

1 **A Role for Acoustic Distortion in Novel Rapid Frequency Modulation**
2 **Behaviour in Free-Flying Male Mosquitoes**

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12 **Running title**

13 Acoustic Behaviour in Male Mosquitoes

14
15 **Keywords**

16 Mosquito, *Culex quinquefasciatus*, mating behaviour, insect hearing, Johnston's
17 organ, acoustic behaviour, phonotaxis, distortion products

18
19 **Summary Statement**

20 Female mosquito flight-tones elicit a novel, stereotypical, auditory behaviour from
21 male mosquitoes, although the male hearing organ is actually tuned to the frequency
22 difference between male and female flight-tones.

23
24 **Abstract**

25 We describe a new stereotypical acoustic behaviour by male mosquitoes in
26 response to the fundamental frequency of female flight-tones during mating
27 sequences. This male-specific free-flight behaviour consists of phonotactic flight
28 beginning with a steep increase in wing-beat frequency (WBF) followed by Rapid
29 Frequency Modulation (RFM) of WBF in the lead-up to copula formation. Male RFM
30 behaviour involves remarkably fast changes in WBF and can be elicited without
31 acoustic feedback or physical presence of the female. RFM features are highly
32 consistent, even in response to artificial tones that do not carry the multi-harmonic
33 components of natural female flight-tones. Comparison between audiograms
34 of the robust RFM behaviour and the electrical responses of the auditory Johnston's

35 organ (JO) reveals that the male JO is tuned not to the female WBF *per se*, but,
36 remarkably, to the difference between the male and female WBFs. This difference is
37 generated in the JO responses due to intermodulation distortion products (DPs)
38 caused through nonlinear interaction between male-female flight-tones in the
39 vibrations of the antenna. We propose that male mosquitoes rely on their own flight-
40 tones in making use of DPs to acoustically detect, locate and orientate towards flying
41 females. We argue that the previously documented flight-tone harmonic
42 convergence of flying male and female mosquitoes could be a consequence of WBF
43 adjustments so that DPs generated through flight-tone interaction fall within the
44 optimal frequency ranges for JO detection.

45

46

47 **Introduction**

48 It has been known since the 19th century that male mosquitoes locate females by
49 homing in on their flight-tones, and that this behaviour can be elicited from males
50 using artificial tones at frequencies within the range of female flight-tones (Child,
51 1894; Mayer, 1874; Maxim, 1901; Roth, 1948; Belton, 1994). More recently, it has
52 been shown in *Culex quinquefasciatus* (Warren et al., 2009) and other mosquito
53 species (Cator et al., 2009; Pennetier et al., 2010) that one component of the mating-
54 chase involves an interactive behaviour that appears to lead to frequency
55 convergence of the harmonic components of the flight-tones of both sexes, possibly
56 as a mechanism for recognition of conspecific mating partners. Earlier reports (Kahn
57 et al., 1945; Roth, 1948; Wishart and Riordan, 1959; Belton, 1994) indicate that the
58 wing-beat frequencies (WBFs) of male mosquitoes fluctuate during the final
59 approach to a female (or artificial sound source simulating a female), but these
60 acoustic changes were not fully described or analysed.

61 Mosquitoes possess one of the most sensitive hearing organs in the animal
62 kingdom (Göpfert et al., 1999; Göpfert and Robert, 2000; Gibson and Russell, 2006);
63 near-field acoustic stimulation causes antennal vibrations which are conducted via
64 an arrangement of extracellular spokes in the pedicel to the many thousands of
65 mechanosensitive scolopidia that constitute the highly sensitive auditory (Johnston's)
66 organ (JO) (Belton, 1974; Clements, 1999; Göpfert et al., 1999; Göpfert and Robert,
67 2000). The antennae also vibrate in response to simultaneous acoustic stimulation
68 from the mosquito's own flight-tones and those of a mosquito of the same or

69 opposite sex; interaction between pairs of tones generates strongly amplified
70 intermodulation distortion products (DPs) that can be detected by measuring the
71 vibrations of the antenna and the electrical responses of the JO (Warren et al., 2009;
72 Pennetier et al., 2010; Lapshin, 2012). The frequency characteristics of the antennal
73 vibrations and the electrical responses of the JO of *C. quinquefasciatus* have been
74 described (Warren et al., 2009). However, to our knowledge, there has not been a
75 quantitative study relating the auditory physiology of *C. quinquefasciatus* with the
76 acoustic behaviour of males in response to the sounds they hear in free-flight during
77 the final phases of mating (i.e. the male's own flight-tones plus those of the female).

78 In this paper we present and characterize a new acoustic behaviour specific
79 to male mosquitoes which is elicited by tones at frequencies that encompass the
80 frequency range of the female flight-tones. We exploited this stereotypical behaviour
81 and made electrophysiological measurements from the male JO to reveal that it is
82 not tuned to the female flight-tones, but it is tuned sharply to, and strongly amplifies,
83 difference-tone DPs generated through interaction between tones at the fundamental
84 frequencies of the flight-tones of each sex. Comparison between the behavioural and
85 physiological audiograms (tuning curves) suggests that male mosquitoes rely on
86 their own flight-tones in making use of DPs to acoustically detect, locate, and
87 orientate towards flying females. Consequences of the findings reported here for
88 mosquito auditory physiology, mosquito behaviour, and particularly harmonic
89 convergence are discussed.

90

91

92 **Materials and Methods**

93 ***Mosquitoes***

94 *Culex quinquefasciatus* Say, "Muheza" strain were obtained from the London School
95 of Hygiene and Tropical Medicine. Mosquitoes were bred in controlled-environment
96 chambers; 70-75% rH, 26±2°C and 12 h light: 12 h dark cycles. Adult mosquitoes
97 between 4 and 14 days post-emergence were tested during the first 3 h of the
98 scotophase.

99

100 ***Behavioural set-up***

101 Sound recordings of single free-flying pairs of a male and a virgin female were made
102 under semi-natural conditions in a large (1.5 m wide and deep, 2 m high) flight arena

103 in a double-skin sound-attenuated booth (IAC Ltd, Winchester, UK). The flight arena
104 was equipped with Basler[®] Pilot Ace, GigE video cameras (Basler AG) and a Røde[®]
105 NT1 pressure microphone mounted at the focal point of an 24" parabolic reflector
106 (Edmunds) to record behaviour (see details below), kept at constant environmental
107 conditions and provided with dusk light-levels typical during natural mating
108 behaviour. A swarming marker (black disk, 13 cm radius) was placed on the floor of
109 the arena to stimulate flight behaviour typical of swarming; an individual mosquito
110 can be said to 'swarm' by flying in controlled loops, in essence 'station-keeping' with
111 respect to the visual environment (Gibson, 1985). For each recording, one male was
112 released into the flight arena first, and within 3 min the male started to swarm
113 spontaneously. Once swarming flight was established, the female was released and
114 also started to swarm over the marker. Their flight tones were recorded throughout
115 this process for further analysis. Copula formation was verified via TrackIt 3D[®]
116 (SciTrackS, GmbH) zoom tracking software that displayed a full screen image of
117 each mosquito in real time.

118 In a separate set-up, the behaviour of free-flying mosquitoes was recorded
119 inside wire-framed arenas of 30 cm sides. Two variations of this flight arena were
120 used; for simultaneous video/audio recordings, the metal frame was covered with
121 matt-black cotton fabric which is non-reflective to infra-red (IR) light, while the front
122 side was covered by transparent acrylic enabling the camera to view the chamber's
123 interior. The ceiling was covered with white cotton gauze to allow the chamber to be
124 illuminated by two IR multi-LED lights positioned 1 m above the cage. For audio-only
125 records, the flight arena consisted of the wire frame covered by white cotton tubular-
126 gauze. The 30 cm sided flight arenas were placed on a vibration damped table
127 (Newport[®], Irvine, Ca, USA) inside an sound attenuated booth (IAC Ltd, Winchester,
128 UK).

129 Artificially generated tone stimuli were delivered to the cage from a sound
130 source consisting of a 0.5 cm diameter plastic probe tip, damped with acoustic foam,
131 connected via a 1 cm diameter polythene tube to an adapted Audio Technica[®] ATH
132 A700AX speaker (5-35,000 Hz range with flat frequency response 100-25,000 Hz).
133 Sound from the speaker and flight-tones from the mosquitoes were monitored using
134 a particle velocity microphone (Knowles NR-3158, Ithaca NY, USA) that was
135 calibrated (Göpfert and Robert, 2001) and mounted ~4 cm from the speaker probe
136 tip. For the small flight arenas, a pressure microphone (Knowles 23132, Ithaca NY,

137 USA) mounted at the focal point of an 18" parabolic reflector (Edmunds), was placed
138 on one side of the arena to monitor the sound inside. Signals from each of the
139 microphones were amplified 100-fold with a purpose built two-channel preamplifier
140 and the output of each channel was digitized at 192 kHz using a Fireface[®] UC sound
141 card. The digital outputs were then recorded using Spectrogram 16 (Visualization
142 Software, LLC) at a sampling rate of 48 kHz and frequency resolution of 5.9 Hz.
143 Spectrogram 16 was also used to analyse and extract data on the time, frequency
144 and amplitude of all acoustic signals.

145 Artificial sound stimuli were generated using the sine wave function of Test
146 Tone Generator 4.4 (EsserAudio[®], 2011) software. With the exception of the
147 behavioural audiograms, all tone bursts had a fixed duration (5 s or 10 s, depending
148 on the experiment) and were cosine windowed at onset and offset to avoid acoustic
149 transients. Calibrated pure tones simulating the sound intensity of the fundamental
150 component of the flight-tones of tethered-flying female mosquitoes were based on
151 measurements with the particle velocity microphone placed 2 cm in front of their
152 heads. The mean \pm s.e.m. particle velocity for this reference distance was $5.7 \times 10^{-5} \pm 1.9 \times 10^{-6} \text{ ms}^{-1}$ (N=23).
153

154 For video recordings, an IR-video camera (Swann[®] Pro-880) was placed 30
155 cm in front of the clear wall of the chamber and connected to the computer. Digital
156 video recordings at 30 FPS of the flying mosquitoes were obtained using Debut
157 Video Capture Software v1.88 (NCH[®] Software). The video recorded flight paths
158 were then digitised using Kinovea (Version 0.8.23) software. The synchronised
159 video-spectrogram sequences in the supplemental movies were composed using
160 Adobe[®] After Effects.

161 Depending on the experiment, a single or several male mosquitoes were
162 placed inside the flight arena at the time of spontaneous circadian activity and left to
163 fly freely during the recordings. After ~10 min period of adaptation to conditions
164 inside the booth, the mosquitoes started to fly spontaneously, whereupon sound
165 recording and stimuli presentation were initiated. All behavioural experiments were
166 conducted at a room temperature of $30 \pm 2^\circ\text{C}$, which is within the range of
167 temperatures of the natural habitat of the *C. quinquefasciatus* mosquitoes (Gokhale
168 et al., 2013).

169

170 ***Electrophysiology***

171 Mosquitoes were immobilized by cold narcosis and fixed with beeswax to a small
172 brass block. The pedicel, head and legs were fixed using superglue (Loctite®).
173 Sound was delivered to the preparation from a pair of modified DT48 headphone
174 speakers, each coupled to the preparation via separate 7 mm (ID) plastic tubes. The
175 point of each tube was positioned 10 mm from the mosquito on opposite sides of the
176 head. Compound extracellular receptor potentials were measured from the JO with
177 tungsten electrode (5-7 M Ω , 1 μ m tip, Microprobes.com. USA, part # WE30032.OH3)
178 that were advanced with a Märzhäuser (GmbH) PM10 manipulator so that the tip of
179 the electrode just penetrated the wall of the pedicel. In this location voltage
180 responses from the JO are dominated by compound, phasic receptor potentials from
181 the scolopidia that are twice the frequency of the acoustic stimulus (Tischner, 1953;
182 Belton, 1974; Clements, 1999). All measurements were made on an isolation table
183 (Newport corporation, Model: M-VW-3036-OPT-99-9-28-92) inside an IAC sound
184 attenuated booth. Signals from the electrodes were amplified (10,000-fold) and low
185 pass filtered (5 kHz) using a laboratory designed and built differential pre-amplifier.
186 Pure tones of 82 ms duration with 8 ms rise/fall time were delivered via a 5 kHz low
187 pass filter and calibrated against a known 94 dB SPL (Bruel & Kjaer 4230) (Göpfert
188 and Robert, 2001). Voltage signals for the sound system were generated and
189 voltage signals from the electrodes were digitized at 250 kHz via a Data translation
190 3010 D/A A/D card using programs written in Matlab. Raw data and online
191 computation of the magnitude and phase of the phasic voltage signals were stored in
192 ASCII files for display and further analysis. All recordings were made under
193 controlled conditions, and within 30 min of preparation to ensure excellent
194 physiological state and hearing sensitivity. Temperature control for the physiological
195 experiments was provided by placing the mosquito in a chamber machined in a
196 Peltier controlled heat-sink (Warren et al., 2010). Current was fed to the Peltier
197 element by a power supply with a negative feedback control from a thermistor (80TK,
198 Fluke©) which was thermally coupled to the chamber.

199

200

201 **Results**

202 ***Acoustic behaviour of free-flying male-female pairs***

203 The wing beat frequency (WBF) of male-female mosquito pairs (N=7) were recorded
204 using a parabolic microphone while free-flying above a visual marker inside a large

205 sound attenuated booth with ambient illumination adjusted to dusk, the natural
206 condition for mating swarms (Gibson, 1985). The spectrograms of these flight
207 sequences (Fig. 1A) showed that the mean \pm s.e.m. WBF of males was 789 ± 10 Hz
208 and of females was 474 ± 10 Hz. After flying simultaneously for a variable length of
209 time, in all cases the WBF of the male followed by that of the female increased
210 steeply, followed by intense frequency oscillations at the elevated frequency which
211 lasted several seconds (4466 ± 883 ms for males, 3939 ± 959 ms for females).
212 Significantly, in all recorded sequences, the steep increase in frequency was initiated
213 by the male mosquito (Fig. 1A – Green arrow), followed 682 ± 120 ms later by an
214 increase in the female's WBF (Fig. 1A – Red arrow). Video-recordings of these
215 mating chases revealed that a copula was formed during these rapid frequency
216 oscillations.

217

218 ***Free-flying male behaviour in the presence of a tethered female***

219 To further examine this free-flight interaction, the flight-tones and 2D flight paths of
220 male mosquitoes (N=9) were recorded in the presence of a tethered-flying female
221 under infra-red (IR) illumination in the smaller flight arena. The duration of the
222 recorded sequences when both sexes were flying simultaneously ranged between
223 ~ 1.5 min and ~ 11 min, during which the mean \pm s.e.m. of the male's WBF was
224 739 ± 5 Hz and that of the tethered female was 411 ± 5 Hz (WBF sampled every 30 s).
225 There was an initial Latency period of variable duration during which the male
226 displayed continuous looping flight, with no obvious deviation towards the tethered-
227 flying female. The male then approached the tethered female repetitively and
228 displayed a characteristic modulation of his WBF while flying in close proximity to or
229 touching the female (Fig. 1B, C). This behaviour was initiated by a steep increase in
230 the male's WBF followed by rapid WBF oscillations while within ~ 4 cm of or touching
231 the tethered female. The male would then cease WBF oscillations and gradually
232 decrease his WBF as he departed from the female (Fig. 1B, C). Each male displayed
233 this behaviour on average 6.2 ± 1.0 times per minute, while flying continuously. In
234 contrast, when the tethered female was prevented from flying by using the tarsal
235 reflex (by positioning a small piece of paper under her legs), males (N=3) did not
236 display any conspicuous changes in WBF or attempt to approach the female during
237 sequences lasting ~ 14 min. The tethered female occasionally increased her mean
238 WBF and also oscillated her WBFs rapidly (Fig. 1B). This was observed to occur,

239 however, only as a direct consequence of physical contact by the male. That the
240 initiation of WBF modulation is a male-specific response was confirmed by releasing
241 a virgin free-flying female in the presence of a tethered-flying male; in all of the
242 recorded sequences (N=3), females displayed continuous looping flight for several
243 minutes without ever being attracted to the tethered male or exhibiting any
244 conspicuous changes in acoustic or flight behaviour in response to the male. It was
245 noted, however, that tethered males did not display rapid modulation of their WBF as
246 they would have done in free-flight. It appears, therefore, that tethering enables
247 sustained flight but impairs the exhibition of the RFM behaviour in male mosquitoes.

248 These observations confirm that male mosquitoes are phonotactically
249 attracted by the flight-tones of females, whereas females are not attracted to the
250 flight-tones of males (Kahn et al., 1945; Roth, 1948; Wishart and Riordan, 1959;
251 Belton, 1994). Moreover, we provide the first quantitative evidence of a complex
252 male-specific acoustic modulation of their flight-tones when in close proximity of a
253 flying female, which we have termed “Rapid Frequency Modulation” (RFM).

254

255 ***Characterization of male acoustic behaviour***

256 To characterize and quantify the acoustic parameters of RFM behaviour, we tested
257 the effect on male free-flight of artificial pure-tone stimuli over a frequency range
258 intended to cover the range of fundamental WBFs of free-flying females (5 s pure
259 tone bursts; 21 different frequencies ranging from 265-525 Hz). The stimuli level was
260 set to that measured 2 cm from the front of the head of a tethered-flying female
261 mosquito (see Materials and Methods). In total, 69 RFM events were observed in 12
262 males. Male responses to pure tones, including RFM of their WBFs, were similar to
263 the responses observed to the flight tones of tethered-flying females (Fig. 1D).

264 The mean \pm s.e.m. WBF measured immediately before tone stimulation was
265 742 ± 9 Hz. These WBFs were ~ 200 Hz higher than those previously reported for
266 tethered-flying males of the same species (Warren et al., 2009), probably because in
267 the current experiments males were free-flying and the ambient temperature was
268 higher (30°C compared with tethered-flight at 22-24°C in Warren et al. (2009)).
269 During a variable period we termed ‘Latency’ (time from tone initiation to the onset of
270 the acoustic behaviour), the male’s WBF remained essentially unchanged
271 (Δ WBF= 2 ± 1 Hz; Fig. 1D). A likely cause of the highly variable Latency (range: 161-
272 3510 ms; mean: 1479 ± 94 ms) was variation in distance between the male and the

273 sound source at tone onset; males flying near the sound source at tone onset would
274 have detected the stimulus sooner than mosquitoes flying near the walls of the
275 arena.

276 The Onset phase of RFM was initiated with a steep increase in WBF of 85 ± 3
277 Hz in 327 ± 37 ms, equivalent to a rate of change of ~ 260 Hz s^{-1} . Onset was followed
278 by the Modulation phase, which lasted 1148 ± 79 ms. During the Modulation phase,
279 the WBFs were frequency modulated as indicated by the greater bandwidth
280 (measured 10 dB from the peaks) of the Fast Fourier Transforms of the fundamental
281 frequencies of the flight-tones compared with that measured during Latency (Fig. 1D,
282 Inset); the 10dB bandwidth during the Modulation phase was 87 ± 6 Hz, significantly
283 higher than the 25 ± 1 Hz during Latency (paired T-student test, $t=12.31$, $N=30$,
284 $P<0.001$). As shown in the spectrograms in Fig. 1, the frequency modulations, which
285 are more clearly visualised in the higher harmonics of the WBFs, comprise fast and
286 variable upward and downward shifts in frequency that ranged from ~ 20 - 200Hz in
287 amplitude at the fundamental frequency (Fig. 1D, Fig. S1A). The number of
288 frequency modulations varied from 3-14 cycles during the Modulation phase. On
289 average, the peak-to-peak interval between the frequency modulations (calculated
290 by dividing the duration of the Modulation phase by the number of peaks, see Fig.
291 S1A) was 204 ± 9 ms, i.e. a rate of ~ 5 s^{-1} . The resulting conversion of these peak-to-
292 peak values indicates that during RFM male mosquitoes, remarkably, modulate their
293 WBF at a rate of up to ~ 1950 Hz s^{-1} .

294 The Modulation phase was followed by the Offset phase, during which the
295 WBFs gradually decreased over a period lasting 1246 ± 64 ms, until they reached a
296 final WBF only 2 ± 1 Hz higher than that during Latency (Fig. 1D). The duration of
297 RFM behaviour, from the Onset (steep frequency spike) until the Offset (end of the
298 final frequency drop) was 2722 ± 104 ms (range: 1141 - 4638 ms). The duration and
299 variation in frequency of RFM and its constituent components (Onset, Modulation
300 Phase and Offset) were independent of the stimulus frequency (ANOVA $F_{1,20}<1.64$;
301 $P>0.081$) and of the initial WBF of the male (ANOVA $F_{1,20}<1.73$; $P>0.075$).

302 In contrast, no conspicuous acoustic interactions or RFM behaviour was
303 observed in virgin free-flying females ($N=7$) stimulated with 5 s artificial pure-tone
304 bursts with frequencies ranging from 200-2000Hz, which further suggests that the
305 changes in WBF observed in free- and tethered- flying females (Figs. 1A and 1B,

306 respectively) were in response to the physical contact with the male, rather than in
307 response to auditory stimuli.

308

309 ***Flight patterns during RFM behaviour***

310 The flight paths of male mosquitoes recorded during RFM behaviour were examined
311 (Fig. 2A; Supplemental Movie S1). Before sound stimulation with artificial pure-tones
312 on the range of fundamental WBFs of free-flying females, males typically flew in
313 large loops around most of the volume of the chamber (white path in Supplemental
314 Movie S1). During Latency, which started at tone initiation (Fig. 2A – yellow path),
315 the flight paths were confined mainly to the centre of the chamber in slow, station-
316 keeping flight without looping. The Onset of the acoustic response (Fig. 2A – orange
317 path) was associated with the beginning of the phonotactic approach to the sound
318 source. The Modulation phase (Fig. 2A – red path) occurred when the male
319 mosquito was within close proximity (~4 cm or less) to the sound source, and
320 displaying tight looping flight paths around the sound source. In some interactions
321 the male touched or even landed on the source without ceasing RFM. The Offset
322 (Fig. 2A – green path) coincided with departure of the male from the vicinity of the
323 sound source. Close-up video recordings of the flight behaviour of males when near
324 the sound source during the Modulation phase confirmed that male mosquitoes
325 displayed a series of short, tight loops around the sound source (Figs. 2B, S1B, S1C;
326 Supplemental Movie S2). During the Modulation phase it was also observed that
327 males extended and moved their legs, trying to grasp the sound source while flying
328 continuously (Supplemental Movie S2).

329 On the basis of these results, we propose that RFM in males is an
330 acoustically driven behaviour in response to female flight tones. This behaviour
331 comprises *i*) the Onset phase, characterised by a steep increase in WBF and
332 associated with phonotaxis towards the sound source, followed immediately by *ii*) the
333 Modulation phase, when the mosquito is in close proximity to the sound source and
334 the elevated WBF is rapidly frequency modulated (appearing in spectrograms as a
335 series of increases and decreases of variable duration about the elevated WBF) and
336 *iii*) the Offset phase, when the male moves away from the sound source and
337 gradually decreases his WBF until it is similar to that during Latency. The total
338 duration of RFM behaviour is variable, from just over one to several seconds.

339

340 ***Frequency range of RFM response***

341 The frequency range of RFM behaviour was obtained by recording the responses of
342 individual free-flying male mosquitoes (N=13) when presented with single-tone
343 bursts between 200-2500 Hz (20 Hz increments for frequencies between 200-800
344 Hz, 100 Hz increments between 800-2500 Hz). The tone burst duration was 10 s,
345 with an inter-burst interval of ~5 s and with a sound intensity equal to that generated
346 2 cm in front of the head of a tethered-flying female ($5.7 \times 10^{-5} \text{ ms}^{-1}$). Each of the
347 resulting 48 stimuli were presented randomly and only once to each male. Males
348 exhibited RFM responses to tones at frequencies between 280-640 Hz and were
349 unresponsive to tone frequencies below and above this range (Fig. 2C, Fig. S2).
350 Within the responsive range, more than 75% of males exhibited an RFM response to
351 tones between 340-540 Hz, a frequency range that encompasses the WBF range of
352 conspecific free-flying female mosquitoes (range=430-527 Hz, N=30), which
353 indicates that the male's RFM response is elicited by the detection of tones of similar
354 frequencies to the fundamental WBF of the female.

355 To determine the percentage of RFM responses within individuals, free-flying
356 males mosquito (N=7) were presented with 7 consecutive tone bursts (10 s duration,
357 inter-trial interval of 5 s). For tones at 460 Hz, each male responded on average to
358 $96 \pm 3\%$ of the presented stimuli, but no responses were observed when the tone was
359 860 Hz. Thus, RFM behaviour is highly repeatable when the stimulus frequency is
360 similar to a female's WBF. When the duration of each of the 460 Hz consecutive
361 tone bursts was reduced to only 1 s, each male responded on average to $45 \pm 7\%$ of
362 the presented stimuli. Although significantly lower than the proportion of responses
363 to the 10 s tone bursts (unpaired T-student test, $t=6.60$, $N=7$, $P<0.001$), the
364 robustness of RFM behaviour to short 1 s tone bursts remains high.

365

366 ***Behavioural threshold of RFM response***

367 Behavioural audiogram of male mosquitoes (i.e. the threshold of the RFM response
368 relative to the particle velocity of the sound stimulus) was obtained for stimulus
369 frequencies between 200-1000 Hz (20 Hz increments). For each replicate (N=6), a
370 group of 7-10 males was placed in the flight arena under illumination simulating
371 dusk, when they are normally active. Upon initiation of spontaneous flight, a
372 continuous tone of fixed frequency was presented to the swarming mosquitoes. The
373 tone level was increased at a rate of 0.4 dB s^{-1} from $\sim 1 \times 10^{-8} \text{ ms}^{-1}$ output until an

374 RFM response was elicited from at least one male or until the maximum operating
375 level ($4 \times 10^{-4} \text{ ms}^{-1}$) was reached. The sound stimulus was then terminated and the
376 particle velocity that elicited the response and the WBF of the responding male
377 immediately before the onset of RFM (see Fig. S1A, red arrow) were stored. Particle
378 velocity values are relative to a reference distance of 2 cm away from the speaker.
379 After a 5-10 s rest period without stimulation, the procedure was repeated for
380 another stimulus frequency. Even when several males were swarming at the same
381 time, the spectrogram analysis permitted the detection and isolation of the RFM
382 response of individual males because the response was much louder (due to their
383 proximity to the particle velocity microphone) than the humming of the swarm in the
384 background. The presence of higher harmonics of flight-tones provided a further
385 basis for distinguishing between the WBFs of individual males.

386 The behavioural audiogram (Fig. 3A) shows that the lowest and highest
387 frequencies that elicited an RFM response were 260 Hz and 720 Hz, respectively.
388 Tone frequencies between 340-560 Hz elicited responses at the lowest thresholds
389 (ANOVA $F_{1,23}=14.64$, $P<0.001$), and encompass the range of WBFs for conspecific
390 free-flying female mosquitoes (430-527 Hz, 492 ± 4 Hz, $N=30$, grey shaded area, Fig.
391 3A). RFM is thus very robust and responses are elicited to tones at frequencies that
392 exceed the upper and lower range of female WBFs by ~ 190 Hz, but only at very high
393 levels. Within the range of the most sensitive frequencies, male mosquitoes
394 responded to particle velocities between $4.8 \times 10^{-7} \text{ ms}^{-1}$ and $1.3 \times 10^{-6} \text{ ms}^{-1}$ (Fig. 3A),
395 which are ~ 40 dB below the average sound intensity generated by tethered-flying
396 females 2 cm in front of their heads ($5.7 \times 10^{-5} \pm 1.9 \times 10^{-6} \text{ ms}^{-1}$, see Materials and
397 Methods).

398 The positive correlation between WBF (measured just prior to the onset of
399 RFM) and the frequency of the stimulus shows that males flying at lower WBFs tend
400 to respond to the lower frequencies of the stimulus range, while males flying at
401 higher WBFs respond more often to higher stimulus frequencies (Fig. 3B;
402 Stimulus = $2.6 \times \text{WBF} - 1553$; Pearson's $r=0.69$). This strong correlation suggests that
403 the detection of female-like tones (and consequently the expression of RFM) by male
404 mosquitoes is dependent on their own WBFs.

405 How might RFM behaviour be related to harmonic convergence of male and
406 female flight-tones, as described for *C. quinquefasciatus* (Warren et al., 2009) and
407 other mosquito species (Cator et al., 2009; Pennetier et al., 2010)? We calculated

408 the Harmonic Convergence Ratio (HCR) by dividing the stimulus frequency (which
409 simulates the WBF of a female) by the male's WBF just prior to the onset of RFM
410 elicited by the stimulus (Fig. S1A). The inverse of the HCR corresponds to the
411 harmonic relation of the two sound frequencies; e.g. $HCR=0.5=1/2$ indicates a 2:1
412 harmonic relation, i.e. the frequency of the 2nd harmonic of the female-like sound is
413 equal to the male's fundamental WBF, whereas, $HCR=0.667=2/3$ indicates a 3:2
414 harmonic relation, which would correspond to a frequency convergence between the
415 3rd harmonic of the stimulus and the 2nd harmonic of the male's WBF. Although the
416 stimulus frequencies were sinusoidal pure tones, harmonics of these pure tones are
417 produced in the vibrations of the male's antenna and JO upon sound detection, so
418 males can potentially use these tones to reach harmonic convergence (Cator et al.,
419 2009; Warren et al., 2009; Pennetier et al., 2010). The HCRs, plotted as a function of
420 the stimulus frequency are not centred on any particular value, but rather increase
421 proportionally with stimulus frequency (Fig. 3C), which indicates that the initiation of
422 the RFM response by males is independent of any harmonic convergence between
423 their flight-tones and the stimulus. Interestingly, the most sensitive RFM responses
424 (elicited by low particle velocity levels, as indicated by bubble areas in Fig. 3C) are
425 distributed between HCRs of 0.5 and 0.667 (Fig.3 – dashed lines), values that
426 correspond respectively, to 2♀:1♂ and 3♀:2♂ harmonic convergences.

427

428 ***Comparison between behavioural and Johnston's organ frequency tuning***

429 We recorded and measured the magnitude of the fundamental frequency component
430 of the extracellular electrical responses from the Johnston's organ (JO) of male
431 mosquitoes (N=6) as function of stimulus level (particle velocity) to tones between 61
432 Hz and 1001 Hz. These extracellular electrical responses are dominated by phasic
433 compound receptor potentials (see Materials and Methods). Threshold frequency
434 tuning curves were obtained by determining, for each stimulus frequency, the particle
435 velocity threshold at which the electrical signal elicited a response 5 dB above noise
436 floor of the recording (Fig. 3D). All measurements were made at the same
437 temperature ($30.0\pm 2^\circ\text{C}$) as the behavioural experiments. At its most sensitive
438 frequency (281 Hz), the JO responded to particle velocities of $2.0\times 10^{-7}\text{ ms}^{-1}$. The JO
439 is most sensitive to frequencies (244-364 Hz; 10dB bandwidth), which are
440 considerably below those of the female free-flight WBF range (Fig. 3D) and to which
441 the behavioural audiogram is most sensitive (Fig. 3A).

442 Following these findings, we investigated the hypothesis that male
443 mosquitoes detect not the female flight-tones *per se*, but the frequency difference
444 between his WBF and that of a flying female mosquito. We re-plotted the behavioural
445 audiogram as a function of the frequency difference between the WBFs of male
446 mosquitoes just prior to the onset of their RFM responses and the tone stimulus (Fig.
447 3E). The quadratic curve fitted to the behavioural audiogram ($\text{dB}=0.001F^2-$
448 $0.689F+77.81$; $R^2=0.761$; $F_2=211.9$, $P<0.001$) indicates a minimum behavioural
449 threshold with a 10dB bandwidth extending between 244-444 Hz that encompasses
450 the 10dB bandwidth of the JO electrical responses (Fig. 3E, grey bar). These results
451 suggest that RFM acoustic behaviour, and consequently the JO of male mosquitoes,
452 is tuned not to the fundamental frequencies of the female WBF, but to the difference
453 in frequency between the fundamental WBFs of the male and female.

454

455 ***The JO of male mosquitoes is tuned to difference tones generated through***
456 ***interaction between male and female flight-tones.***

457 To test the hypothesis that hearing in male mosquitoes is tuned to the frequency
458 difference between male and female flight-tones, we recorded the phasic compound
459 receptor potential from male JO to continuous pairs of pure tones (N=9). The first
460 tone (f_1), with fixed frequency (796 Hz) and level ($4.3 \times 10^{-3} \text{ ms}^{-1}$, measured 2 mm
461 from the tip of the antenna; mean from 10 tethered flying male mosquitoes) intended
462 to simulate the average male flight-tone, was presented simultaneously with a
463 second tone (f_2) which varied in frequency and level and was intended to simulate
464 the flight-tone of a female mosquito. The two tones were delivered through separate
465 speakers, each placed 10mm from the antennae. The system distortion was 50dB
466 below the primaries. An example of the resulting compound electric intermodulation
467 distortion product (DP) (f_1-f_2) of a pair of tones is shown in Fig. 4A (Inset). The DP is
468 sometimes masked by spontaneous oscillations that occur in the vibrations of the
469 antenna and the electrical responses of the JO (Göpfert and Robert, 2001).
470 Examples of the magnitude of the compound electric DPs above the recording noise
471 floor are shown as a function of the particle velocity level of the female-like tone (f_2)
472 for different DP frequencies in Fig. 4A. Low frequency DPs (100–250 Hz), which
473 would occur via the interaction between the WBF of a male and those of a female
474 with unusually or unrealistically high WBF, have slopes close to unity throughout the
475 intensity range (Fig. 4A). The DPs generated through interaction between male and

476 female mosquitoes flying at their characteristic WBFs are > 100 times more
477 sensitive, with slopes ~ 0.4 and tend to saturate at high stimulus levels (Fig. 4A). The
478 DPs with frequencies between 440 – 700 Hz that would occur through interaction
479 between the flight-tones of a male and those of a female with unusually low and
480 unrealistic WBFs are very compressive with shallow slopes (Fig. 4A).

481 The quadratic curve fit derived from the behavioural threshold as a function of
482 the frequency difference (from Fig. 3E) was superimposed on the iso-level plots of
483 the magnitude of the JO compound electrical DPs as a function of frequency
484 difference ($f_1 - f_2$) (Fig. 4B). The central, most sensitive frequencies of both the
485 behavioural response and of the iso-level plots overlap noticeably, which suggests
486 that the JO of the male mosquito is tuned to detect DPs generated through the
487 frequency difference of male-female flight-tones and not to the female flight-tones
488 themselves. The conjunction of these behavioural and electrophysiological results,
489 including the correlation found between the male WBFs and the frequency of the
490 tones to which they are most strongly attracted (Fig. 3B), support the hypothesis that
491 male mosquitoes rely on their own flight-tones in making use of DPs to acoustically
492 detect and locate flying females by their flight-tones that fall within this frequency
493 'sweet spot'.

494

495

496 **Discussion**

497 We describe and quantify a new stereotypical behaviour of free-flying male *Culex*
498 *quinquefasciatus* mosquitoes in response to tone stimulation at frequencies within
499 the range of conspecific female flight-tones. Rapid Frequency Modulation (RFM),
500 which involves the fastest changes in WBF yet reported for a flying animal (~ 1950 Hz
501 s^{-1}), precedes copula formation but it is not dependent on acoustic feedback from the
502 female or her physical presence. The features and pattern of RFM are highly
503 conserved and consistent across males, even in response to artificial acoustic
504 signals that do not carry the multi-harmonic components of natural female flight-
505 tones. Significantly, RFM is not dependent on any specific frequency convergence of
506 the harmonic components of male WBFs and the sound source. This suggests that
507 RFM is a different behavioural process to that of harmonic convergence (Cator et al.,
508 2009; Warren et al., 2009; Pennetier et al., 2010).

509 Notably, the JO of male mosquitoes is tuned to frequencies around 280 Hz,
510 thus to frequencies ~150 Hz below the flight-tones of free-flying female mosquitoes.
511 These electrophysiological measurements are in accord with those obtained from the
512 closely related *C. pipiens pipiens* (Lapshin, 2012), and in part, with previous
513 measurements made from *C. quinquefasciatus* with lower sensitivity and higher
514 detection threshold criteria (Warren et al., 2009). The electrophysiological responses
515 recorded from the JO by Warren et al. (2009), Lapshin, (2012), and here are more
516 narrowly tuned than the nonlinear antennal mechanical responses that provide a
517 source for the DPs (Warren et al., 2009). While the bandwidth of the antennal
518 mechanical vibrations encompasses the flight-tones and their lower order harmonics
519 of male and female mosquitoes, the difference tone DPs generated by the nonlinear
520 vibration of the antenna, due to interaction between the male and female flight-tones,
521 fall within the sensitive frequency range of the JO, where they are amplified (Warren
522 et al., 2009; Pennetier, 2010). The frequency tuning of the JO compound receptor
523 potentials reported here is closely correlated with behavioural audiograms based on
524 the difference in frequency between the male and female flight-tones. The finding
525 that the JO is tuned sharply to intermodulation DPs at the difference frequency
526 between male and female flight-tones lends further support to the contention that
527 male mosquitoes detect females and exhibit stereotypical RFM behaviour by
528 detecting the frequency difference between their own and female flight-tones. The
529 observation that female-like artificial tones fail to elicit any response or attraction in
530 resting male mosquitoes (i. e., not flying) (Wishart and Riordan, 1959), which agrees
531 with our own unpublished observations, provides further support for this hypothesis.
532 It suggests that males must use their own flight-tones in order to acoustically detect,
533 recognize and locate flying females.

534 Electrical responses to DPs measured in the JO, and generated by the male-
535 female flight-tone frequency difference, become compressive with increasing
536 stimulus level. They are >100-fold more sensitive than those generated more than a
537 half octave lower in frequency, which increase linearly with level. The appearance of
538 compression in the DP level functions, which increases with frequency and level
539 from frequencies just below the resonant frequency, is reminiscent of non-linear
540 amplification and compression in the active mechanics of the mammalian cochlea
541 (Robles and Ruggero, 2001); perhaps an indication of shared principles of operation
542 in structures that share function but differ profoundly in structure and underlying

543 mechanisms. Mosquitoes are thus remarkable, if not unique, in exploiting their own
544 flight-tone to acoustically detect, locate and orientate towards flying females. In this
545 context, swarming behaviour expressed by some mosquito species, such as *C.*
546 *quinquefasciatus* (Gibson, 1985), could enable males to use the flight-tones created
547 by their station-keeping flight to detect and locate females as the latter join swarms.

548 How are these findings related to the harmonic convergence observed
549 between males and females of *Culex* (Warren et al., 2009) and other mosquito
550 species (Cator et al., 2009; Pennetier et al., 2010)? The fact that RFM appears as a
551 robust, open loop behaviour without the need for female interaction indicates is a
552 different behavioural process to that of harmonic convergence, which is a dynamic
553 interaction between male-female pairs. Another possibility arising from our results is
554 that harmonic convergence is based on the detection of intermodulation DPs
555 generated as a consequence of interaction between the fundamental frequencies of
556 the flight-tones of the two flying mosquitoes. In this case, harmonic convergence
557 might be an epiphenomenon; the unintended consequence of adjustments in the
558 fundamental flight-tones so that the resulting DPs fall within the optimal frequency
559 ranges for JO detection. In this way, pairs of fundamental frequencies that would
560 generate the most sensitive DPs to the male mosquito will convert, by multiplication,
561 to a particular integer-based convergence of the harmonics (e.g. 2♀:1♂, 3♀:2♂,
562 5♀:3♂ relations).

563 RFM behaviour is characterised by phonotaxis by the male towards the sound
564 source, around which it flies in tight loops. The exact function of the male's RFM
565 flight remains uncertain, but it is clearly a significant component of mosquito mating
566 behaviour and is likely to represent a pre-copulatory controlled flight to maintain a
567 close-range position while attempting to seize and engage terminalia with the female
568 (Roth, 1948; Wishart and Riordan, 1959; Charlwood and Jones, 1979) and/or a
569 specific and open loop sexual signal to the nearby female. Nonetheless, this highly
570 robust and stereotypical behaviour has enabled us to elucidate the sensory
571 mechanisms by which males detect the presence of females and could provide an
572 unusual opportunity to further investigate how mosquitoes integrate the demands of
573 flight and orientation with those for communication and hearing while on the wing.
574 Due to its extraordinary reliability, the RFM response has the potential to be the
575 basis for an acoustic trap for male mosquitoes and an important behavioural
576 assessment assay for the mating fitness of laboratory bred male mosquitoes,

577 especially in the context of quality control in programs based on male release
578 methods (Condon et al., 2007; Carvalho et al., 2014; Gilles, 2014; Lees et al., 2014;
579 Benelli, 2015; Diabaté and Tripet, 2015).

580

581

582 **List of abbreviations**

583 DP - Distortion Products

584 HCR - Harmonic Convergence Ratio

585 IR - Infra-Red

586 JO - Johnston's Organ

587 RFM - Rapid Frequency Modulation

588 WBF - Wing-Beat Frequency

589

590

591 **Acknowledgments**

592 We thank James Hartley for technical assistance and Andrei Lukashkin for
593 comments on the manuscript.

594

595

596 **Funding**

597 This work was supported by the Leverhulme Trust grant RPG/2012/783.

598

599

600 **Competing Interests**

601 No competing interests declared.

602

603

604 **Author contributions**

605 The experiments were conceived and designed by PMVS, GG and IJR. Experiments
606 were conducted and data was analysed by PMVS, GG and RAI. PMVS and IJR
607 wrote the paper with contributions from GG and RAI.

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Figure Legends

Figure 1. Acoustic behaviour of male *C. quinquefasciatus* in the presence of flying females and female-like artificial tone. A) Spectrogram of acoustic interactions in a male-female pair of free-flying mosquitoes. Right-side labels identify fundamental (f) and harmonic (nf) components of wing-beat frequencies (WBFs). The male's first rapid increase in WBF (green arrow) is followed by the rapid increase in the female's WBF (red arrow). Rapid Frequency Modulation (RFM) shows rapid oscillations around new higher mean WBF in male and female for several cycles. RFM always initiated by the male. B) Flight path and C) Spectrogram of male and tethered-flying female. White and green paths in B represent, respectively, spatial position of male before and after RFM (red path). Duration of these periods are indicated in the coloured bars above spectrogram in C. D) Spectrogram of fundamental WBF and lower harmonics of male during 5s, 500 Hz tone (lowest trace) that evoked RFM. White bars indicate duration of Latency, Onset (On.), Modulation phase (MP) and Offset. Inset: Fast Fourier Transforms of flight-tones during Latency, Onset, and Modulation phase of RFM. Stimulus tone shown at 500 Hz. Flight-tone peaks increase in frequency from Latency (blue dotted line) to Onset and broaden during Modulation phase as a result of oscillating WBFs.

Figure 2. Characterization of the Rapid Frequency Modulation (RFM) in free-flying male *C. quinquefasciatus*. A) Flight path and spectrogram of a male mosquito in flight arena during stimulation with 5s, 450Hz tone (Stim.). Right-side labels on spectrogram show fundamental (f) and harmonic (nf) components of male wing-beat frequencies (WBFs). Colour codes represent position of male during the Latency (yellow), Onset (orange), Modulation phase (red), and Offset (green). All sides of flight arena = 30 cm. See also Supplemental Movie S1. B) Close-up of flight path (blue line) near sound source and spectrogram during stimulation with 10s, 400Hz tone. Arrows on flight path indicate direction of flight. Lightened rectangle in spectrogram corresponds to the illustrated flight path. See also Supplemental Movie S2. C) Percentage of free-flying male mosquitoes (N=13) displaying RFM as a function of the stimulus frequency (10 s pure tone stimulation between 200-2500 Hz; sound intensity equal to that generated by tethered flying females: $\sim 5.7 \times 10^{-5} \text{ ms}^{-1}$ at a reference distance of 2 cm. See Materials and Methods).

Figure 3. Behavioural and electrophysiological tuning curves for male *C. quinquefasciatus*. A) Threshold of Rapid Frequency Modulation (RFM) behaviour (mean \pm s.e.m. expressed as the particle velocity of the sound stimulus measured 2 cm from the front of the speaker) as a function of stimulus frequency (N=6). Bandwidth measured 10 dB from the best frequency: 338-562 Hz. Grey shading: frequency range of free-flying female wing-beat frequencies (WBFs). ♀WBPv : mean particle velocity generated by the wing beats of tethered-flying females when measured 2 cm in front of the head ($5.7 \times 10^{-5} \text{ ms}^{-1}$). B) Correlation between WBF of responding males in (A) and stimulus frequency (Stimulus = $2.6 \times \text{♂WBF} - 1553$; Pearson's $r=0.69$). C) Relation between stimulus frequency that elicited RFM response and the Harmonic Convergence Ratio. Bubble areas are proportional to stimulus intensity. Dashed lines at the ratios 0.5 and 0.667 are equivalent, respectively, to $2\text{♀}:1\text{♂}$ and $3\text{♀}:2\text{♂}$ harmonic convergence. D) Detection threshold (measured 5 dB above noise floor) of the JO electrical response (mean \pm s.e.m. of particle velocity of the stimulus tone) as a function of tone frequency. Bandwidth measured 10 dB from the best frequency: 244-364 Hz. Grey shading: frequency range of WBF of free-flying females. E) Threshold (dB relative to the ♀WBPv ; $5.7 \times 10^{-5} \text{ ms}^{-1}$) of RFM behaviour as a function of stimulus tone frequency (blue curve) and as a function of the difference between the male's WBF measured before the onset of the acoustic behaviour and stimulus tone frequency (red scatter plot fitted with quadratic curve). Grey shading: 10 dB bandwidth of JO electrical response tuning curve.

Figure 4. Electric intermodulation distortion products (DPs) recorded from the JO of male *C. quinquefasciatus* in response to pairs of stimulus tones. A) Magnitude of the compound electrical DPs above the recording noise floor as a function of the particle velocity level of the variable tone (f_2) for different DP frequencies. The DPs were generated through interaction between the fixed male-like tone ($f_1=796 \text{ Hz}$, particle velocity = $4.3 \times 10^{-3} \text{ ms}^{-1}$) and the variable f_2 (range: 98.7 - 648.0 Hz). The two tones were delivered through separate speakers. System distortion was 50dB below primaries. Each measurement is the mean of 20 averages, and each point is the mean \pm s.e.m. of measurements from 9 mosquitoes. Inset: Example of a Fast Fourier Transform of the electrical response recorded from

the JO when stimulated by two tones ($f_1=796$ Hz, 4.3×10^{-3} ms⁻¹ and $f_2=500$ Hz, 8.5×10^{-4} ms⁻¹). The trace has peaks at f_1 , f_2 and at the frequency corresponding to DP of f_1-f_2 (296 Hz). B) Iso-level curves of the magnitude of the JO compound electrical DPs as a function of frequency difference (f_1-f_2). The dashed line represents the quadratic fit curve from the behavioural threshold as a function of the difference between WBF and stimulus frequency as in Fig. 3E.

Supplemental Figure S1. Further Rapid Frequency Modulation (RFM) characteristics and close-ups of flight paths during acoustic stimulation. A) Spectrogram of the fundamental wing-beat frequency (WBF) and lower harmonics of a male mosquito during a 5s, 300 Hz tone burst stimulation that evoked a RFM response. Grey bar corresponds to the duration of the Modulation phase; asterisks correspond to peaks of a single upward and downward cycle of frequency shift. The mean peak-to-peak duration of each cycle was calculated by dividing duration of Modulation phase by the number of cycle peaks. The Harmonic Convergence Ratio (HCR) was calculated by dividing stimulus frequency (green arrow) by the fundamental WBF of the free-flying male just prior to the onset of RFM elicited by the stimulus (red arrow). B) and C) Close-ups of the male flight paths (blue lines) near the sound source and their respective spectrograms during stimulation with a 10s at 450Hz or 500Hz tones. Arrows in the flight paths indicate direction of flight. Lightened rectangles in spectrograms correspond to the illustrated flight paths. Scale represents distance at the plane of the microphone and speaker, i.e. at the middle of the arena. See also Supplemental Movie S2.

Supplemental Figure S2. Spectrograms of the wing-beat frequencies (WBFs) of a single male *C. quinquefasciatus* in response to pure tone stimulation with different frequencies. A-H) WBF of male stimulated with 10 s of pure tones bursts at 260 Hz, 420 Hz, 520 Hz, 580 Hz, 900 Hz, 1200 Hz, 1700 Hz and 2100 Hz, respectively. The rapid frequency modulation (RFM) response is evident when the male is stimulated with 420 Hz, 520 Hz and 580 Hz. Acoustic responses were not elicited by the other stimulus frequencies and the WBF of the male remained stable.

Supplemental Movie S1. Video, audio and spectrogram recording of the flight of a male *C. quinquefasciatus* mosquito during Rapid Frequency Modulation

(RFM). The flight path during RFM is played at 50% speed. The LED represents the onset of a 5 s pure tone burst at 500 Hz.

Supplemental Movie S2. Three close up video, audio and spectrogram recordings of the flight of *C. quinquefasciatus* males during Rapid Frequency Modulation (RFM). Males were stimulated with pure tones at 400Hz, 450Hz and 500Hz, respectively. The LED represents the onset of a 10 s tone burst.







