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Harmonic convergence in the love songs of the dengue vector mosquito

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Abstract

The familiar buzz of flying mosquitoes is an important mating signal, with the fundamental frequency of the female's flight tone signalling her presence. In the yellow fever and dengue vector, *Aedes aegypti*, both sexes interact acoustically by shifting their flight tones to match, resulting in a courtship duet. Surprisingly, matching is made not at the fundamental frequency of 400 Hz (female) or 600 Hz (male), but at a shared harmonic of 1200 Hz, which exceeds the previously known upper limit of hearing in mosquitoes. Physiological recordings from Johnston's organ (the mosquito's "ear") reveal sensitivity up to 2000 Hz, consistent with our observed courtship behavior. These findings revise widely accepted limits of acoustic behavior in mosquitoes.

Mosquito-borne diseases such as malaria, yellow fever, and dengue continue to afflict millions, even after decades of work to control vector populations. Despite this effort, basic aspects of mosquito biology are not fully understood, including mating behavior, an important target for vector control. We now describe investigations in *A. aegypti* that require revision of the current understanding of mosquito mating behavior. Since Johnston(1) first suggested in 1855 that mosquitoes could perceive sound, over 14 studies have been published on sound production and hearing in *A. aegypti* (2-17)(Table S1). The buzz of a flying female mosquito acts as a mating signal, attracting males. Typically, the behaviourally salient frequency component of flight tone is the fundamental frequency of wing beat, between 300-600 Hz depending on species (8). However, mate attraction is not simply a matter of a male passively hearing and homing in on a 400 Hz tone. For example, males and females of the non-blood feeding mosquito, *Toxorhynchites brevialpis*, modulate their 300-500 Hz wing beat frequencies to match each other (18). Thus, acoustically-mediated mate attraction involves active modulation by both sexes, creating a duet.

We show here that males and females of the dengue and yellow fever vector *A. aegypti* also modulate their flight tones when brought within a few centimeters of each other. This modulation, however, does not match the fundamental wing beat frequency of around 400 Hz (female) or around 600 Hz (male), but a shared harmonic of around 1200 Hz (Fig 1). Consistent with this, a neurophysiological examination of the ears of *A. aegypti*, shows response in both males and females up to 2000 Hz (Fig 2). These results are surprising, since over decades of behavioral and physiological studies had concluded that male mosquito ears (antennae and associated Johnston's organ) are tuned to 300-800 Hz and deaf to frequencies above 800 Hz (8,19). The present study also directly addresses the issue of auditory competence in female

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Behavioral and physiological experiments demonstrate intersexual acoustic interactions by the yellow fever and dengue vector mosquito, *Aedes aegypti*, that call for a revision of our understanding of the role of acoustic signals in mosquitoes.

mosquitoes. Acoustic duetting behavior in the non-vector mosquito, *T. brevipalpis* (18) would seem to imply active audition in both sexes and laser vibrometry studies of the Johnston's organ in the same species and *A. aegypti* (16,17) indicate it responds mechanically to salient sounds. Moreover, female frog-biting mosquitoes are reported to be attracted by the sounds of their singing hosts (20). The auditory physiology on *A. aegypti*, here, provide the first direct evidence that females can hear and puts to rest “textbook wisdom” that females are deaf (8,9).

For behavioural experiments, we tethered each mosquito to the end of an insect pin. When suspended in midair, flies initiated bouts of wing-flapping flight. We recorded flight tones with a particle velocity microphone. Acoustic interaction was demonstrated by moving a tethered flying mosquito past a stationary tethered flying partner (see movie S1 with audio in supplemental materials). Females were brought in and out of the male hearing range (2 cm) for 10 sec “fly bys”. Recordings revealed acoustic interaction: in 14 of 21 (67%) pairs, both sexes altered their flight tones so that the male's second harmonic ($F_0 = 636.7 \pm 15.1$, $F_1 = 1238.3 \pm 31.0$ (SEM)) matched the female's third harmonic ($F_0 = 430.6 \pm 10.8$, $F_2 = 1356.2 \pm 29.2$ (SEM)) (Fig 1A-C). The period of synchronization lasted an average of 9.71 ± 1.05 (SEM) sec with the synchronization frequency averaging 1354.5 ± 31.5 (SEM) Hz. *A. aegypti* do not shift their flight tones in the absence of acoustic stimulation, as tested both by deafening the mosquitoes (and stimulating with tones, Fisher's Exact test, males $P = 0.02$, females $P = 0.04$, see supplement) and flying intact control subjects in silence (Fisher's Exact test, males $P = 0.02$, females $P = 0.04$, see supplement).

The presence of the fundamental frequency tone was not necessary for harmonic matching. We stimulated tethered mosquitoes with electronically-generated pure sinusoidal tones as well as with harmonic combinations of pure tones lacking the fundamental frequency (Fig. 1D-F). The intensity of the pure tones was set at a particle velocity of 0.024 mm/sec corresponding to 54 dB (SPL) when played through an ear bud speaker positioned 1.5 cm in front of the test mosquito. This intensity is well within the response range of *Ae. aegypti*'s Johnston's organ, as measured by Doppler vibrometry (17). Stimuli were played in 10-15 sec bursts with 5-20 sec recovery periods. Play-back experiments with pure tone combinations demonstrated that 11 of 28 (39%) males could synchronize their 1200 Hz second harmonic to the simulated female's 1200 Hz third harmonic in the absence of the 400 Hz fundamental tone of an actual female. Furthermore, 12 of 54 (22%) males could match a pure 1200 Hz tone (the third harmonic of female flight tone) in the absence of the fundamental and any other harmonic components. We also tested the ability of females to synchronize to playback of pure tones mimicking male sound. Six of 20 (30%) unmated females matched the second harmonic of their flight tone to a complete stack of tones (F_0 to F_3 , 700 to 2800 Hz). When unmated females were stimulated with a pure 1400 Hz tone, 7 of 20 (35%) responded by matching with their third harmonic. In contrast, only 2 of 18 (11%) previously-mated females, confirmed by the presence of sperm in the female's sperm storage organs, performed a frequency match to playback of complete male songs, suggesting that mating decreases sensitivity to male stimuli.

Physiological data from the mosquito's auditory organ were obtained by impaling Johnston's organ with tungsten electrodes and recording acoustically-evoked field potentials. The Johnston's organ of both males and females responded to 0.5-sec cosine-enveloped pure tone pulses at all frequencies tested (125-2000 Hz), including the shared harmonic of their flight tones at 1200 Hz (Fig. 2). The response consists of a sustained voltage deflection, which is negative with respect to the thoracic ground electrode, and a concurrent periodic oscillation at the stimulation frequency and its harmonics, a form of neural encoding which bears striking resemblance to that seen in the mammalian cochlea (21). Importantly, it is the amplitude of the sustained deflection that remains significantly higher than pre-stimulus background noise and thoracic control recordings up to 2000 Hz (t test, $p < 0.001$ in both males and females); the amplitude of the periodic oscillation is about an order of magnitude smaller and remains higher

than background and controls only up to 1000 Hz, in the case of the stimulus' fundamental, and 700 Hz, for its second harmonic (t test, $p < 0.01$). These recordings confirm earlier studies that the mosquito Johnston's organ is sensitive to 100-500 Hz tones and extend the upper limit of hearing to at least 2000 Hz. Earlier physiological studies of Johnston's organ that failed to report a high frequency response are likely due to filter bandwidths set at the time of recording. We recorded high frequency responses only when we set the high-pass filter to 1 Hz or less, not the customary 100 Hz or higher used in extracellular recording.

Tone matching behavior does not require that a tethered, flying mosquito hear a live partner. Either a male or a female *A. aegypti* can modulate its flight tone harmonics to match electronically-generated pure-tone probes (Fig. 1). Frequency match is elicited to a probe that simulates a natural flight tone stripped of its fundamental frequency and even to a probe that contains only a single harmonic. Moreover, mosquitoes can modulate their flight tone harmonics to match the probe tone whether the probe frequency is set above or below the fly's actual flight tone. This directly demonstrates that both sexes of this species (and likely others) can hear and respond to high frequency tones alone. These novel behavioral findings are supported by sensory physiology (Fig. 2). Extracellular recordings of acoustically evoked field potentials from the Johnston's organ elicited clear responses not only to frequencies of the fundamental (400-600 Hz), as expected, but into the kilohertz range, where male and females perform active acoustic modulation of their flight tone harmonics. Thus, both behavioral and physiological experiments establish that *A. aegypti* signal to each other using frequencies in the kilohertz range and can detect these with their auditory organs. Taken together, these data call for revision of our understanding of acoustically-mediated mating behavior in mosquitoes, and in particular, removal of the long-accepted benchmark ceiling for hearing in mosquitoes.

Finally, and perhaps most intriguingly, once mated, females are much less responsive to male flight tones and less likely to perform tone matching. These results are consistent with the observation that once mated, *A. aegypti* females are not responsive to additional matings for the duration of one or more egg laying cycles (22,23). This implies that an initial mating depresses the likelihood of subsequent matings. Thus, releasing sterile male mosquitoes into the wild might adversely affect the reproductive potential of virgin females, providing a rationale for controlling these disease vectors through diminishing mating potential. Other vector and pest species of flies have been controlled by the release of sterile males (24-26). Our investigation connects control to signal function. We hypothesize that the ability of males to modulate their flight tones to females is the result of sexual selection. Hence, harmonic convergence could be a measure of a male's reproductive fitness and the ability of lab-reared, sterilized or genetically modified males to modulate their flight tones could be a useful behavioral bioassay for the sterilization program (27,28). At the very least, our findings open the door to a new understanding of their mating behavior—one that stresses acoustic interactivity between the sexes at frequencies thought previously to be beyond their range of hearing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(A) An oscillogram from a sound clip of a tethered male and female duetting. (B) A spectrogram depicting the harmonic stack of the same sound clip. The male was held in a fixed position and sang continuously for nearly two minutes. The female was brought within two centimeters of the male on three separate occasions. (C) An expanded view of B showing the synchronization of the flight tone at the second harmonic of the male (blue) and the third of the female (red). At $t=45$ sec, the two tones converge to the extent that they cannot be readily distinguished. (D) A separate recording of a tethered male (blue) flying solo and synchronizing to a loud speaker stimulus consisting of a simulated female flight tone with a missing fundamental (red). (E) A recording of a tethered male flying solo (blue) modulating his flight tone to match the playback

of a simulated third harmonic of a female. (F) A female flying solo (red) matches a simulated second harmonic of a male (blue) playback tone.

Figure 2.

(A) Acoustically-evoked field potentials recorded from Johnstone's organ exhibit periodic oscillations (inset, F0-4) riding on top of a sustained deflection (SD). Shown are averages of 10 and 5 repetitions to 1200 and 400 Hz, respectively, in a male. Thoracic control recordings are in black; the stimulus envelope is at bottom. (B) Spectral analysis of the response to the 400 Hz tone in A shows substantial power at low frequencies (SD) during the stimulus (blue) compared to a pre-stimulus background period (black), as well as peaks at multiple harmonics (F1-4) of the stimulus' fundamental frequency (F0). (C) Averaged across 12 males (blue) and 15 females (red), the amplitude (mean $\pm$ SEM) of the sustained deflection (SD, solid squares) remains higher than pre-stimulus background noise (dashed line at zero) up to 2000 Hz,

whereas the amplitudes of F0 and F1 (open and solid circles, respectively), are substantially smaller.