM being expressed towards the masking speaker.

231 To address these possibilities, Experiment 2 was conducted with a second particle
232 velocity microphone placed close to the masking speaker, in addition to the one located near
233 the probe speaker. This arrangement enabled us to identify to which of the two speakers
234 males directed their RFM responses (Figure S1). The same probe frequencies were used as in
235 Experiment 1 and the masking frequencies ranged between 200-550 Hz. The masking tone
236 frequency limits were based on the results from Experiment 1 (Supplemental Table 2).

237 The effect of simultaneous acoustic masking on the proportion of RFM response to
238 each sound source is shown in Figure 2. Probe tone-only presentations elicited a high
239 proportion of RFM responses towards the probe speaker (Figure 2A-C; probe 340 Hz: 75%;
240 400 Hz: 81%; 450 Hz: 84%), in agreement with the results from Experiment 1. Similarly, the
241 presentation of probe/masking tone pairs caused significant suppression of the RFM response
242 towards the probe speaker (Figure 2, range of blue bars) when compared to probe-only
243 presentations (Figure 2A-C, probe 340 Hz: blue bar = 300-500 Hz, $G \geq 5.31$, $p \leq 0.021$; probe
244 400 Hz: blue bar = 320-550 Hz, $G \geq 4.37$, $p \leq 0.037$; probe 450 Hz: blue bar = 250-500 Hz,
245 $G \geq 9.01$, $p \leq 0.003$).

246 Instead of being attracted towards the probe speaker, as indicated by exhibiting RFM
247 behaviour, male mosquitoes can instead direct their response towards the masking speaker or
248 they can display no conspicuous response, flying without frequency modulation (Figure S1).
249 Suppression of attraction towards the probe appears to be dominated by competition from
250 tones emitted by the masking speaker; indeed, attraction (i.e. the RFM response) towards the

251 masking speaker occurred significantly more often than towards the probe speaker (Figure 2,
252 red shading) for masking frequencies between 360 Hz and 470 Hz (Figure 2A-C, probe 340
253 Hz: red shading = 360-450 Hz, $G \geq 4.98$, $p \leq 0.026$; probe 400 Hz: red shading = 390-470 Hz,
254 $G \geq 18.22$, $p \leq 0.001$; probe 450 Hz: red shading = 400-470 Hz, $G \geq 5.15$, $p \leq 0.023$).

255 However, the competition effect, i.e. the attractiveness of the masking frequency
256 relative to the probe frequency, does not account for all the observed behavioural masking
257 because masking tones caused significant RFM suppression to *either* speaker (Figure 2, grey
258 shading). This interference effect by the masking tones on the overall RFM response was
259 observed for all probe frequencies (Figure 2A-C, probe 340 Hz: grey shading = 320-400 Hz,
260 $G \geq 11.53$, $p \leq 0.001$; probe 400 Hz: grey shading = 320-470 Hz, $G \geq 6.14$, $p \leq 0.013$; probe 450
261 Hz: grey shading = 280-470 Hz, $G \geq 4.85$, $p \leq 0.028$).

262

263 *Acoustic masking relative to JO tuning*

264 Maximum masking of the behavioural responses to the probe tones (Figure 3A) coincides
265 with the frequency range of the flight-tones of female *C. quinquefasciatus* mosquitoes but
266 outside the 10dB bandwidth of the JO (244-364 Hz) [9]. A possible hypothesis for this
267 mismatch is that male mosquitoes do not detect probe-tones *per se* but detect their difference
268 in frequency with respect to their own WBF. To test this, the difference between the WBF of
269 the responding males, measured just prior to the onset of an RFM, and the masking tone
270 frequency was calculated for each response. For non-responding males, the WBF was
271 measured ~ 1 s after the start of stimulation. The calculated differences were binned in 25 Hz
272 intervals (50 Hz intervals in the extreme differences) and the proportion of RFM response re-
273 plotted for these groups (Figure 3B).

274 When the suppression of the RFM response is plotted as a function of the difference
275 between WBF and the masking tone, maximum masking is within the 10dB bandwidth of the

276 JO, regardless of the probe tone frequency (Figure 3B). Masking tone frequencies that cause
277 maximum attraction towards the masking speaker also fall within the 10dB bandwidth of the
278 JO, when plotted as the difference between WBF and masking tone. The maximum is centred
279 on the JO 10dB bandwidth when using the 450 Hz probe tone, but moves to the low-
280 frequency boundary that bandwidth for the 350 and 400 Hz probe tones (Figure 3B). These
281 relations indicate that the masking tones suppress the formation of DPs in the vibrations of
282 the antenna [11] or the detection of DPs by the JO.

283 These results imply that RFM behaviour (and its suppression) in male mosquitoes
284 may be dependent on adjustment of their WBF in relation to the frequencies of the stimulus
285 tones. Analysis of variance (Supplemental Table 3) indicates that the WBF during
286 simultaneous probe/masking tone stimulation differed significantly between probe tones, but
287 not between masking tones. Crucially, the WBF of males when stimulated with a probe tone
288 of 340 Hz was 722 ± 1.7 Hz (average \pm S.E.M.), which is a significantly lower WBF than
289 those observed for 400 Hz (732 ± 2.0 Hz) and 450 Hz (735 ± 2.0 Hz) probe tone stimulation.
290 Overall, these results suggest that male mosquitoes may adjust their WBF with respect to the
291 stimulus tones to maintain the difference tone DP within the most sensitive bandwidth of the
292 JO.

293

294 *Attenuation of DPs generated by difference tone in the compound electrical responses of* 295 *the JO*

296 The particle velocity level required to suppress the magnitude of DP electric responses by 10
297 dB and 15 dB as a function of the masking tone frequency are shown in Figure 4. Analysis of
298 variance revealed a significant effect of the masking tone frequency on the suppression of the
299 DP electrical response (ANOVA; 10 dB: $F=7.34$, d.f.=13, $p<0.001$; 15 dB; $F=2.77$, d.f.=10,
300 $p=0.031$). Both suppression tuning curves have their minima outside the range female WBFs,

301 but centred within the 10dB bandwidth of the JO threshold tuning curve (Figure 4). This
302 finding supports the hypothesis from the behavioural experiments that acoustic masking of
303 the RFM behaviour is due to the suppression of the DPs generated at frequencies in the most
304 sensitive frequency range of the JO.

305

306

307 **Discussion**

308 We report here that the RFM behaviour of free-flying *C. quinquefasciatus* male mosquitoes
309 can be significantly suppressed by simultaneous pure tone acoustic masking. Although
310 background noise masking has been reported in *Drosophila* [30], from our knowledge this is
311 the first study describing pure tone-on-tone acoustic masking in insects with antennal
312 hearing. RFM behaviour represents an acoustic measure of a mating attempt by a male, which
313 in mosquitoes is a function mediated essentially by sound [9-13, 22-25, 31, 32]. The most
314 effective masking frequency range encompasses the fundamental frequency range of female
315 flight-tones (430-527 Hz), which are similar to the most sensitive frequencies of the male
316 behavioural audiograms (340-560 Hz) [9]. Acoustic masking is mediated by both competition
317 and interference processes; a masking pure tone can significantly suppress the RFM response
318 by being more attractive than a female-like probe tone and/or by interfering with the ability
319 of the males to detect or locate the probe tone.

320 Significantly, suppression by acoustic masking of RFM behaviour towards pure tone
321 sources provides direct evidence that male mosquitoes hear females through detection of
322 difference tone DPs [9, 11-14]. Maximum RFM suppression occurred at similar masking
323 frequencies for the three probe tones and within the range of the most sensitive frequencies of
324 male behavioural audiogram [9]. Had the male mosquitoes been listening to the probe tones
325 *per se* then the acoustic responses towards the probe tones would have been expected to be

326 suppressed maximally at masking frequencies centred on the probe tones [1, 2]. This is
327 because in nonlinear systems, such as the electrical responses of hair cells in the mammalian
328 cochlea, probe and masking tones suppress themselves mutually when these tones both fall
329 within the sensitive bandwidth of the receptor [33, 34]. In the case of male mosquitoes, this
330 should occur for tone frequencies falling within the sensitive 10 dB bandwidth of the JO
331 (244-364 Hz) [9]. However, RFM responses to the 340 Hz probe tone were completely
332 suppressed by masking tones between 400 and 450 Hz. Crucially, behavioural suppression
333 (for all probe tones) and the 10dB bandwidth of the JO coincide only if it is expressed as a
334 function of the frequency difference between the male fundamental flight-tone and the
335 masking stimulus.

336 This behavioural finding was supported by the electrophysiology; DPs in the
337 electrical responses of the JO generated by two tones, simulating the fundamental frequencies
338 of the male and female flight-tones, were maximally suppressed by masking tones with
339 frequencies within the 10dB bandwidth of the JO. Auditory masking is likely to occur at the
340 level of the antennae where the male and female flight-tones interact non-linearly to generate
341 difference tones in the antennal vibrations [11]. Given that maximum suppression by pure
342 tone acoustic masking is centred on the most sensitive frequency of the auditory receptor [1,
343 2], masking of acoustic behaviour in male mosquitoes, as confirmed by the
344 electrophysiology, is due to suppression of the DPs that are generated at frequencies in the
345 most sensitive frequency range of the JO.

346 The WBF of males differed significantly between probe tones; their WBF, measured
347 during the final approach phase just before RFM, is lowest for the 340 Hz tone and highest
348 for the higher probe tones. This result, in conjunction with the finding that maximum RFM
349 suppression occurred at similar masking frequencies indicates that male mosquitoes may
350 adjust their WBF with respect to the stimulus tones to maintain the difference tone DP within

351 the most sensitive bandwidth of the JO. In this context, it is also possible that harmonic
352 frequency matching [11, 13, 32] could be a consequence of the attempts by the male (and
353 perhaps female) mosquito to maintain the difference tones DP within the “sweet spot” of the
354 JO.

355 Acoustic masking in free-flying male mosquitoes is due not only to suppression of the
356 RFM behaviour but is also due to attraction to masking tone. We have separated these two
357 processes to understand the basis for the suppression. Attraction of male mosquitoes to the
358 masking sound source, i.e. competition, is likely to be related to the free-flight paradigm;
359 probe and masking sound sources are spatially separated, so if both tone frequencies are
360 attractive, males can respond towards whichever tone appears loudest. Evidently, the
361 perceived sound level will be dependent on the spatial location of the mosquito relative to the
362 sound sources when stimulation occurs. It may also depend on the WBF of the male; slight
363 changes in WBF will alter the frequency of the difference tone DP and could alter the
364 apparent loudness of one tone relative to the other. It is also possible that a mechanism like
365 the one found in the *Ormia ochracea* flies is present [8]; in these parasitoid flies the
366 localization of two conflicting, spatially separated, sound sources is solved by a precedent
367 effect, whereby the detection of small time differences (~10 ms) in sound reception are used
368 to determine location of the first source detected.

369 Under natural conditions, *C. quinquefasciatus* males form relatively dense swarms
370 while waiting for sexually receptive females [26, 27]. Given that masking frequencies above
371 600 Hz did not suppress RFM behaviour, male-male acoustic interactions within the swarm
372 should not impair the ability of an individual male mosquito to detect and locate potential
373 mates and perhaps other males [11, 12, 31]. In other words, from a male mosquito’s
374 perspective, swarms appear not to be a source of acoustic noise, although one-to-one
375 encounters between pairs of males may cause them to shift apart their WBFs [11, 31].

376 Acoustic masking of RFM behaviour is most effective for masking frequencies
377 similar to those of the female flight-tones. In this way, the extreme sensitivity of male
378 mosquitoes to these frequencies brings with it the potential cost of high susceptibility to
379 signal distortion and attenuation if two similar, female-like, tones were to be detected
380 simultaneously. Under natural conditions, this would occur only if a male within a swarm
381 was to detect the flight-tones of two nearby females at the same time and for a sustained
382 period. This situation, however, would occur only with unrealistically high densities of
383 unmated females nearby or within the swarm. Wishart and Riordan [23] studied the
384 attractiveness to various sounds in *Aedes aegypti* males and found the most attractive
385 frequencies were, as in *C. quinquefasciatus* [9] and *A. gambiae* species [10], centred on the
386 female fundamental frequency and ranged optimally between 400-600 Hz. Crucially, their
387 work showed that two or more pure tones, which are each attractive on their own, are not
388 attractive when presented together in the same speaker; in some frequency pairs
389 (450Hz/500Hz and 500Hz/550Hz), this resulted in a > 95% reduction in the number of males
390 trapped by their sound-lure vacuum trap. The cause for this marked decrease was not
391 determined, but it appears that, as presented here, acoustic masking could be the underlying
392 process.

393 The findings reported here support the hypothesis that mosquitoes must fly to hear
394 and that hearing in male mosquitoes is an active process mediated by the detection of
395 intermodulation distortion products. Nonetheless, a more complete model of acoustic
396 masking in male mosquitoes could lead to the development of new strategies to control
397 mosquitoes based on acoustic tools capable of disrupting swarming and mating in nature.

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402 **Author contributions**

403 Experiments were conceived and designed by P.M.V.S., G.G. and I.J.R. Experiments were
404 conducted and data was analysed by P.M.V.S. and R.I. Manuscript was written by P M.V.S.
405 and I.J.R. with contributions from G.G. and R.I.

406

407 **Ethics**

408 No vertebrate animals were used in this research. Mosquitoes were cold anaesthetized prior to
409 preparation for electrophysiological recordings. All applicable international, national, and
410 institutional guidelines for the care and use of animals were followed.

411

412 **Data accessibility**

413 Data supporting this article can be accessed at Dryad Digital Repository:
414 <http://dx.doi.org/XXXXXX/dryad.XXXX> (to be added after submission) and have also been
415 uploaded as electronic supplementary material.

416

417 **Competing interests**

418 We have no competing interests.

419

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422

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428

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FIGURE LEGENDS

Figure 1. Acoustic masking of RFM behaviour of free-flying male mosquitoes to a speaker emitting a probe tone. The proportion of mosquitoes initiating an RFM response towards the probe speaker is plotted as a function of the masking frequency. Probe tone: A) 340 Hz; B) 400 Hz; C) 450 Hz. Horizontal dashed line: proportion of male mosquitoes expressing the RFM response to probe tone-only. Closed symbols: Response proportions significantly lower than responses to probe tone-only.

Figure 2. Interference and competition of masking tone. Probe tone: A) 340 Hz; B) 400 Hz; C) 450 Hz. The proportion of male mosquitoes exhibiting RFM behaviour towards the probe speaker (blue line) or masking speaker (red line) plotted as function of the masking frequency. The black line represents the proportion of response to either speaker. Horizontal line: Proportion of response to probe tone-only. Blue bar: masking frequencies causing significant acoustic masking towards the probe speaker. Red shading: masking frequencies causing a significantly higher proportion of RFM response towards the masking speaker than to the probe speaker. Grey shading: masking frequencies causing a significantly lower proportion of RFM response to either speaker relatively to the probe-only presentations.

Figure 3. Acoustic masking of an RFM response correlates with JO tuning when the proportion of response is calculated as function of frequency difference between the male's WBF and the masking frequency. A) Proportion of RFM response to the probe tone (blue curve) as a function of the masking tone frequency (as in Figure 2), is strongly reduced in the presence of the masking tone, especially for masking frequencies between 350-450 Hz, regardless of probe tone frequency. The proportion of RFM response to the masking tone speaker is greatly increased (red curve) during maximum masking. B) Proportion of RFM response calculated as a function of the frequency difference between the male's WBF measured immediately prior the RFM response and the masking frequency. Grey range: 10 dB bandwidth of the JO frequency threshold tuning curve (244-364 Hz) [9].

Figure 4. Attenuation of difference tone distortion products generated in the compound electrical responses of the JO. Difference tones ($DP = f_1 - f_2 = 300$ Hz) were generated by the simultaneous presentation of two tones simulating male ($f_1 = 700$ Hz, 4×10^{-4} ms⁻¹) and female ($f_2 = 400$ Hz, 1×10^{-5} ms⁻¹) flight-tones. Curves represent the masking tone levels (ms⁻¹) required to suppress the magnitude of the DP response by 10dB and 15dB. Each point is mean \pm SD from 4 preparations. Grey range: 10 dB bandwidth of the JO frequency threshold tuning curve (244-364 Hz) [9].







