

RESEARCH ARTICLE

A role for acoustic distortion in novel rapid frequency modulation behaviour in free-flying male mosquitoes

Patrício M. V. Simões^{1,*}, Robert A. Ingham¹, Gabriella Gibson² and Ian J. Russell^{1,*}

ABSTRACT

We describe a new stereotypical acoustic behaviour by male mosquitoes in response to the fundamental frequency of female flight tones during mating sequences. This male-specific free-flight behaviour consists of phonotactic flight beginning with a steep increase in wing-beat frequency (WBF) followed by rapid frequency modulation (RFM) of WBF in the lead up to copula formation. Male RFM behaviour involves remarkably fast changes in WBF and can be elicited without acoustic feedback or physical presence of the female. RFM features are highly consistent, even in response to artificial tones that do not carry the multi-harmonic components of natural female flight tones. Comparison between audiograms of the robust RFM behaviour and the electrical responses of the auditory Johnston's organ (JO) reveals that the male JO is tuned not to the female WBF per se but, remarkably, to the difference between the male and female WBFs. This difference is generated in the JO responses as a result of intermodulation distortion products (DPs) caused by non-linear interaction between male–female flight tones in the vibrations of the antenna. We propose that male mosquitoes rely on their own flight tones in making use of DPs to acoustically detect, locate and orientate towards flying females. We argue that the previously documented flight-tone harmonic convergence of flying male and female mosquitoes could be a consequence of WBF adjustments so that DPs generated through flight-tone interaction fall within the optimal frequency ranges for JO detection.

KEY WORDS: *Culex quinquefasciatus*, Mating behaviour, Insect hearing, Johnston's organ, Acoustic behaviour, Phonotaxis, Distortion products

INTRODUCTION

It has been known since the 19th century that male mosquitoes locate females by homing in on their flight tones, and that this behaviour can be elicited from males using artificial tones at frequencies within the range of female flight tones (Child, 1894; Mayer, 1874; Maxim, 1901; Roth, 1948; Belton, 1994). More recently, it has been shown in *Culex quinquefasciatus* (Warren et al., 2009) and other mosquito species (Cator et al., 2009; Pennetier et al., 2010) that one component of the mating chase involves an interactive behaviour that appears to lead to frequency convergence of the harmonic components of the flight tones of the two sexes, possibly as a mechanism for recognition of conspecific mating partners. Earlier reports (Kahn et al., 1945; Roth, 1948; Wishart and

Riordan, 1959; Belton, 1994) indicate that the wing-beat frequencies (WBFs) of male mosquitoes fluctuate during the final approach to a female (or artificial sound source simulating a female), but these acoustic changes were not fully described or analysed.

Mosquitoes possess one of the most sensitive hearing organs in the animal kingdom (Göpfert et al., 1999; Göpfert and Robert, 2000; Gibson and Russell, 2006); near-field acoustic stimulation causes antennal vibrations which are conducted via an arrangement of extracellular spokes in the pedicel to the many thousands of mechanosensitive scolopidia that constitute the highly sensitive auditory (Johnston's) organ (JO) (Belton, 1974; Clements, 1999; Göpfert et al., 1999; Göpfert and Robert, 2000). The antennae also vibrate in response to simultaneous acoustic stimulation from the mosquito's own flight tones and those of a mosquito of the same or opposite sex; interaction between pairs of tones generates strongly amplified intermodulation distortion products (DPs) that can be detected by measuring the vibrations of the antenna and the electrical responses of the JO (Warren et al., 2009; Pennetier et al., 2010; Lapshin, 2012). The frequency characteristics of the antennal vibrations and the electrical responses of the JO of *C. quinquefasciatus* have been described (Warren et al., 2009). However, to our knowledge, there has not been a quantitative study relating the auditory physiology of *C. quinquefasciatus* with the acoustic behaviour of males in response to the sounds they hear in free flight during the final phases of mating (i.e. the male's own flight tones plus those of the female).

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

MATERIALS AND METHODS

Mosquitoes

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

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List of abbreviations

DP	distortion product
HCR	harmonic convergence ratio
IR	infra-red
JO	Johnston's organ
RFM	rapid frequency modulation
WBF	wing-beat frequency

Behavioural set-up

Sound recordings of single free-flying male and virgin female pairs were made under semi-natural conditions in a large (1.5 m wide and deep, 2 m high) flight arena in a double-skin sound-attenuated booth (IAC Ltd, Winchester, UK). The flight arena was equipped with Basler[®] Pilot Ace, GigE video cameras (Basler AG) and a Røde[®] NT1 pressure microphone mounted at the focal point of a 24 in (~610 mm) parabolic reflector (Edmunds) to record behaviour (see details below), kept at constant environmental conditions and provided with dusk light levels typical during natural mating behaviour. A swarming marker (black disc, 13 cm radius) was placed on the floor of the arena to stimulate flight behaviour typical of swarming; an individual mosquito can be said to 'swarm' by flying in controlled loops, in essence 'station-keeping' with respect to the visual environment (Gibson, 1985). For each recording, one male was released into the flight arena first, and within 3 min the male started to swarm spontaneously. Once swarming flight was established, the female was released and also started to swarm over the marker. Their flight tones were recorded throughout this process for further analysis. Copula formation was verified via TrackIt 3D[®] (SciTrackS, GmbH) zoom tracking software that displayed a full-screen image of each mosquito in real time.

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

Artificially generated tone stimuli were delivered to the cage from a sound source consisting of a 0.5 cm diameter plastic probe tip, damped with acoustic foam, connected via a 1 cm diameter polythene tube to an adapted Audio Technica[®] ATH A700AX speaker (5–35,000 Hz range with flat frequency response 100–25,000 Hz). Sound from the speaker and flight tones from the mosquitoes were monitored using a particle velocity microphone (Knowles NR-3158, Ithaca, NY, USA) that was calibrated (Göpfert and Robert, 2001) and mounted ~4 cm from the speaker probe tip. For the small flight arenas, a pressure microphone (Knowles 23132) mounted at the focal point of an 18 in (~457 mm) parabolic reflector (Edmunds), was placed on one side of the arena to monitor the sound inside. Signals from each of the microphones were amplified 100-fold with a purpose-built two-channel preamplifier and the output of each channel was digitized at 192 kHz using a Fireface[®] UC sound card. The digital outputs were then recorded using Spectrogram 16 (Visualization Software, LLC) at a sampling rate of

48 kHz and frequency resolution of 5.9 Hz. Spectrogram 16 was also used to analyse and extract data on the time, frequency and amplitude of all acoustic signals.

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

For video recordings, an IR-video camera (Swann[®] Pro-880) was placed 30 cm in front of the clear wall of the chamber and connected to the computer. Digital video recordings at 30 frames s^{-1} of the flying mosquitoes were obtained using Debut Video Capture software v1.88 (NCH[®] software). The video-recorded flight paths were then digitized using Kinovea (v0.8.23) software. The synchronized video-spectrogram sequences in the supplementary movies were composed using Adobe[®] After Effects.

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

Electrophysiology

Mosquitoes were immobilized by cold narcosis and fixed with beeswax to a small brass block. The pedicel, head and legs were fixed using superglue (Loctite[®]). Sound was delivered to the preparation from a pair of modified DT48 headphone speakers, each coupled to the preparation via separate 7 mm (i.d.) plastic tubes. The point of each tube was positioned 10 mm from the mosquito on opposite sides of the head. Compound extracellular receptor potentials were measured from the JO with tungsten electrodes (5–7 M Ω , 1 μm tip, part no. WE30032.OH3, MicroProbes, Gaithersburg, MD, USA) that were advanced with a Märzhäuser (GmbH) PM10 manipulator so that the tip of the electrode just penetrated the wall of the pedicel. In this location, voltage responses from the JO are dominated by compound, phasic receptor potentials from the scolopidia that are twice the frequency of the acoustic stimulus (Tischner, 1953; Belton, 1974; Clements, 1999). All measurements were made on a vibration-damped table (model: M-VW-3036-OPT-99-9-28-92, Newport Corporation) inside an IAC sound-attenuated booth. Signals from the electrodes were amplified (10,000-fold) and low-pass filtered (5 kHz) using a laboratory designed and built differential pre-amplifier. Pure tones of 82 ms duration with 8 ms rise/fall time were delivered via a 5 kHz low-pass filter and calibrated against a known 94 dB sound pressure level (SPL; Bruel & Kjaer 4230; Göpfert and Robert, 2001). Voltage signals for the sound system were generated and voltage signals from the electrodes were digitized at 250 kHz via a Data Translation 3010 D/A A/D card using programs written in Matlab. Raw data and online computation of the magnitude and phase of the phasic voltage signals were stored in ASCII files for display and further analysis. All recordings were made under

controlled conditions, and within 30 min of preparation to ensure excellent physiological state and hearing sensitivity. Temperature control for the physiological experiments was provided by placing the mosquito in a chamber machined in a Peltier-controlled heat sink (Warren et al., 2010). Current was fed to the Peltier element by a power supply with a negative feedback control from a thermistor (80TK, Fluke[®]) which was thermally coupled to the chamber.

RESULTS

Acoustic behaviour of free-flying male–female pairs

The WBF of male–female mosquito pairs ($N=7$) was recorded using a parabolic microphone while mosquitoes were free-flying above a visual marker inside a large sound-attenuated booth with ambient illumination adjusted to dusk, the natural condition for mating swarms (Gibson, 1985). The spectrograms of these flight sequences (Fig. 1A) showed that the mean \pm s.e.m. WBF of males was 789 ± 10 Hz and that of females was 474 ± 10 Hz. After flying simultaneously for a variable length of time, in all cases the WBF of the male and subsequently that of the female increased steeply, followed by intense frequency oscillations at the elevated frequency which lasted a few seconds (4466 ± 883 ms for males, 3939 ± 959 ms for females). Significantly, in all recorded sequences, the steep increase in frequency was initiated by the male mosquito (Fig. 1A, green arrow), followed 682 ± 120 ms later by an increase in the female's WBF (Fig. 1A, red arrow). Video recordings of these mating chases revealed that a copula was formed during these rapid frequency oscillations.

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

To further examine this free-flight interaction, the flight tones and 2D flight paths of male mosquitoes ($N=9$) were recorded in the

presence of a tethered-flying female under IR illumination in the smaller flight arena. The duration of the recorded sequences when the male and female were flying simultaneously ranged between ~ 1.5 and ~ 11 min, during which the mean \pm s.e.m. of the male's WBF was 739 ± 5 Hz and that of the tethered female was 411 ± 5 Hz (WBF sampled every 30 s). There was an initial latency period of variable duration during which the male displayed continuous looping flight, with no obvious deviation towards the tethered-flying female. The male then approached the tethered female repetitively and displayed a characteristic modulation of his WBF while flying in close proximity to or touching the female (Fig. 1B,C). This behaviour was initiated by a steep increase in the male's WBF followed by rapid WBF oscillations while he was within ~ 4 cm of or touching the tethered female. The male would then cease WBF oscillations and gradually decrease his WBF as he departed from the female (Fig. 1B,C). Each male displayed this behaviour on average 6.2 ± 1.0 times per minute, while flying continuously. In contrast, when the tethered female was prevented from flying by using the tarsal reflex (by positioning a small piece of paper under her legs), males ($N=3$) did not display any conspicuous changes in WBF or attempt to approach the female during sequences lasting ~ 14 min. The tethered female occasionally increased her mean WBF and also oscillated her WBF rapidly (Fig. 1B). However, this was observed to occur only as a direct consequence of physical contact by the male. That the initiation of WBF modulation is a male-specific response was confirmed by releasing a virgin free-flying female in the presence of a tethered-flying male; in all of the recorded sequences ($N=3$), females displayed continuous looping flight for several minutes without ever being attracted to the tethered male or exhibiting any conspicuous changes in acoustic or flight behaviour in response to the male. It was noted, however, that tethered males did not display rapid

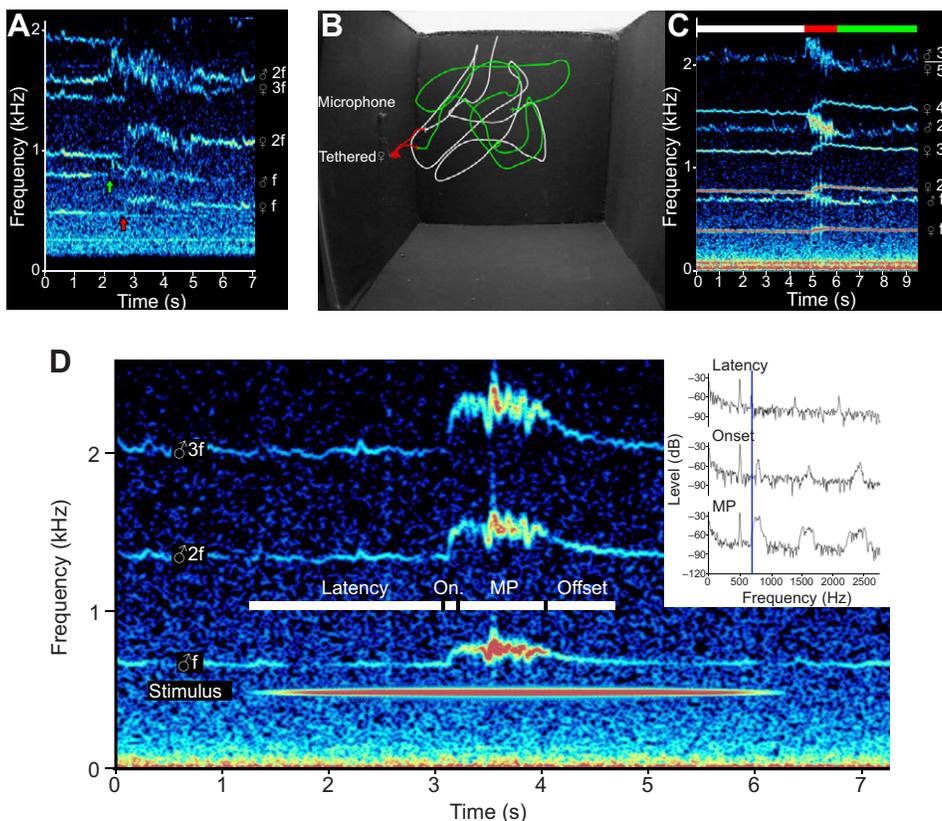


Fig. 1. Acoustic behaviour of male *Culex quinquefasciatus* in the presence of flying females and female-like artificial tone.

(A) Spectrogram of acoustic interactions in a male–female pair of free-flying mosquitoes. Labels on the right 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 a rapid increase in the female's WBF (red arrow). Rapid frequency modulation (RFM) shows rapid oscillations around the new higher mean WBF in the male and female for several cycles. RFM was always initiated by the male. (B) Flight path and (C) spectrogram of a male and tethered-flying female. White and green paths in B represent, respectively, the spatial position of the male before and after RFM (red path). The duration of these periods is indicated in the coloured bars above the spectrogram in C. (D) Spectrogram of fundamental WBF and lower harmonics of a male during a 5 s, 500 Hz tone (lowest trace) that evoked RFM. White bars indicate the duration of latency, onset (On.), modulation phase (MP) and offset. Inset: fast Fourier transforms of flight tones during latency, onset and modulation phase of the RFM. Stimulus tone is 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.

modulation of their WBF as they would have done in free flight. It appears, therefore, that tethering enables sustained flight but impairs the exhibition of the RFM behaviour in male mosquitoes.

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

Characterization of male acoustic behaviour

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

The mean \pm s.e.m. WBF measured immediately before tone stimulation was 742 \pm 9 Hz. These WBFs were \sim 200 Hz higher than those previously reported for tethered-flying males of the same species (Warren et al., 2009), probably because in the current experiments males were free flying and the ambient temperature was higher (30°C compared with tethered flight at 22–24°C in Warren et al., 2009). During a variable period that we termed ‘latency’ (time from tone initiation to the onset of the acoustic behaviour), the male’s WBF remained essentially unchanged (Δ WBF=2 \pm 1 Hz; Fig. 1D). A likely cause of the highly variable latency (range: 161–3510 ms, mean: 1479 \pm 94 ms) was variation in the distance between the male and the sound source at tone onset; males flying near the sound source at tone onset would have detected the stimulus sooner than mosquitoes flying near the walls of the arena.

The onset phase of RFM was initiated with a steep increase in WBF of 85 \pm 3 Hz in 327 \pm 37 ms, equivalent to a rate of change of \sim 260 Hz s $^{-1}$. Onset was followed by the modulation phase, which lasted 1148 \pm 79 ms. During the modulation phase, the WBFs were frequency modulated as indicated by the greater bandwidth (measured 10 dB from the peaks) of the fast Fourier transforms of the fundamental frequencies of the flight tones compared with that measured during latency (Fig. 1D, inset); the 10 dB bandwidth during the modulation phase was 87 \pm 6 Hz, significantly higher than the 25 \pm 1 Hz during latency (paired Student’s *t*-test, *t*=12.31, *N*=30, *P*<0.001). As shown in the spectrograms in Fig. 1, the frequency modulations, which are more clearly visualized in the higher harmonics of the WBFs, comprise fast and variable upward and downward shifts in frequency that ranged from \sim 20 to 200 Hz in amplitude at the fundamental frequency (Fig. 1D; Fig. S1A). The number of frequency modulations varied from 3 to 14 cycles during the modulation phase. On average, the peak-to-peak interval between the frequency modulations (calculated by dividing the duration of the modulation phase by the number of peaks; see Fig. S1A) was 204 \pm 9 ms, i.e. a rate of \sim 5 s $^{-1}$. The resulting conversion of these peak-to-peak values indicates that during RFM male mosquitoes, remarkably, modulate their WBF at a rate of up to \sim 1950 Hz s $^{-1}$.

The modulation phase was followed by the offset phase, during which the WBFs gradually decreased over a period lasting

1246 \pm 64 ms, until they reached a final WBF only 2 \pm 1 Hz higher than that during latency (Fig. 1D). The duration of RFM behaviour, from the onset (steep frequency spike) until the offset (end of the final frequency drop) was 2722 \pm 104 ms (range: 1141–4638 ms). The duration and variation in frequency of the RFM and its constituent components (onset, modulation phase and offset) were independent of the stimulus frequency (ANOVA *F*_{1,20}<1.64, *P*>0.081) and of the initial WBF of the male (ANOVA *F*_{1,20}<1.73, *P*>0.075).

In contrast, no conspicuous acoustic interactions or RFM behaviour were observed in virgin free-flying females (*N*=7) stimulated with 5 s artificial pure-tone bursts with frequencies ranging from 200 to 2000 Hz, which further suggests that the changes in WBF observed in free- and tethered-flying females (Fig. 1A and B, respectively) were in response to the physical contact with the male, rather than in response to auditory stimuli.

Flight patterns during RFM behaviour

The flight paths of male mosquitoes recorded during RFM behaviour were examined (Fig. 2A; Movie 1). Before sound stimulation with artificial pure tones on the range of fundamental WBFs of free-flying females, males typically flew in large loops around most of the volume of the chamber (white path in Movie 1). During latency, which started at tone initiation (Fig. 2A, yellow path), the flight paths were confined mainly to the centre of the chamber in slow, station-keeping flight without looping. The onset of the acoustic response (Fig. 2A, orange path) was associated with the beginning of the phonotactic approach to the sound source. The modulation phase (Fig. 2A, red path) occurred when the male mosquito was within close proximity (\sim 4 cm or less) to the sound source and displaying tight looping flight paths around the sound source. In some interactions, the male touched or even landed on the source without ceasing RFM. The offset (Fig. 2A, green path) coincided with departure of the male from the vicinity of the sound source. Close-up video recordings of the flight behaviour of males when near the sound source during the modulation phase confirmed that male mosquitoes displayed a series of short, tight loops around the sound source (Fig. 2B; Fig. S1B,C, Movie 2). During the modulation phase, it was also observed that males extended and moved their legs, trying to grasp the sound source while flying continuously (Movie 2).

On the basis of these results, we propose that RFM in males is an acoustically driven behaviour in response to female flight tones. This behaviour comprises (i) the onset phase, characterized by a steep increase in WBF and associated with phonotaxis towards the sound source, followed immediately by (ii) the modulation phase, when the mosquito is in close proximity to the sound source and the elevated WBF is rapidly frequency modulated (appearing in spectrograms as a series of increases and decreases of variable duration about the elevated WBF) and (iii) the offset phase, when the male moves away from the sound source and gradually decreases his WBF until it is similar to that during latency. The total duration of RFM behaviour is variable, from just over one to several seconds.

Frequency range of RFM response

The frequency range of RFM behaviour was obtained by recording the responses of individual free-flying male mosquitoes (*N*=13) when presented with single-tone bursts between 200 and 2500 Hz (20 Hz increments for frequencies between 200 and 800 Hz, 100 Hz increments between 800 and 2500 Hz). The tone burst duration was 10 s, with an inter-burst interval of \sim 5 s and with a sound intensity equal to that generated 2 cm in front of the head of a tethered-flying female (5.7 \times 10 $^{-5}$ m s $^{-1}$). Each of the resulting 48 stimuli was

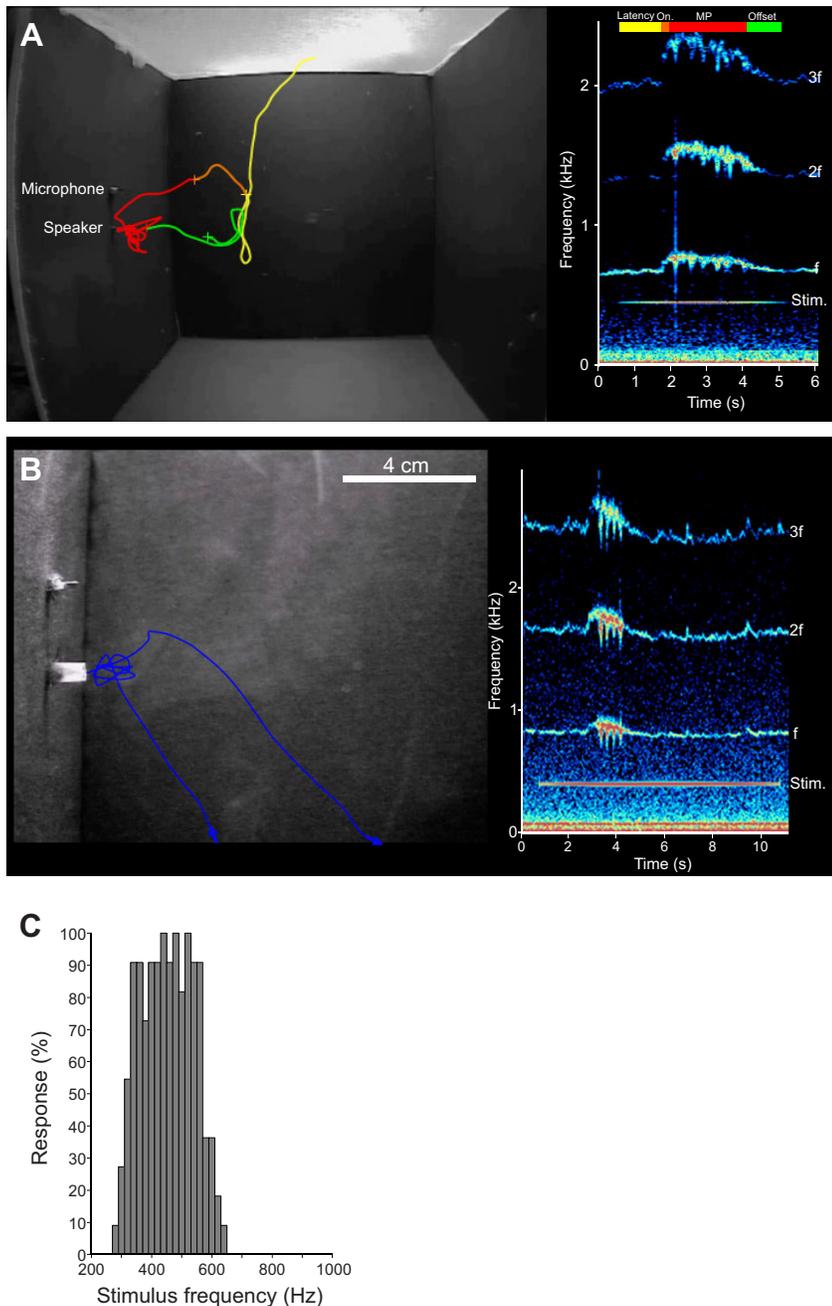


Fig. 2. Characterization of the RFM in free-flying male *C. quinquefasciatus*. (A) Flight path and spectrogram of a male mosquito in the flight arena during stimulation with a 5 s, 450 Hz tone (Stim.). Labels on the right of the spectrogram show fundamental (f) and harmonic (nf) components of male WBFs. Colour codes represent the position of the male during the latency (yellow), onset (orange), modulation phase (red) and offset (green). All sides of the flight arena were 30 cm in length. See also Movie 1. (B) Close-up of the flight path (blue line) near the sound source and spectrogram during stimulation with a 10 s, 400 Hz tone. Arrows on the flight path indicate the direction of flight. The lightened rectangle in the spectrogram corresponds to the illustrated flight path. See also Movie 2. (C) Percentage of free-flying male mosquitoes ($N=13$) displaying RFM as a function of stimulus frequency (10 s pure-tone stimulation between 200 and 2500 Hz; sound intensity equal to that generated by tethered-flying females: $\sim 5.7 \times 10^{-5} \text{ m s}^{-1}$ at a reference distance of 2 cm. See Materials and methods).

presented randomly and only once to each male. Males exhibited RFM responses to tones at frequencies between 280 and 640 Hz and were unresponsive to tone frequencies below and above this range (Fig. 2C; Fig. S2). Within the responsive range, more than 75% of males exhibited an RFM response to tones between 340 and 540 Hz, a frequency range that encompasses the WBF range of conspecific free-flying female mosquitoes (430–527 Hz, $N=30$), which indicates that the male's RFM response is elicited by the detection of tones of similar frequencies to the fundamental WBF of the female.

To determine the percentage of RFM responses within individuals, free-flying male mosquitoes ($N=7$) were presented with seven consecutive tone bursts (10 s duration, inter-trial interval of 5 s). For tones at 460 Hz, each male responded on average to $96 \pm 3\%$ of the presented stimuli, but no responses were observed when the tone was 860 Hz. Thus, RFM behaviour is highly repeatable

when the stimulus frequency is similar to a female's WBF. When the duration of each of the 460 Hz consecutive tone bursts was reduced to only 1 s, each male responded on average to $45 \pm 7\%$ of the presented stimuli. Although significantly lower than the proportion of responses to the 10 s tone bursts (unpaired Student's t -test, $t=6.60$, $N=7$, $P<0.001$), the robustness of RFM behaviour to short (1 s) tone bursts remains high.

Behavioural threshold of RFM response

A behavioural audiogram of male mosquitoes (i.e. the threshold of the RFM response relative to the particle velocity of the sound stimulus) was obtained for stimulus frequencies between 200 and 1000 Hz (20 Hz increments). For each replicate ($N=6$), a group of 7–10 males was placed in the flight arena under illumination simulating dusk, when they are normally active. Upon initiation of spontaneous flight, a continuous tone of fixed frequency was

presented to the swarming mosquitoes. The tone level was increased at a rate of 0.4 dB s^{-1} from $\sim 1 \times 10^{-8} \text{ m s}^{-1}$ output until an RFM response was elicited from at least one male or until the maximum operating level ($4 \times 10^{-4} \text{ m s}^{-1}$) was reached. The sound stimulus was then terminated and the particle velocity that elicited the response and the WBF of the responding male immediately before the onset of RFM (see Fig. S1A, red arrow) were stored. Particle velocity values are relative to a reference distance of 2 cm away from the speaker. After a 5–10 s rest period without stimulation, the procedure was repeated for another stimulus frequency. Even when several males were swarming at the same time, the spectrogram analysis permitted the detection and isolation of the RFM response of individual males because the response was much louder (because of their proximity to the particle velocity microphone) than the humming of the swarm in the background. The presence of higher harmonics of flight tones provided a further basis for distinguishing between the WBFs of individual males.

The behavioural audiogram (Fig. 3A) shows that the lowest and highest frequencies that elicited an RFM response were 260 and 720 Hz, respectively. Tone frequencies between 340 and 560 Hz elicited responses at the lowest thresholds (ANOVA $F_{1,23}=14.64$, $P<0.001$), and encompass the range of WBFs for conspecific free-flying female mosquitoes (430–527 Hz, $492 \pm 4 \text{ Hz}$, $N=30$; Fig. 3A, grey shaded area). RFM is thus very robust and responses are

elicited to tones at frequencies that exceed the upper and lower range of female WBFs by $\sim 190 \text{ Hz}$, but only at very high levels. Within the range of the most sensitive frequencies, male mosquitoes responded to particle velocities between 4.8×10^{-7} and $1.3 \times 10^{-6} \text{ m s}^{-1}$ (Fig. 3A), which are $\sim 40 \text{ dB}$ below the average sound intensity generated by tethered-flying females 2 cm in front of their heads ($5.7 \times 10^{-5} \pm 1.9 \times 10^{-6} \text{ m s}^{-1}$, see Materials and methods).

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

How might RFM behaviour be related to harmonic convergence of male and female flight tones, as described for *C. quinquefasciatus* (Warren et al., 2009) and other mosquito species (Cator et al., 2009; Pennetier et al., 2010)? We calculated the harmonic convergence ratio (HCR) by dividing the stimulus frequency (which simulates the WBF of a female) by the male's WBF just prior to the onset of RFM elicited by the stimulus (Fig. S1A). The inverse of the HCR corresponds to the harmonic

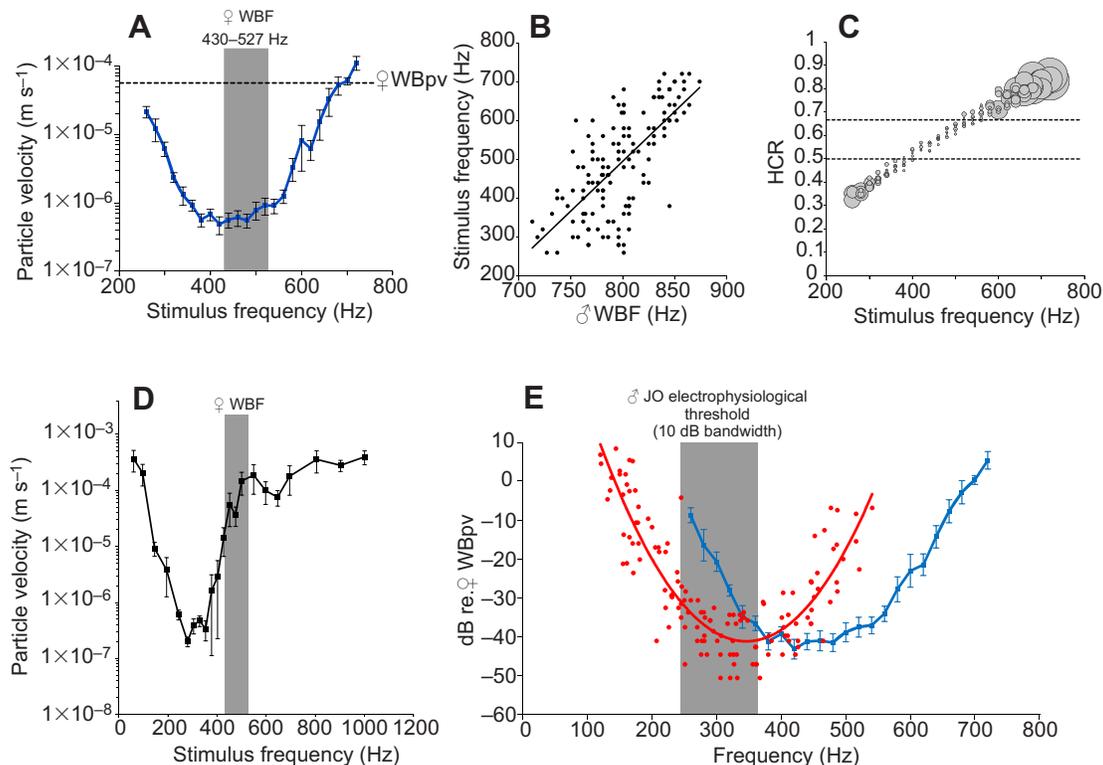


Fig. 3. Behavioural and electrophysiological tuning curves for male *C. quinquefasciatus*. (A) Threshold of RFM behaviour (means \pm s.e.m. expressed as the particle velocity of the sound stimulus measured 2 cm from the front of the speaker) as a function of stimulus frequency ($N=6$). Bandwidth measured 10 dB from the best frequency: 338–562 Hz. Grey shading: frequency range of free-flying female WBFs. φ WBPv: mean particle velocity generated by the wing beats of tethered-flying females when measured 2 cm in front of the head ($5.7 \times 10^{-5} \text{ m s}^{-1}$). (B) Correlation between WBF of responding males in A and stimulus frequency (stimulus = $2.6 \times \delta \text{WBF} - 1553$; Pearson's $r=0.69$). (C) Relationship between the stimulus frequency that elicited the 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 $2\varphi:1\delta$ and $3\varphi:2\delta$ harmonic convergence. (D) Detection threshold (measured 5 dB above noise floor) of the Johnston's organ (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 φ WBPv: $5.7 \times 10^{-5} \text{ m s}^{-1}$) 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.

relationship of the two sound frequencies; for example, $HCR=0.5=1/2$ indicates a 2:1 harmonic relationship, i.e. the frequency of the 2nd harmonic of the female-like sound is equal to the male's fundamental WBF, whereas $HCR=0.667=2/3$ indicates a 3:2 harmonic relationship, which would correspond to a frequency convergence between the 3rd harmonic of the stimulus and the 2nd harmonic of the male's WBF. Although the stimulus frequencies were sinusoidal pure tones, harmonics of these pure tones are produced in the vibrations of the male's antenna and JO upon sound detection, so males can potentially use these tones to reach harmonic convergence (Cator et al., 2009; Warren et al., 2009; Pennetier et al., 2010). The HCRs, plotted as a function of the stimulus frequency are not centred on any particular value, but rather increase proportionally with stimulus frequency (Fig. 3C), which indicates that the initiation of the RFM response by males is independent of any harmonic convergence between their flight tones and the stimulus. Interestingly, the most sensitive RFM responses (elicited by low particle velocity levels, as indicated by bubble areas in Fig. 3C) are distributed between HCRs of 0.5 and 0.667 (Fig. 3, dashed lines), values that correspond, respectively, to 2♀:1♂ and 3♀:2♂ harmonic convergences.

Comparison between behavioural and JO frequency tuning

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

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

The JO of male mosquitoes is tuned to difference tones generated through interaction between male and female flight tones

To test the hypothesis that hearing in male mosquitoes is tuned to the frequency difference between male and female flight tones, we recorded the phasic compound receptor potential from male JO to

continuous pairs of pure tones ($N=9$). The first tone (f_1), with fixed frequency (796 Hz) and level ($4.3\times 10^{-3}\text{ m s}^{-1}$, measured 2 mm from the tip of the antenna; mean from 10 tethered-flying male mosquitoes) intended to simulate the average male flight tone, was presented simultaneously with a second tone (f_2) which varied in frequency and level and was intended to simulate the flight tone of a female mosquito. The two tones were delivered through separate speakers, each placed 10 mm from the antennae. The system distortion was 50 dB below the primaries. An example of the resulting compound electric intermodulation DP (f_1-f_2) of a pair of tones is shown in Fig. 4A (inset). The DP is sometimes masked by spontaneous oscillations that occur in the vibrations of the antenna and the electrical responses of the JO (Göpfert and Robert, 2001). Examples of the magnitude of the compound electric DPs above the

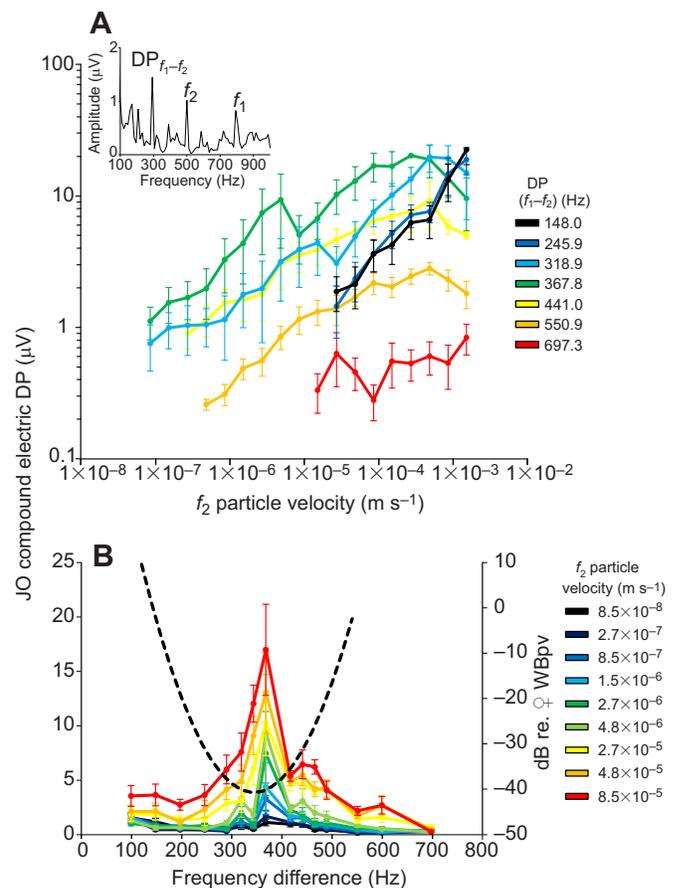


Fig. 4. Electric intermodulation distortion products recorded from the JO of male *C. quinquefasciatus* in response to pairs of stimulus tones.

(A) Magnitude of the compound electrical distortion products (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= $4.3\times 10^{-3}\text{ m s}^{-1}$) and the variable f_2 (range: 98.7–648.0 Hz). The two tones were delivered through separate speakers. System distortion was 50 dB below primaries. Each measurement is the mean of 20 averages, and each point is the mean \pm s.e.m. of measurements from nine mosquitoes. Inset: example of a fast Fourier transform of the electrical response recorded from the JO when stimulated by two tones ($f_1=796\text{ Hz}$, $4.3\times 10^{-3}\text{ m s}^{-1}$ and $f_2=500\text{ Hz}$, $8.5\times 10^{-4}\text{ m s}^{-1}$). The trace has peaks at f_1 , f_2 and at the frequency corresponding to the 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.

recording noise floor are shown as a function of the particle velocity level of the female-like tone (f_2) for different DP frequencies in Fig. 4A. Low-frequency DPs (100–250 Hz), which would occur via the interaction between the WBF of a male and those of a female with unusually or unrealistically high WBF, have slopes close to unity throughout the intensity range (Fig. 4A). The DPs generated through interaction between male and female mosquitoes flying at their characteristic WBFs are >100 times more sensitive, with slopes of ~ 0.4 and tend to saturate at high stimulus levels (Fig. 4A). The DPs with frequencies between 440 and 700 Hz that would occur through interaction between the flight tones of a male and those of a female with unusually low and unrealistic WBFs are very compressive with shallow slopes (Fig. 4A).

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

DISCUSSION

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

Notably, the JO of male mosquitoes is tuned to frequencies around 280 Hz, and thus to frequencies ~ 150 Hz below the flight tones of free-flying female mosquitoes. These electrophysiological measurements are in accord with those obtained from the closely related *Culex pipiens pipiens* (Lapshin, 2012) and, in part, with previous measurements made from *C. quinquefasciatus* with lower sensitivity and higher detection threshold criteria (Warren et al., 2009). The electrophysiological responses recorded from the JO by Warren et al. (2009) and Lapshin, (2012), and here are more narrowly tuned than the non-linear antennal mechanical responses that provide a source for the DPs (Warren et al., 2009). While the bandwidth of the antennal mechanical vibrations encompasses the flight tones and their lower order harmonics of male and female mosquitoes, the difference tone DPs generated by the non-linear vibration of the antenna, as a result of interaction between the male and female flight tones, fall within the sensitive frequency range of

the JO, where they are amplified (Warren et al., 2009; Pennetier et al., 2010). The frequency tuning of the JO compound receptor potentials reported here is closely correlated with behavioural audiograms based on the difference in frequency between the male and female flight tones. The finding that the JO is tuned sharply to intermodulation DPs at the difference frequency between male and female flight tones lends further support to the contention that male mosquitoes detect females and exhibit stereotypical RFM behaviour by detecting the frequency difference between their own and female flight tones. The observation that female-like artificial tones fail to elicit any response or attraction in resting male mosquitoes (i.e. not flying; Wishart and Riordan, 1959), which agrees with our own unpublished observations, provides further support for this hypothesis. It suggests that males must use their own flight tones in order to acoustically detect, recognize and locate flying females.

Electrical responses to DPs measured in the JO, and generated by the male–female flight-tone frequency difference, become compressive with increasing stimulus level. They are >100-fold more sensitive than those generated more than half an octave lower in frequency, which increase linearly with level. The appearance of compression in the DP level functions, which increases with frequency and level from frequencies just below the resonant frequency, is reminiscent of non-linear amplification and compression in the active mechanics of the mammalian cochlea (Robles and Ruggero, 2001) – perhaps an indication of shared principles of operation in structures that share function but differ profoundly in structure and underlying mechanisms. Mosquitoes are thus remarkable, if not unique, in exploiting their own flight tone to acoustically detect, locate and orientate towards flying females. In this context, swarming behaviour expressed by some mosquito species, such as *C. quinquefasciatus* (Gibson, 1985), could enable males to use the flight tones created by their station-keeping flight to detect and locate females as the latter join swarms.

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

RFM behaviour is characterized by phonotaxis of the male towards the sound source, around which it flies in tight loops. The exact function of the male’s RFM flight remains uncertain, but it is clearly a significant component of mosquito mating behaviour and is likely to represent a pre-copulatory controlled flight to maintain a close-range position while attempting to seize and engage terminalia with the female (Roth, 1948; Wishart and Riordan, 1959; Charlwood and Jones, 1979) and/or a specific and open-loop sexual signal to the nearby female. Nonetheless, this highly robust and stereotypical behaviour has enabled us to elucidate the sensory

mechanisms by which males detect the presence of females and could provide an unusual opportunity to further investigate how mosquitoes integrate the demands of flight and orientation with those for communication and hearing while on the wing. Because of its extraordinary reliability, the RFM response has the potential to be the basis for an acoustic trap for male mosquitoes and an important behavioural assessment assay for the mating fitness of laboratory-bred male mosquitoes, especially in the context of quality control in programmes based on male release methods (Condon et al., 2007; Carvalho et al., 2014; Lees et al., 2014; Benelli, 2015; Diabate and Tripet, 2015).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

The experiments were conceived and designed by P.M.V.S., G.G. and I.J.R. Experiments were conducted and data was analysed by P.M.V.S., G.G. and R.A.I.P. M.V.S. and I.J.R. wrote the paper with contributions from G.G. and R.A.I.

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Supplementary information

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