Title

Behavioural measurements of hearing-sensitivity in swarming *Anopheles coluzzii* mosquitoes reveal an unprecedentedly low threshold

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Abstract

Background Male and female mosquitoes of many species mate in swarms consisting of males and uninseminated females; males form station-keeping swarms and mating chases ensue as soon as a male detects the flight tones of a female. The auditory organs of mosquitoes have been reported to be the most sensitive among arthropods, enabling them to track their flying mate by the sound of the female's flight tones during mating chases. Previous studies on hearing thresholds have mainly used electrophysiological methods, which unfortunately limit the natural behaviour of the mosquitoes. This study explores male auditory behavioural sensitivity to female flight tones.

Methods We developed a behavioural method for measuring hearing sensitivity in swarming male mosquitoes. Free-flying male *Anopheles coluzzii* mosquitoes were released in a large arena (~2 m high x 2 m x 1 m) with a visually conspicuous object on the ground that stimulates swarming behaviour consisting of elliptical flight pattern over the object. Males were exposed to a range of natural and synthetic played-back sounds of female flight emanating from a speaker located at the same height as the swarm centre and 0.9 m away from it. We monitored the responses of males to female sound by recording the flight-tone and flight-dynamic responses of males. Calibrated sound-levels were measured at the swarm centre; the changing distances between the speaker and the flying males as well as other measurement uncertainties were taken into account to estimate the sound levels that males were effectively exposed to.

Results We show that the mean male behavioural threshold of particle-velocity hearing lies between 13-20 dB SVL (95%-CI). A conservative estimate of 20 dB SVL (i.e., less than a particle velocity of 0.5 μm/s) is already 12 to 26 dB less than most of the published electrophysiological measurements on Johnston's organ sensilla. Our results confirm, under laboratory conditions, the high behavioural hearing-sensitivity of swarming mosquitoes recently found with swarming *Aedes communis* in the field, and suggest a hearing threshold at least two times lower than that of published results (26 dB SVL or 1 μm/s particle-velocity). In addition, we established that 1) the first harmonic of *Anopheles coluzzii* female flight sound is sufficient for males to detect her presence, 2) males respond with a greater amplitude to single-female sounds than to group-female sounds and 3) males respond to the playback of single-female sound as much as to pure-sound at a constant frequency and amplitude.

Keywords

Auditory processing, free-flying, Johnston's organ, insect sensory system, sensory ecology, sound sensitivity.

MAIN TEXT

Introduction

Hearing is a key sensory modality for mosquito mating; it enables males to detect females at a distance through the combined sounds of their respective flapping wings *(01)(02)(03)*. The more sensitive males are, the further away they can hear a female and the sooner they detect and close in on a nearby female in the context of highly-competitive mating-swarms. Male hearing organs are the most sensitive known so far among arthropods *(04)*, however, the measurement of hearing sensitivity is usually performed on tethered male, which constrains natural body movement and wing flapping behaviour in response to female sound. Only a few studies have measured hearing thresholds in a behavioural context *(03)(05)(06).* The measurement of behavioural sound-sensitivity in flying mosquitoes faces the difficulty of monitoring how much sound energy actually reaches their antennae, because the energy meter is at a fixed-position, whereas the position of a mosquito is continuously changing during its flight. The aim of this study was to quantify behaviourally the sound-level threshold at which males can hear females. Accordingly, we had to determine the components of female-wingbeat sound that male mosquitoes are the most responsive to, in order to consider only the audible frequency bands in the definition of the sound level.

Mosquitoes hear airborne sound by detecting air-particle velocity through friction between air particles and the mosquito's fibrillae located on the flagellum of their antennae. Unfortunately, there are no instruments which can truly measure particle-velocity on the market yet *(07)*, however, it can be estimated by using pressure-gradient microphones (commonly called 'particle-velocity microphones'). Another strategy to estimate particle-velocity is to use pressure microphones located in the far-field of the sound source, i.e., where the sound pressure level (SPL) can be approximated to that of sound particle velocity level (SVL). Many studies have published SPL hearing thresholds without the far-field condition being fulfilled *(08)(09)(10)*, which means that hearing thresholds have been under-estimated as elaborated in the Discussion section.

Hearing thresholds can be assessed by measuring a physiological or behavioural response to a given stimulus sound level and sound frequency. Electrophysiological methods enable us to measure the mechanical response of the flagellum and the electrical response of the Johnston's Organ (JO) to sound stimuli, without interference of mosquito body movements. Laser vibrometry records the vibration of the flagellum *(11)(12)*, however, it is limited when assessing hearing threshold because the recorded vibration only refers to the input of the hearing chain (i.e., flagella movement) and does not provide any indication as to whether or not the signal reached the mosquito electrical sensory network.

On the contrary, electrical responses of the JOs to airborne sound stimuli result from the complete sensory chain of the auditory system (i.e., from the mechanical vibration of the flagella to the electrical response of the JOs). With this method, the electrical response-threshold in male *Culex pipiens pipiens* JOs showed a mean sensitivity of 32 dB SVL per JO unit (22-44 dB SVL) *(13)* and a mean of 44 dB SVL per mosquito in three male *Culex*

quinquefasciatus JOs (36-52 dB SVL) *(01).* In *Aedes aegypti,* male JO nerves were shown to respond to a mean of 40 dB SVL (31-50 dB SVL) *(05)*. In some species, such as *Anopheles coluzzii*, the antennal fibrillae are extended only during their active phase which improves their JO hearing sensitivity by 17 dB in terms of SVL *(12)*. Under this antennal physiological state, Pennetier *et al. (12)* measured a JO response-threshold in two male *An. coluzzii* of only 10 dB SVL (particle velocity of $1.5 \pm 0.6\ 10^{-7}$ m/s).

Normally, a mosquito's hearing sensitivity is further enhanced by beating its wings due to antennal distortion products created by the combination of wing-beat frequencies of a male and a female in flight *(14)(15)*. However, electrophysiological methods usually prevent mosquitoes from beating their wings, so in order to simulate the effect of male flight on the male auditory organ, it is possible to add the male's flight sound-frequency to the female stimulus sound. For example, male *Cx. pipiens pipiens* JO sensitivity was improved by 7 dB with the addition of simulated flight sound at the main frequency optimum *(15).*

Electrophysiology results can be difficult to compare against each other due to different methodology of assessing threshold responses (e.g. statistical definitions of threshold, location of the electrodes, temperature). In addition, the main goal of these studies are often not about measuring absolute hearing thresholds and as a consequence, often, they only use few mosquitoes.

Behavioural methods to assess the physiological responses to sound stimuli offer more natural physiological contexts that release more natural responses to sound, and along with a statistical analysis, behavioural responses provide acceptable evidence of hearing

because the whole auditory chain plus the motor response are involved. To our knowledge, there are only three published behavioural studies of mosquito sensitivity to sound intensity. First, Menda *et al. (05)* measured the behavioural response of *Ae. aegypti* by monitoring the take-off of resting mosquitoes in a cage located in the far-field of the sound-source. They measured a taking-off response at 40 and 65 dB SVL in males but did not test lower sound levels. However, their behavioural methodology does not refer to the natural physiological context of swarming mosquito species because in the field both males and females fly (i.e., do not rest) when the males detect the female's flight tones. Indeed, male responsiveness to sound was found to be reduced when they were not flying *(15)*. Second, Feugère *et al. (03)* measured the flight and wingbeat frequency response of free-flying, swarming male *An. coluzzii* to a range of sound levels of a played-back group of females and found a response at 33±3 dB SPL. However, males may respond better to the sound of individual females rather than a group of females occupying a relatively wide spread of wingbeat frequencies, as it has been described with *Ae. aegypti (26)*. Third, Lapshin and Vorontsov *(06)* showed an increase in flight speed in swarming male *Aedes communis* in response to the sound frequency of females in the field, with a hearing sound-level threshold of 26 dB SVL on average (26 dB SPL under far-field conditions).

The aim of our study was to investigate the behavioural hearing threshold of *An. coluzzii* males, which JOs are believed to be very sensitive to (i.e., up to 10 dB SVL) *(12)*. In addition and as suggested 70 years ago by Roth *(16)*, male hearing may be enhanced during swarming behaviour (i.e., flying in loops over a floor marker, but station-keeping while they wait for females to join the swarm) when male sensitivity to the sound of flying females is expected to be maximised. To do so, we used a modified approach of Lapshin *et al.* who worked in the field with *Ae. communis (06).* Our study was performed with the

following conditions: 1) in a laboratory sound-proof chamber, under controlled measurement of sound levels; 2) with a greater range of type of sounds to be exposed to males; 3) by monitoring both the male flight-tone and the flight-dynamic quantitatively; and 4) with *An. coluzzii*, a swarming species belonging to the *Anopheles gambiae* complex. 'Sound-level values' depend on how sound level is defined and on the type of sound stimuli, therefore, a meaningful sound-level definition should be related to the sound-frequency band and temporal patterns which mosquitoes are sensitive to. For this reason, our main question of quantifying hearing threshold was inter-connected with the following questions:

- Is the second harmonic of female flight tones necessary to stimulate a response in males? We need this information to establish the frequency band(s) for which the sound level is defined to be appropriate to mosquito hearing.
- Is temporal variation in natural female sound required for males to detect females or is a pure sound sufficient?
- Do the flight tones of a group of females have the same effect on male hearing as those of a single female, over a range of sound levels? The main interest in the last two questions is to investigate whether we can use pure-frequency sounds to mimic female sound, which will make the hearing threshold easier to estimate in future studies.

Materials and Methods

Mosquitoes

All experiments were performed with virgin *An. coluzzii* Coetzee & Wilkerson. The colony was established at the Natural Resources Institute (NRI), University of Greenwich (UK) from eggs provided by the Institut de Recherche en Sciences de la Santé (IRSS), Burkina Faso. Eggs were obtained from a colony established in 2017 from wild gravid females collected from inhabited human dwellings in Bama, Burkina Faso (11°23'14"N, 4°24'42"W). Females were identified to species level by PCR *(17)*. The NRI colonies were kept in environmentally controlled laboratory rooms with a 12h:12h light:dark cycle (lights went off at 15h00), >60% relative humidity and ~24-26°C. Larvae were fed Tetramin® fish-flakes and rice powder. Adult males and females were separated < 12h post-emergence and fed a solution of 10% sucrose in an isotonic saline *ad libitum*.

Experimental setup

The experimental setup (Fig. 1) was the same as for *An. coluzzii* in a previous study *(03)*.

Sound-proof chamber. All experiments were conducted in a sound-proof chamber to limit interference from external sounds. The chamber consisted of double-skin sound-proof walls, ceiling and floor (L x W x H = 2.7 m x 1.9 m x 2.3 m), producing a reverberation time ≤ 0.07 s for frequencies above 200 Hz (IAC Acoustics, manufacturers). Fig. S1 B displays the sound-pressure level per octave band when the sound-proof chamber was silent. Between 176 Hz (lower limit of the 250-Hz octave band) and 1.4 kHz (upper limit of the 1-kHz octave band), i.e., the frequency range within the *An. coluzzii* mosquito's hearing sensitivity is the highest *(01)*, the SPL in the sound-proof room without any playback was always quieter than that with playback of the sound stimuli.

Swarming arena. The swarming arena in the sound-proof chamber was designed to include the key environmental conditions and sensory cues known to control mating and swarming flight in the field. A large mosquito bed-net enclosure (NATURO, *L* x *W* x *H* = 1.8 m x 1.7 m x 2 m) filling most of a sound-proof chamber (Fig. 1) enabled mosquitoes to fly freely in a volume 100 times greater than that covered by the swarming space. Lighting was provided by an artificial-sunlight system to imitate natural daylight, sunrise and sunset (LEDs 5630, HMCO FLEXIBLE dimmer, and PLeD software, custom-built). Dimming the ambient light level at the appropriate circadian time elicits mosquitoes to take-off in random flight at first, followed by swarming flight in response to the presence of a visually conspicuous matt-black marker on the floor; both males and females fly in loops above the marker (swarming behaviour; Fig. 1).

Sound recording and monitoring. The wingbeats (aka, 'flight tones') of mosquitoes in the laboratory were recorded with a weatherproof microphone (Sennheiser MKH60; RF-condenser; super-cardioid polar pattern at 0.5-1 kHz, with amplitude decrease of > 15 dB beyond 90° from the microphone head) directed toward the swarm location. The microphone was located at a distance of 0.89 m from the centre of the swarm area for the tested male mosquitoes and the sound recording of the 30-female swarm stimulus (Fig. 1), except for the recording of the 1-female sound-stimulus for which the microphone was located at 0.75 cm from the centre of the swarm area. Microphone were plugged into a Scarlett 18i8 audio interface on a Windows7 computer running Pro Tools First 12.8 (Avid Technology, Inc).

Flight track recording. The 3D flight trajectories of male mosquitoes were recorded at a sampling rate of 50 Hz with Trackit software (SciTrackS GmbH, Switzerland *(18)*). Two

video cameras (Basler, ace A640-120gm) were fitted with wide-angle lenses (Computar, T3Z3510CS, 1/3" 3.5-10.5mm f1.0 Varifocal, Manual Iris) to maximize 3D volume of video-tracking. IR lights (Raytec RM25-F-120 RAYMAX 25 FUSION) enabled the tracking system to detect flying mosquitoes as silhouettes against an IR-illuminated white back-wall made of thin cotton cloth (Fig. 1). The 3D-flight trajectories were smoothed using a cubic spline interpolation at a sampling frequency of 200 Hz on Matlab (version R2017a).

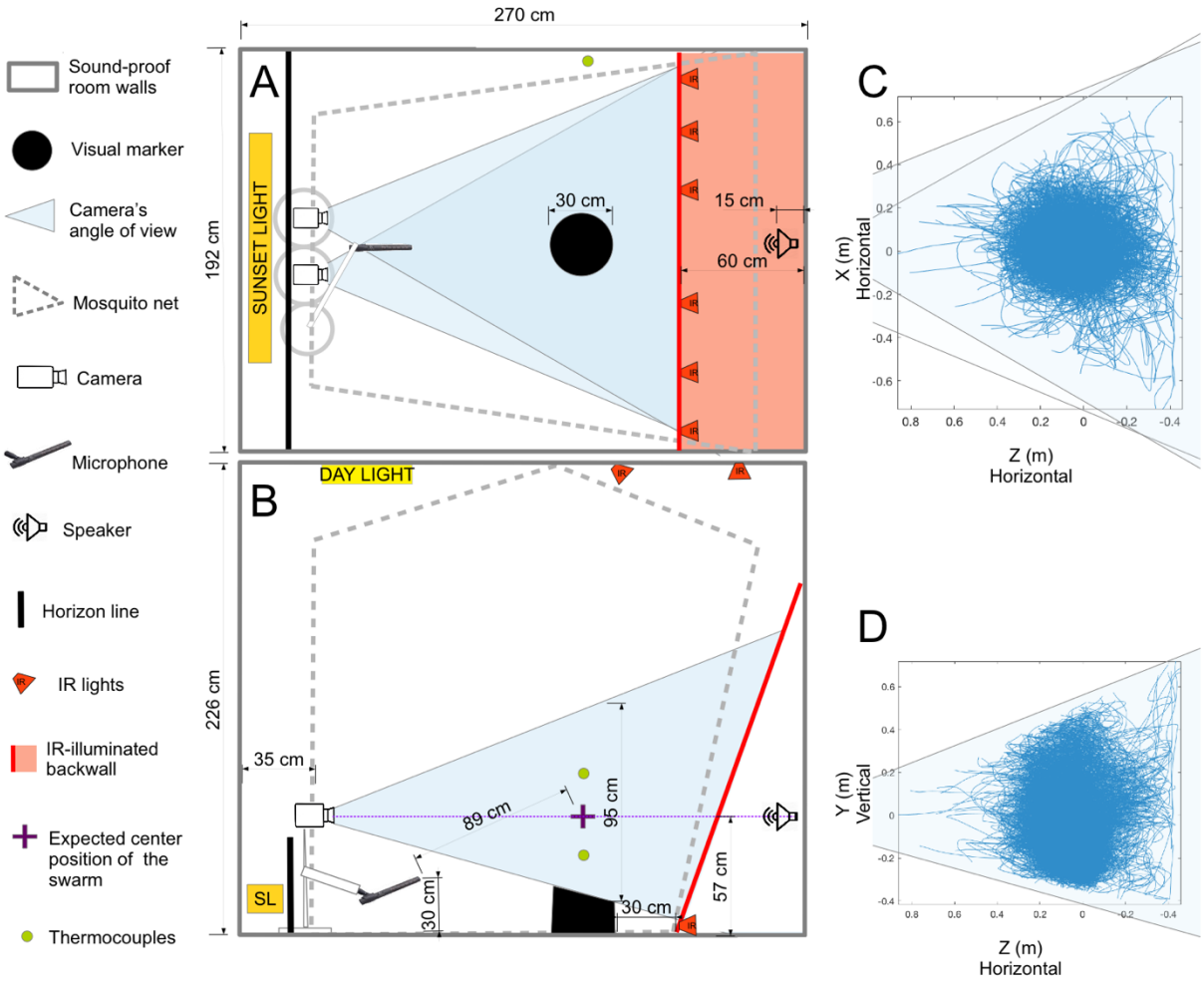

Fig. 1. Sound-proof chamber setup for recording sound and video of *An. coluzzii* behaviour (modified version from *(03)*).

(A) Bird's-eye and (B) side views of sound-proof chamber. Blue shaded areas indicate the 3D fields-of-view of cameras recording mosquito flight paths. Two IR-sensitive cameras fitted with IR pass filters recorded flying mosquitoes as black silhouettes against evenly lit

IR- background. A separate lighting system provided gradual semi-natural dusk visible to mosquitoes, consisting of dispersed dim white lights on ceiling and 'sunset' lighting below horizon (opaque wall ~40 cm tall). A microphone recorded flight sounds of mosquitoes swarming directly above black swarm marker. A thermocouple (85 cm above ground level) recorded temperature at ~ mean swarm height. A speaker located behind IR-illuminated thin-cotton sheet, outside net enclosure played back sound stimuli.
(C) Bird's-eye and (D) side views of the superimposed flight tracks of the entire dataset.

Sound stimuli

Recording context. Two recordings of the natural flight-sounds of 3-6 days-old swarming females were recorded and used to produce the played-back stimuli for the behavioural assays. These recordings consisted of 1) a single swarming female and 2) a group of 30 swarming females which were released two days before the experiment to acclimatize. The standard environmental conditions in the room were: 12h:12h light:dark cycle with a 1h artificial dawn/dusk transition in light intensity and ~60-75% RH.

Signal generation. We generated a total of 10 stimuli; 9 derived from the two recordings plus one recording of synthetic sound. First, we selected the first 7s section of the sound of a single female swarming over the marker. Similarly, a 7s section of the sound of 30 swarming females was selected, ~10 min after the first female started to swarm. Four sound levels for each of the 1- and 30-female sounds were selected (10-45 dB SPL, Table S1), based on results of preliminary experiments. These 8 stimuli contained the two first harmonics. A high-pass filter was added to all the stimuli to remove the electrical noise below the first harmonic. In addition, we generated a 33 dB SPL stimulus, which has been

shown in preliminary experiments to be the lowest level sound stimulus that females detect in the sound-proof chamber (but see Method section 'Corrected SPLs for estimating the hearing threshold' below*)*. This sound stimulus included only the first harmonic because it has been shown electrophysiologically that the male auditory organ is more sensitive to the first harmonic than higher harmonics *(01)*. Finally, we generated a synthetic 1-harmonic sound at the same peak amplitude as the 1-harmonic 1-female sound. A gradual increase/decrease over 1 s in the level of the start and end sounds were added to avoid creating sound artefacts due to the signal truncation, and to make the stimulus more natural. The ten stimuli were played sequentially, with a 10 s interval of silence to be played-back during the behavioural assays. To avoid an effect of the order of in which stimuli were played, ten different sequences were generated, each containing the ten sounds in random order. All stimuli were sampled at 8 kHz / 24 bits and designed on Matlab (R2017a, The Mathworks Inc, Natick, USA). Fig. 2 summarizes the time and spectral characteristics of each stimulus. Table S1 gives the sound levels for each of them. Audio S1 and Audio S2 are the 1-female and 30-female 2-harmonic sound-stimuli, respectively.

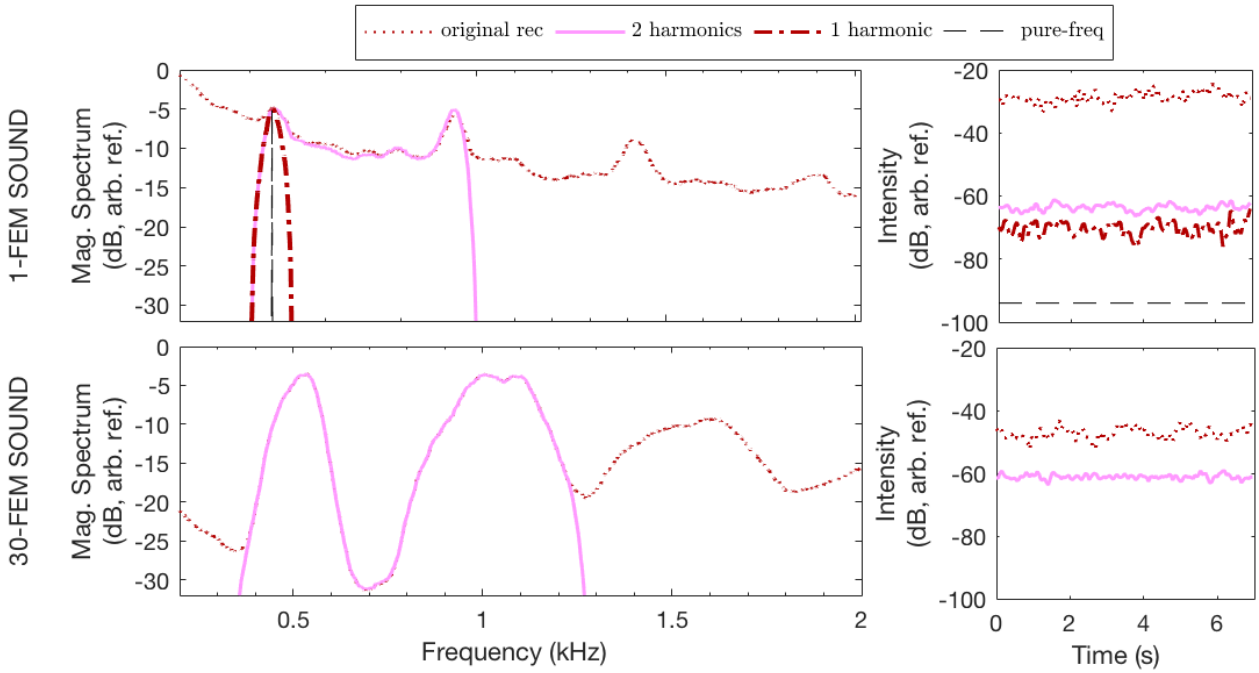

Fig. 2. Spectral and temporal properties of sound stimuli. Spectral (first column) and temporal (second column) properties of sound stimuli of one single swarming *An. coluzzii* female (top row) compared to that of 30 females (bottom row). The originally recorded sounds (not used) are represented with a dotted line. The first-harmonic sounds are shown as a semi-dotted-dashed red line, while the second-harmonic sound is represented by a solid pink line (Audio S1 and Audio S2 for a 1-female and 30-female, respectively). Magnitude spectrum were calculated over 7 s and averaged over 50-Hz windows. The root-rean-square pressure levels were computed over a 0.1 s time window with 0.05 s overlap, along the 7 s duration of the stimuli.

***Sound diffusion*.** Sequences of sound stimuli were played-back from a speaker (Genelec 8010A) plugged into a Scarlett 18i8 sound card running pro-Tools First and Audacity on Windows 7. The speaker's membrane centre was located 57 cm above the floor, 15 cm from the back wall and 0.9 m from the swarming centre (Fig. 1).

Data Subsets. While stimuli were played-back in random order during a single experiment, they can be grouped into three overlapping subsets (Fig. 3), each of them corresponding to one of the questions presented at the end of the Introduction. Subset A: study of the effect of the second harmonic on male hearing (1-harmonic versus 2-harmonic stimuli)'. Subset B: investigation of the effect of 'type of sound stimulus' (pure-sound versus pre-recorded played-back sound). Subset C: effect on male hearing of sound stimuli of different numbers of females (1 versus 30) as well as different sound levels of female sound to estimate the hearing threshold.

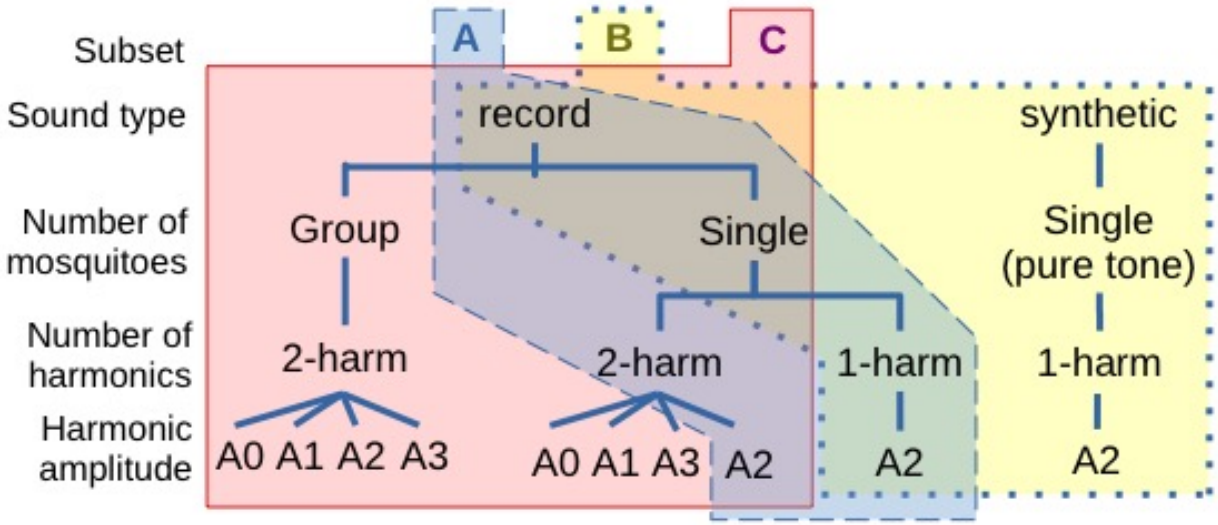

Fig. 3. Data subsets for our analysis (A, B, C). Subset A was used to study the effect of the number of harmonics in the sound stimuli. Subset B was used to compare the sound type (playback of female sound or pure sound of the same wingbeat frequency). Subset C was used to study the effect of number of mosquito(es) (1 female or 30 females).

Behavioural assays

To investigate the sensitivity of swarming males to female sounds, we played-back the female sound stimuli to swarming males in the sound-proof chamber. About twenty 3-4

days-old males were released the day prior to experiments at ~ 18h00 in the sound recording flight arena. At 15h00, after the ceiling lights had dimmed to the lowest intensity, the horizon light completed a 10 min dimming period and then was kept at a constant dim light intensity until the experiment was finished. When at least one male started to swarm robustly over the marker, the first sequence of sound stimuli was played-back from the speaker (see Movie S1 with 4 males exposed to one sound stimulus). After 10 stimuli were played and if the male(s) was still swarming, or as soon as at least one male started swarming, a new sequence of 10 stimuli was immediately played and so on, until all 10 stimuli sequences were played or after 50 min of constant horizon light, either of which marking the end of the experiment (= 1 replicate). Males were then collected and removed from the flight arena. A new group of ~20 male mosquitoes were released in the sound-proof chamber, to be used for a new replicate the next day (one replicate per day, for 15 days in August-September 2018).

Sound pressure level (SPL)

Measurement. Stimulus SPLs were measured at the mean male swarming position with a sound meter (Casella, CEL633C1, Class 1) set as follows: reference pressure of 20 µPa; no octave weighting (i.e., dB Z); slow octave time-constant (IEC 61672-1: 2002); octave and third-octave bands; calibrated twice a day (CEL-120/1, Class 1, at 94 dB / 1 kHz) before and after each measurement. The speaker and the software/soundcard gains were set to be the same as during the behavioural experiment.

Third-octave bands. All SPLs reported in this study included only the frequency bands that are audible to male mosquitoes, i.e., mostly the first harmonic of the female *(01)*.

They were calculated as follows: $10log_{10}(10^{0.1L_{B1}} + 10^{0.1L_{B2}})$ where L_{B1} and L_{B2} are SPL measurements in frequency bands *B1* and *B2*; *B1*=500 Hz and *B2*=630 Hz are the third-octave bands nearest the female's wingbeat frequency of the first harmonic (Table S1; and Fig. S1 for all third-octave values).

Corrected SPLs for estimating the hearing threshold. The sound of a 1-female was recorded at a distance of 0.7±0.2 m, which gave a relatively low signal-to-noise ratio compared to the high signal-to-noise ratio of the sound of 30-females recorded at 0.9±0.2 m. As explained in the Method section 'Sound stimuli', noise was removed below the first harmonic and above the second harmonic but not in-between to limit any artefacts in the sound stimulus. SPL was computed over the frequency-band of the first harmonics, which, for the 2-harmonic 1-female sound, included a part of the noise between the first and second harmonics. Results from Subset A suggested that this noise was not heard by males because males reacted to the 2-harmonic 1-female sound as much as to the 1-harmonic 1-female sound. Since these two stimuli had the same first-harmonic amplitude but a SPL difference of 8 dB (Table S1), and because SPL was defined over a frequency band below the second harmonic, we established that the noise between the first and second harmonics is responsible for 8 dB in our SPL measurements. In order to estimate an accurate hearing threshold, we applied a correction of 8 dB to the sound level of the 2-harmonic 1-female stimuli (Subset C). All sound levels, with correction or not, are summarized in Table S1.

Control of distance between live mosquito and playback speaker. Swarming mosquitoes confine themselves to a limited area of the flight arena naturally, which enables us to control the SPL they are subjected to, because the distance between swarming mosquitoes and the sound stimulus source was limited to a known range. The speaker (Genelec

8010A) that reproduced the females' flight tones was placed 0.9 m from the centre of the swarm marker. Their flight positions were recorded by 3D-tracking Trackit Software *(18)* (Fig. 4 A, Fig. 4 B) which enabled us to determine the distance between a mosquito and the speaker emitting mosquito sound to be 0.9±0.2 m, 95%-CI (Fig. 4 C).

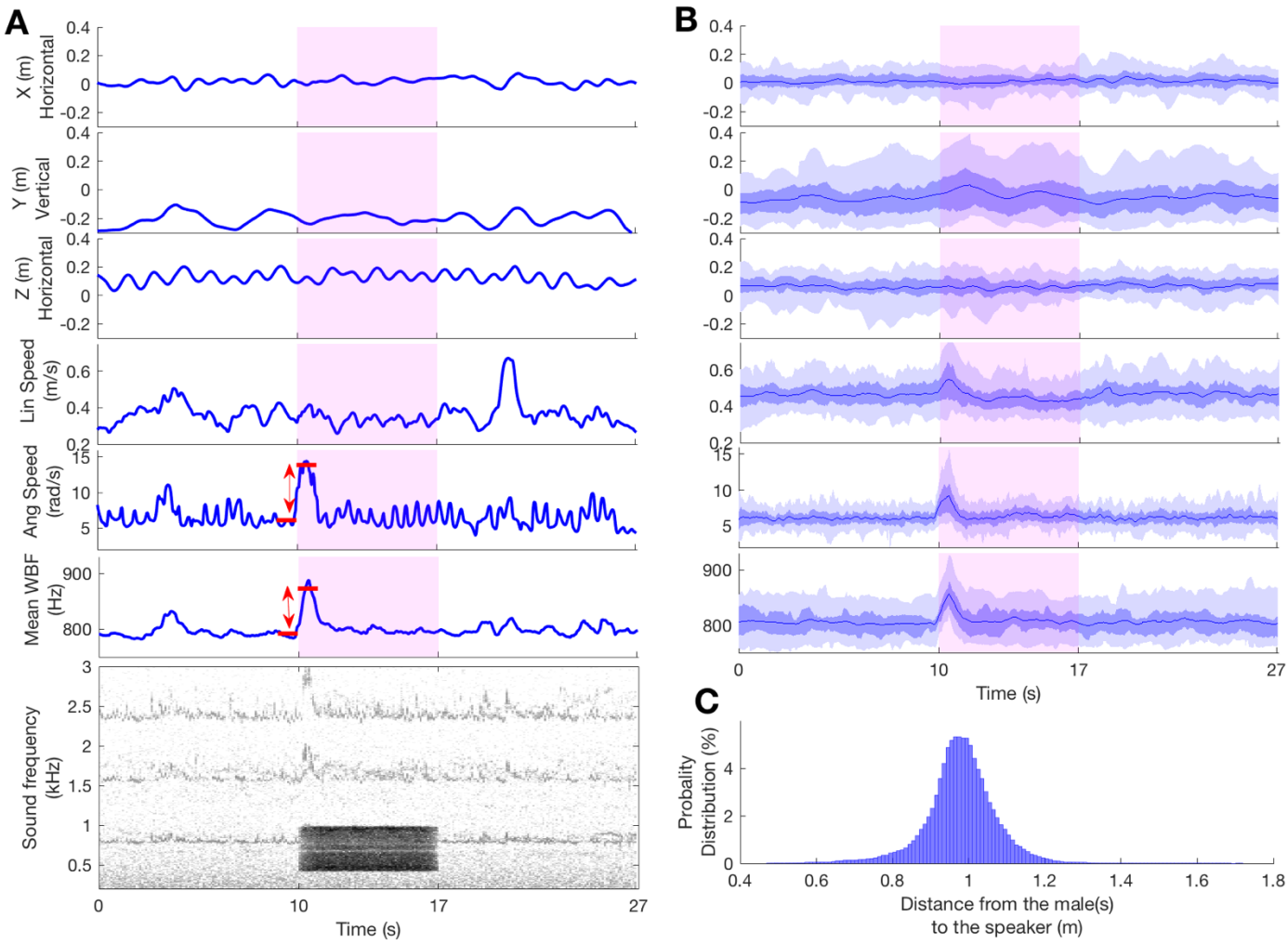

Fig. 4. Flight and sound responses of *An. coluzzii* males to sound-stimuli. Male flight-characteristics and wingbeat-frequencies (blue) before, during and after playback of female (red rectangle) sound stimuli.

(A) Example of male response to the loudest 2-harmonic 1-female sound-stimulus (Audio S1) over 27 s of recording. Stimulus was played-back 10 s from beginning of flight

recording and lasted 7 s (red rectangular shading). First five rows show flight parameters (relative X,Y and Z positions, plus linear and angular flight speeds). 'Z' dimension represents relative distance to the speaker (located 0.9 m from *Z*=0). Last row shows mean wingbeat frequency (WBF). Periodic flight pattern, typical of swarming behaviour, is evident in X, Y and Z plots. In the angular-speed and wingbeat frequency plots, the two red lines correspond to the upper-quartile over 1s and the arrows represent the differences between the two red lines, which are the parameters computed for monitoring the male response (see Methods section 'Extraction of traits used to quantify male responses'). Last row shows the spectrogram of sound recordings before, during and after the sound stimulus; the colour gradient represents the sound level given a frequency and a time (the darker the colour, the louder the frequency). Movie S1 gives the associated raw image and sound recording.

(B) Same as (A) but without spectrogram and for all-male responses to the loudest 2-harmonic 1-female sound-stimulus. Darkest coloured lines represent running median, darkest areas represent second and third quartiles and light areas represent the 90th percentile of data. Distribution of flight coordinates and velocities were recorded for 104 male tracks, and the WBF distribution plot is based on mean WBFs over the number of mosquitoes per swarm (61 swarms). Linear and angular speed, and wingbeat frequency clearly increased in response to the onset of this sound stimulus, plus there was a slight tendency to increase in flight height (Y (m)).

(C) Probability distribution of distance between a male and the speaker during sound stimulus playback for all stimuli; distances ranged between 0.9±0.2 m. This distance interval was used to estimate the uncertainties of the acoustic prediction in Table S1.

Estimate of SPL errors at mosquito's location. Two types of SPL errors were taken into account. The first is related to the time variation of the sound stimulus levels which were between ±0.3 dB and ±0.9 dB, depending on the stimulus considering maximum error (see Fig. 2 for an example of stimulus sound-level along time). The second type of measurement uncertainty arises when the sound-level should be estimated from the mosquito position, and not from the fixed microphone position. Indeed, SPLs were measured at the expected centre of the station-keeping swarm-flight of the mosquito. However, the distance between the female and the speaker varied as 0.9±0.2 (95%-CI, Fig. 4 C), due to the males' swarming-flight pattern, which changes the sound level they are exposed to, accordingly. We evaluated this error by playing-back the *An. coluzzii* female sound stimulus and measured the sound level in a sphere around the expected swarming area centre: the maximum error was ±2 dB. This error is considered to be conservative (at least 95%-CI) and was used to interpret the results of the experiments (see Table S1).

Physical sound quantities produced by a speaker and sensed by mosquitoes. We monitored the sound level of the played-back stimuli by recording the sound pressure level (SPL), however, mosquito hearing organs are sensitive to particle velocity level (SVL) *(19)*. The root-mean square value (RMS) particle velocity v_{RMS} and the RMS sound pressure p_{RMS} can be related as follows, assuming the speaker to be a point source radiating spherically a sound frequency f at a distance r from the measurement (air impedance Z_{air}(28°C) = 408 N.s.m^{-3}; sound speed c(28°C)=348 m/s) *(20)*:

$$v_{RMS}(r) = \frac{p_{RMS}(r)}{Z_{air}} \sqrt{1 + \left(\frac{c}{2\pi f r}\right)^2} \qquad (1)$$

The SPL $L_p \stackrel{\text{def}}{=} 20log_{10}(p_{RMS}/p_0)$ and the associated particle-velocity level $L_v = 20log_{10}(v_{RMS} Z_{air} / p_0)$ (reference p_0 = 2.0 10^{-5} Pa) can be calculated as follows:

$$L_v(r) = L_p(r) + 10log_{10}\left(1 + \left(\frac{c}{2\pi fr}\right)^2\right) \qquad (2)$$

In our case, considering that the female sound stimulus does not have any frequency components below $f = 440$ Hz (the smallest frequency value of the group of first harmonics of the swarming females at -12 dB below the peak at 536 Hz, Fig. 2), the SVL is equal to the SPL at 0.9±0.2 m away from the speaker, under a negligible error of 0.1 dB. As a consequence, and since mosquitoes are sensitive to SVL and for easier comparison with other studies, we report the SPL as SVL. Note that the monopole assumption for mosquito wing-flapping is conservative since higher orders (dipole, quadripole) produce sound levels that decrease more rapidly with distance *(21)*.

Extraction of traits used to quantify male responses

Following the results of preliminary experiments, we used two components of male flight: 1) **angular-speed**, calculated from their 3D trajectories and 2) **wingbeat frequency**, extracted from sound recordings (see Fig. 4 B for example and statistics of wingbeat and flight dynamic characteristics before, during and after exposure to the loudest 1-female sound-stimuli (44±2 dB SVL)). The two components were synchronized using the same techniques as in a previously published study *(22)*.

Angular-speed refers to how much the mosquito flight direction changes per unit time. It was calculated from the linear-velocity components provided by the Trackit software as follows: $avel = \Delta\theta / \Delta t$, where $\Delta t = t_n - t_{n+1}$ is the duration between two consecutive time indexes n and n+1, and $\Delta\theta$ is the turn angle defined as:

$$\Delta\theta = acos\frac{\boldsymbol{v_n} \cdot \boldsymbol{v_{n+1}}}{|\boldsymbol{v_n}| \cdot |\boldsymbol{v_{n+1}}|} \qquad (3)$$

where $\boldsymbol{v_n}$ is the three-dimensional linear velocity vector of the mosquito at time index n and $|\boldsymbol{v}_n|$ is its magnitude. The criteria used to include a tracked flight in the data analysis were that the mosquito was swarming over the marker for at least 1 s before and after the sound stimulus onset.

Wingbeat frequency. Only the first and/or the second harmonic of female sound stimuli were played-back (~400-1200 Hz) in order to free the frequency domain of the male's third harmonic from the female's sound. This allowed us to capture the male's third harmonic without overlapping with the sound stimulus (example of spectrogram in Fig. 4 A). The peak of the third harmonic was detected every 40 ms between 2190 and 2920 Hz using the Fast Fourier Transform algorithm (256-ms FFT-window, Hanning-windowed). When several mosquitoes (from 1 to 6) were present over the swarming marker, the detected value was the peak of the energy in the frequency band 2190-2920 Hz and not the mean of the peak from individual mosquitoes (because it was not possible to track the wingbeat frequencies of individual mosquitoes). Then, the male's third-harmonics (i.e., 3 x wingbeat frequency) were divided by 3 to get the wingbeat frequency (i.e., the first-harmonic frequency). Finally, a 3-point median filter was applied over time to reduce wingbeat tracking error. Fig. 4 A gives an example of detected wingbeat frequencies of males while Fig. 4 B shows the distribution of the detected wingbeat frequency over time for all recordings.

Upper-quartile difference. Since preliminary experiments suggested that mosquitoes responded to sound by increasing their wingbeat frequency and their angular speed somewhere during the first second of the sound stimuli, the upper-quartile angular-speeds

and the upper-quartile wingbeat frequencies were automatically detected during the first 1s stimulus time interval. Indeed, 'upper-quartile' is 1) a more robust metric than median or mean to measure the amplitude of a short peak, which the onset time cannot be predictable precisely and 2) a more reliable metric than maximum to avoid false detection. Then, this value was subtracted from the upper-quartile value computed during the 1s segment just before the stimulus onset, for each individual recording to reduce noise related to individual mosquito variability (Fig. 4 A shows graphically how the parameters were computed).

Statistics

Wingbeat-frequency and angular-speed values for a given stimulus were averaged over the different responses of the same day to form a replicate. The wingbeat and angular-speed response-parameters were analysed using a Bayesian Linear Mixed-Effects Model (*blmer* function, *lme4* package, R), assuming normal distributions. Stimulus sound levels (continuous), number of females in the recording (1 or 30), number of harmonics (1 or 2) and sound type (recording or synthesis) and their interaction were considered as fixed effects. Days, for which replicates were performed, were considered random effects. The dataset was split into the 3 subsets A, B, C, as shown in Fig. 3. A total of 6 models were built (2 parameters x 3 subsets). Stepwise removal of terms was used for model selection, followed by likelihood ratio tests. Term removals that significantly reduced explanatory power ($p<0.05$) were retained in the minimal adequate model *(23)*. For subsets A and B, an additional one-sample t-test (with BF-correction for multiple comparisons) was performed independently for each distribution to measure the significance of the mean to 0, which is the "no response" reference. For subset C, the quietest 2-harmonic 30-female

sound-stimulus was not included in the model because its sound level was too close to the background noise level to be corrected as the three other 2-harmonic 30-female sound-stimuli. The hearing threshold was estimated by the crossing of the X axis (i.e., no response) with the prediction of the fixed-effect components of the mean and associated 95%-CI (*bootMer* function with *nsim*=500*, lme4* package, R). All analyses were performed using R (version 3.5.3).

Results

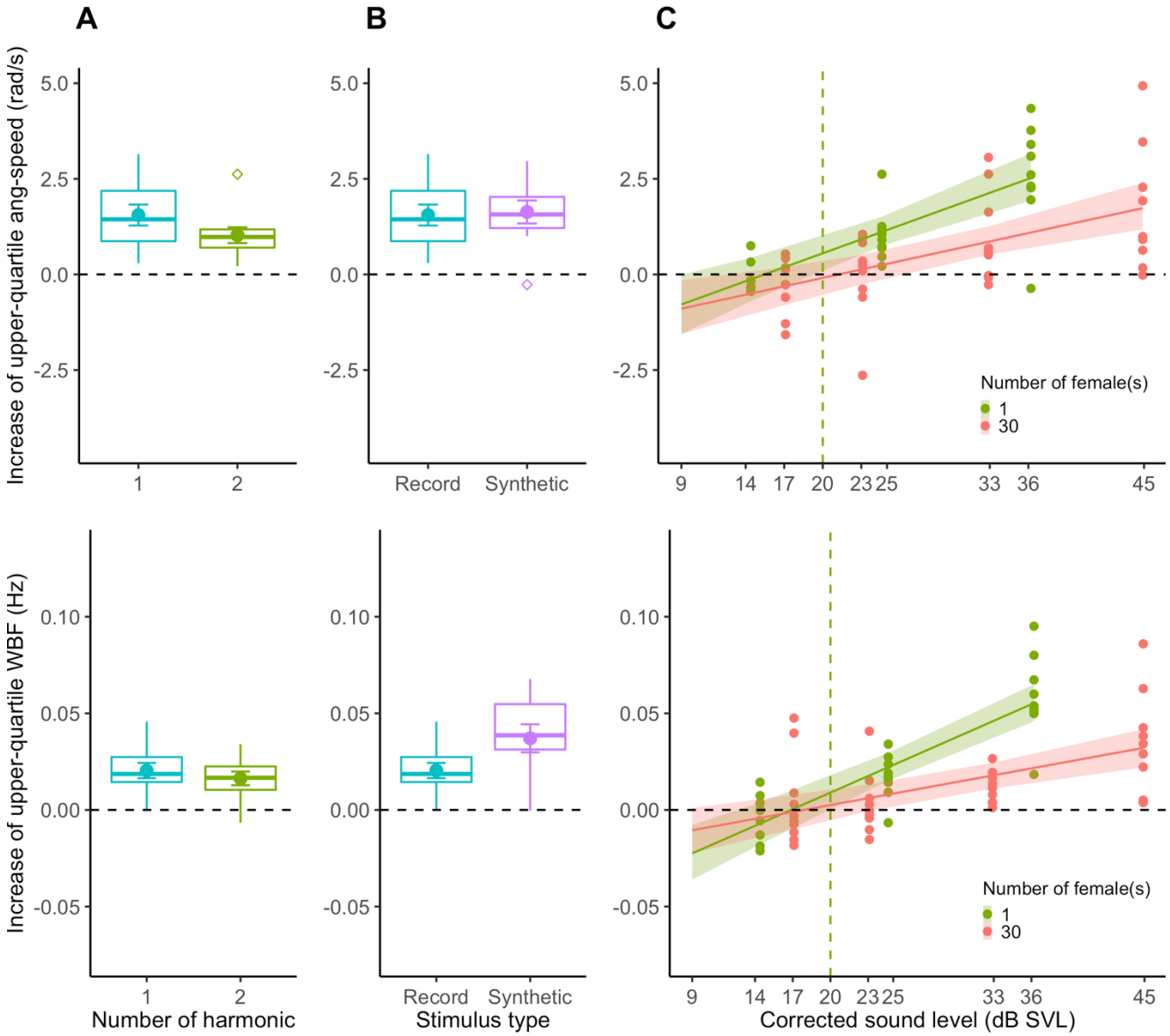

Fig. 5. Results of behavioural experiment. Top and bottom rows show the increase in upper-quartile angular-speed and wingbeat frequency, respectively, when playing-back a given sound stimulus. Black dotted lines represent the absence of change in parameters before and during the stimuli.

(A) Male *An. coluzzii* responses to 1- or 2-harmonic sounds of a single female (data subset A). Boxplots of the parameters show the median, 2nd and 3rd quartiles. Outliers shown as diamond shapes are outside the interval [Q1 – 1.5 * IQD, Q3 +1.5 * IQD] which is represented by whiskers (Q1 = first quartile; Q3 = third quartile and IQD = interquartile distance). Disk and error bars in each distribution show mean and standard error.

(B) Male *An. coluzzii* responses to 1-harmonic sound of a single female or pure sound (data subset B). Boxplots, disk and error bars have the same meaning as in (A).

(C) Male *An. coluzzii* responses to 2-harmonic sounds of single or 30 females along SVLs (data subset C). Each point represents a daily replicate averaged over several trials on potentially same mosquitoes. Continuous lines and associated coloured areas represent the mean and 95%-CI. SVL were corrected as explained in Method section 'Corrected SPLs for estimating the hearing threshold'. The green dotted lines represent the lowest estimate of the hearing threshold from the response to 1-female 2-harmonic sound-stimuli.

Males mostly use the female's first harmonic to hear their flight tone (subset A)

Subset-A sound-stimuli with one or two harmonics were heard by males as the response distributions are different from the null distribution (Fig. 5 A), both for the angular-speed (upper-quartile angular-speed difference: one-sample t=5.7, df=9, BH-corrected p<0.001, mean=1.5 rad/s; one-sample t=5.0, df=9, BH-corrected p<0.001, mean=1.0 rad/s, respectively) and for the wingbeat frequency (upper-quartile wingbeat-frequency

difference: one-sample t=5.2, df=9, BH-corrected p<0.001, mean=16 Hz; one-sample t=4.6, df=9, BH-corrected p=0.0013, mean=13 Hz, respectively).

Our results show that males did not differentiate between the first-harmonic's sound and the combination of the first and second harmonic sounds of a female flight tone (upper-quartile angular-speed difference: LRT, χ^2=2.6, df=1, p=0.11; upper-quartile wingbeat-frequency difference: LRT, χ^2=1.1, df=1, p=0.29).

Males react to a pure tone at least as much as to a female flight tone (subset B)

Subset-B stimuli, i.e. 1-harmonic 1-female sound and pure tone sound, were both heard by the males because the response distributions were different from the null distribution (Fig. 5 B), both for the angular-speed (upper-quartile angular-speed difference: one-sample t=5.7, df=9, BH-corrected p<0.001, mean=1.5 rad/s; one-sample t=5.4, df=38, BH-corrected p<0.001, mean=1.6 rad/s, respectively) and for the wingbeat frequency (upper-quartile wingbeat-frequency difference: one-sample t=5.2, df=9, BH-corrected p<0.001, mean=16 Hz; one-sample t=5.1, df=38, BH-corrected p<0.001, mean=30 Hz, respectively).

Our results show there was little difference in the male response between the female's 1-harmonic sound-stimulus and the pure sound of the same frequency/SVL. While males changed their angular-speed with the same amplitude in response to these two stimuli, they changed their wingbeat frequency two times more with the pure sound (upper-quartile angular-speed difference: LRT χ^2=0.052, df=1, p=0.82; upper-quartile wingbeat-frequency difference: LRT χ^2=4.5, df=1, p=0.033, respectively).

Males react to the 1-female sound more than to the 30-female sound, with a hearing threshold less than 20 dB SVL (subset C)

Using data subset C (ranges of SVLs of Audio S1 and Audio S2; see Table S1 for sound levels), our results (Fig. 5 C) show that free-flying males responded to the sound stimuli providing enough sound level, by increasing both their angular-speed and their wingbeat frequency as the tested sound levels increased (upper-quartile angular-speed difference: LRT χ^2=36.8, *df*=1, p<0.001, effect size=0.12 rad/s per dB SVL; and upper-quartile wingbeat-frequency difference: LRT χ^2=20.7, *df*=1, p<0.001, effect size=2.3 Hz per dB SVL, respectively). The number of females had a negative effect, but this was not interpretable, because of distinct values of sound levels for each number of females (upper-quartile angular-speed difference: LRT χ^2=3.3, *df*=1, *p*<0.001, 1.2 rad/s for 1-female versus 0.6 rad/s for 30-female stimuli; and upper-quartile wingbeat-frequency difference: LRT χ^2=4.7, *df*=1, *p*=0.031, 20 Hz for 1-female versus 10 Hz for 30-female stimuli, respectively). However, globally, the males responded more to the 1-female sound than to the 30-female sound as the sound level increased (i.e. interaction between the sound level and the number of female; upper-quartile angular-speed difference: LRT χ^2=3.3, *df*=1, p=0.070, effect size = additional 0.05 rad/s per dB SVL for 1-female sound-stimulus; and upper-quartile wingbeat-frequency difference: LRT χ^2=12.7, *df*=1, p<0.001, effect size = additional 1.3 Hz per dB SVL for 1-female sound-stimulus, respectively).

For 2-harmonic 30-female sound-stimuli (Fig. 5 C, red colour), the mean sound-level threshold was 21 dB SVL with an 14-25 dB SVL 95%-CI, if considering the angular-speed as response parameter. Using the wingbeat frequency parameter, the mean sound-

level threshold was 18 dB SVL with a 9-23 dB SVL 95%-CI. For 2-harmonic 1-female sound-stimuli (Fig. 5 C, green colour), the mean sound-level threshold was 15 dB SVL with an 9-20 dB SVL 95%-CI, if considering the angular-speed to be a response parameter. Using the wingbeat frequency parameter, the mean sound-level threshold was 17 dB SVL with a 13-20 dB SVL 95%-CI. Considering these latter stimuli, which are the most ecological ones, a conservative estimate of the hearing threshold is then 20 dB SVL.

Discussion

Behavioural assessment of hearing threshold in swarming mosquitoes

Inter-mosquito acoustic communication is believed to occur at short range only *(03)*, during mating behaviour when mosquitoes are flying in loops near a visual marker. In *An. coluzzii*, males gather in tens to thousands over station-keeping swarm sites, while virgin females join the swarm in much fewer numbers as they mate only once in a life-time. Once a male detects a female's presence from her wing-flapping sound, the male starts to chase the female. Thus, there is strong competition between males to detect relatively rare females. Accordingly, acute hearing sensitivity is highly advantageous to males.

Under laboratory conditions, we show that male *An. coluzzii* responds strongly to pure sound of 26±2 dB SVL at the female's wingbeat frequency (Fig. 5 B and Table S1) and we estimate the hearing threshold to be 20 dB SVL or less with a 95%-CI using 2-harmonic 1-female sounds (13-20 dB SVL). Researchers have used electrophysiological mosquito preparations to measure hearing threshold in the Johnston's organ, which does

not involve free-flying, pre-mating behaviour such as swarming (but see *(03)(06)*). This may explain why they found far higher sound thresholds (see Introduction section). However, in one published study and with only 3 male mosquitoes, thresholds in *An. coluzzii* measured in the Johnston's organ nerve in response to sound stimuli were as small as 5-12 dB SVL (1.5±0.7 10^{-7} m/s) with the antenna fibrillae extended *(12)*. Apart from this case, however, lower hearing thresholds measured electrophysiologycally can partly be explained by the absence of flight tones in males, which is known to be important to enhance the sensitivity in males to female sound. This creates mixed-harmonics for which the JO is tuned to, as shown by electrophysiology mosquito preparations exposed to flight sound simulation, which lowers the hearing threshold by 7 dB in *Cx. pipiens pipiens (15)*. However, this may not be the only explanation. Mosquitoes exhibit active hearing, which can be triggered only during specific physiological states *(24)(25)*, which swarming may be one of them, and this could enhance hearing to detect a female when approaching the male swarm before she is chased by a competitor.

The only other species to have been tested while flying/swarming is *Ae. communis*; in the field, the mean hearing-threshold of males at the female's wingbeat frequency was shown to be particularly low, 26 dB SVL. However, their method consisted in monitoring flight-speed changes in natural swarms by eye, which may not have enabled them to detect the smallest response amplitudes, thereby over-estimating the threshold *(06)*. On the contrary, we measured both flight dynamics and wingbeat frequency from quantitative measurements.

Finally, electrophysiology measurements in JO are usually averaged over JO units, however, this misrepresents the effective signal which could trigger a behavioural

response. Indeed, in addition to individual sensitivity in frequency and threshold, JO units are sensitive to the direction of the sound wave, and then only the JO units which are aligned with the sound wave-front display a low response-threshold. As a consequence, averaging all JO-units threshold may over-estimate hearing threshold *(13).*

Male response to sound and the effect of number of females

Males changed their wingbeat frequency with a greater amplitude when exposed to 1-female sound than to 30-female sound, however, the change in angular-speed was small and its statistical significance was marginal (Subset C). Two comments merit emphasis; the first is that a group of frequencies that are attractive alone (e.g., grouped-female sounds) have a masking effect for mosquito auditory perception. These results support reports published 80 years ago on *Ae. aegypti* males which were observed not to be attracted to two or more sounds that are attractive alone *(26).* Second, it is interesting to see that males respond more with their wingbeat frequency than with their flight trajectory or dynamics. The wingbeat-frequency change is consistent with a current theory which suggests that during a chase between a male and a female, the male moves to the sound source by locating the female from her wingbeat sound and that the male adjusts its own wingbeat frequency to hear her better, through an auditory mechanism based on antennae distortion products *(01)(02)*. In our case, the sound wave-front is almost planar at the male position, due to the distance and membrane dimension of the speaker, contrary to the sound wave of a female of same sound level which would be far more spherical. This may create contradictory signals in the mosquito auditory system, i.e., the sound level suggests that the female is very close, but the sound wave shape gives poor information about her actual location.

The question of hearing higher harmonics

Males are known to use mainly the female's first harmonic to hear her flight tone. Indeed, *Ae. aegypti* respond (with clasping and seizing movements) to low frequencies under 500 Hz (i.e., pure tones, or 1-harmonic sounds) using tuning forks *(16)*, while other species, such as *Cx. pipiens pipiens*, have a narrower frequency range of response (500-600 Hz) when swarming *(27)*. In *Toxorhynchites brevipalpis, Cx. pipiens pipiens,* and *An. gambiae s.l.*, electrophysiology revealed that male antennae are sensitive to a large frequency-band up to 2 kHz that encompasses the two first harmonics, however, the electrical tuning of their JO is very narrow and centred on the difference wingbeat frequency of the two sexes which is close to the female's first harmonic *(28)*. Male sensitivity to higher-harmonics of the female wingbeat-frequency without the first harmonic is known to be null since early mosquito studies. Using sound playback of female recordings for which the first harmonics were removed, *Ae. aegypti* males were shown not to respond anymore, but the authors reported their results without any further information *(26)*. The absence of male response if removing the female first-harmonic from the stimulus is complementary with our results which shows similar male response if removing the second harmonic of the female flight-tone. The contrary (i.e., males can hear the female second-harmonic) has been reported more recently without statistical proof *(29)* and also contested using arguments on auditory processing of phasic information in the JO nerves *(01)*. However, the image channel resulting from the non-linear vibration of the antennae from the sound of the two sexes was shown to reinforce the hearing sensitivity of males around (slightly above) the frequency of the female's second harmonic in electrophysiology measurements on *Cx. pipiens pipiens(15)* and *Ae. communis (06)*. The results of our behavioural assay suggest that this reinforcement is negligible in practice, at least in *An. coluzzii.*

Pure sound versus natural sound

Pure sounds and female pre-recorded flight-tones are known for a long time to trigger a response in mosquitoes *(16)(30).* However, to our knowledge no comparison has been formally done between pre-recorded sounds and pure sound of the same frequency. Our experiment shows that the pure sound slightly behaved as a supernormal stimulus (for the wing-beat frequency response-parameter), at least at 26 dB SVL. This means that the information carried in the sound that elicits the male response is mostly the mean wingbeat frequency. A proper study could be carried out with pure-sounds to control better the sound level than with pre-recorded sounds, as well as with larger ranges of frequencies and sound levels than in the present study.

Monitoring SVL from SPL measurements

Many studies report hearing thresholds based on SPL, which is a physical quantity that mosquitoes do not sense. We monitored sound level with SPL, but we paid attention to fulfil the experimental conditions to have equivalence between SPL and SVL (see Methods section). Some studies reported SPL values as hearing thresholds even though the equivalence conditions were not fulfilled or were unknown. Wishart and Riordan *(26)* estimated that *Ae. aegypti* responds to a sound of approximately 20 dB SPL from experiments involving 30cm-side netting cages and sound stimuli presented through a diffuse speaker held against the cage netting. However, mosquitoes could be located at a couple of centimetres from the loudspeaker where SPL and SVL are not equivalent at this distance, i.e., when SPL cannot be a good physical quantity to describe what the mosquito

auditory organs are exposed to. Another example is Belton *et al. (08)* who measured a response threshold in the Johnston's organs of male *Ae. aegypti* between 0 and 10 dB SPL; he related SPL to SVL using a formulae that assumed far-field condition (without saying so), unfortunately, he did not provide enough details of the experimental setup to know the distance between the pressure microphone and the loudspeaker; thus, the thresholds were probably underestimated. More recently, Dou *et al. (10)* put their loudspeaker at 2.5 cm against their 30cm-side cage to measure the response of mosquitoes and monitored the sound level with a SPL meter in the middle of the cage. They measured flight response to sound in *Ae. aegypti* females for the first time, from a threshold of 79 dB SPL, which could be far more in terms of SPL since it was measured in the middle of the cage and mosquitoes were free to move against the side of the loudspeaker. In addition, SVL may have been far greater than SPL at this distance from the speaker. All taken together, such SVLs may never occur in the field.

Data Accessibility

Raw response files (sound and tracked flight trajectories) are available at: https://doi.org/10.5061/dryad.9cnp5hqhj *(31)*. Sound stimuli are available as supplementary material. Custom audio–video code for parameter-extraction and audio–video synchronization (modified Matlab files from https://doi.org/10.17632/hn3nv7wxpk.3 *(32)*), custom statistics code for data analysis and figure plot (R files) and dataset (Text files) are available at https://doi.org/10.17632/6w5jttwkj8.1 *(33)*.

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Natalie Morley (insect rearing), Stephen Young (discussion about statistics).

Author contributions

Conceptualization LF, GG; Methodology LF, GG; Software LF; Formal Analysis LF; Investigation LF; Data Curation LF; Writing – Original Draft LF; Writing – Review & Editing OR, GG; Visualization LF; Supervision GG, OR; Funding Acquisition OR, GG, LF.

Competing interests

The authors declare no conflict of interest.

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SUPPLEMENTARY MATERIALS

Supplementary figures

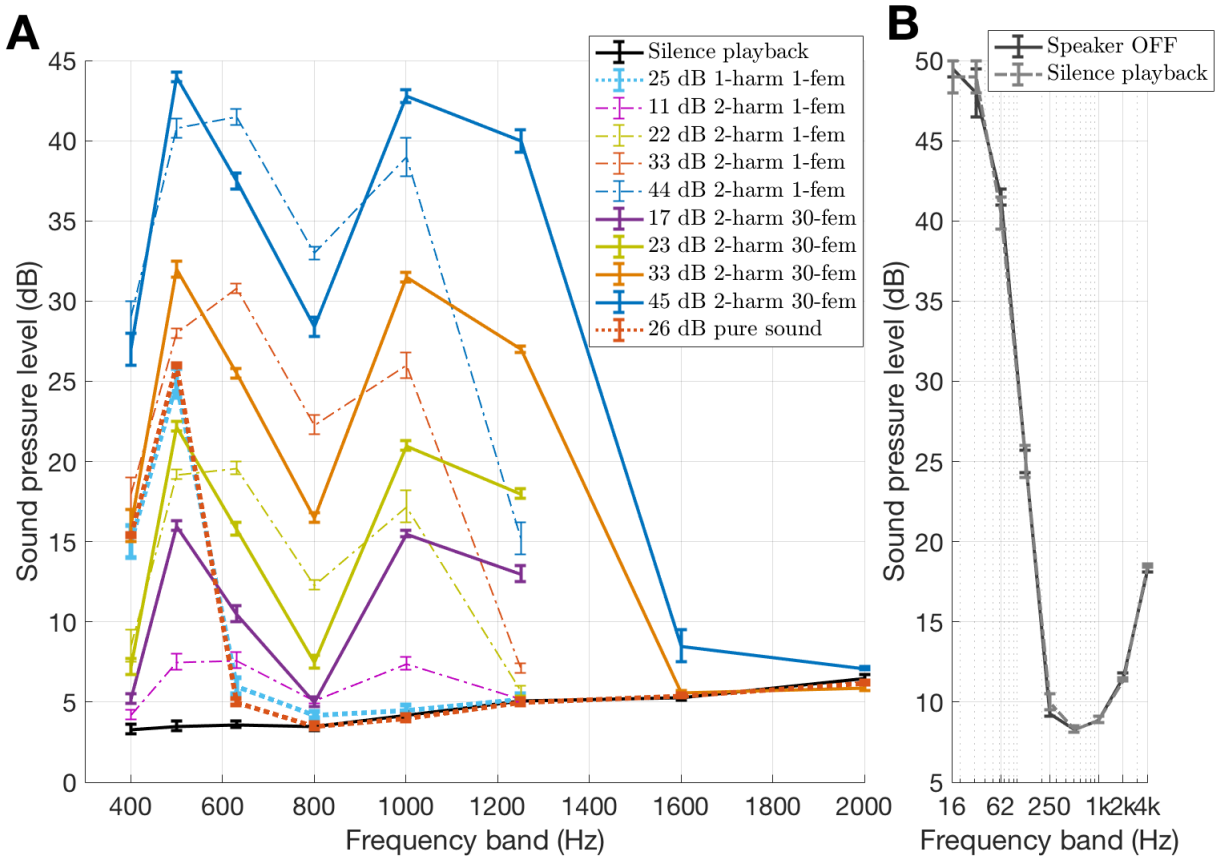

Fig. S1. Calibrated sound-level measurements of played-back sounds and background noise. SPLs (ref 20 µPa) were measured at the *Anopheles coluzzii* swarming position 1) to estimate the sound level received by the tested mosquito and 2) to know the background noise level of the sound-proof chamber. The X-axis values represent the central frequency f_c of the octave or 1/3-octave band filters. Each filter has a lower limit of $2^{-1/6}f_c$ and an upper limit of $2^{1/6}f_c$. For example, X=800 Hz represents the sound pressure level between 713 Hz and 898 Hz. The Y-axis error-bar represents maximum and minimum values measured during stimulus duration under a time constant of 1 s (slow mode, according to IEC 61672-1: 2002). SPLs are equal to SVLs in our setup (see Method section 'Monitoring SVL from SPL measurements').

(A) SPL measurements of female sound stimuli as a function of frequency, limited to a frequency range audible to *An. coluzzii* males *(01)*, and plotted at 1/3 octave steps reveals first and second harmonic of the female wingbeat sound. Black dashed line shows sound level when playing-back 'silent' (i.e. just speaker noise), with the same settings as during the experiment. The four coloured solid lines correspond to the sound levels at the mean male's 'XYZ' position during play-back of the female stimuli (related to four sound levels). Dashed lines represent the same, but for the 1-female sound-stimuli.

(B) SPL measurements in the sound-proof chamber without playback and with 'silence' playback, along octave bands from 16 Hz to 4 kHz. The black dashed line shows the sound level when playing-back silence (i.e. speaker noise), while the plain black line corresponds to when the speaker is off (i.e. showing noise floor of sound-proof room). Both are the same, i.e. they show that the speaker has a very low noise level enabling us to playback low level sounds.

Supplementary tables

	Sound stimuli						
Subset	**Single/ Group**	**Number of harmonics**	**Recording/ Synthetic**	**Sound level**			
				SPL measurement of the two 1/3-octave bands closest to the first-harmonic (dB SPL) at fixed distances from the speaker (0.9 m)			**Error due to ±0.2 m oscillation (and total error)**
				Mean value	**Corrected mean value**	**Error over the 7 s**	
NA	Silence playback	NA		6.9		±0.3	±0.3
A	Single	1	Recording	25.0		±0.9	±2
		2		32.6		±0.3	±2
B	Single	1	Recording	25.0		±0.9	±2
			Synthetic	26.0		±0.2	±2
C	Single	2	Recording	10.6	NA	±0.5	±2
				22.4	14.4	±0.4	±2
				32.6	24.6	±0.3	±2
				44.2	36.2	±0.6	±2
	Group (~30)			17.1		±0.5	±2
				23.1		±0.4	±2
				32.9		±0.5	±2
				44.9		±0.5	±2

Table S1. Description of stimulus sound-levels. This table gives the sound pressure levels (SPL ref 20 µPa) and associated errors of all played-back sound stimuli at the male's mean location in the frequency range of the female's first harmonic. See Methods 'Corrected SPLs for estimating the hearing threshold' for the corrected SPL mean value and Methods section 'Estimate of SPL errors at mosquito's location' for last two columns. SPLs are equal to SVLs in our setup (see Method section 'Monitoring SVL from SPL measurements).

Other supplementary files

Audio S1 (AudioS1.mp3). Sound stimulus recording of the 1-female *An. coluzzii* (7 s) before any filtering and level adjustment. Related to Fig. 2.

Audio S2 (AudioS2.mp3). Sound stimulus recording of the 30-female *An. coluzzii* (7 s) before any filtering and level adjustment. Related to Fig. 2.

Movie S1 (MovieS1.mp4). Audio-video recording of a *An. coluzzii* male exposed to the loudest *An. coluzzii* 1-female sound (10-s silence + 7-s sound exposition + 10-s silence). Related to Fig. 4 A.

Supplementary information

Temperature monitoring. The temperature was monitored by type-T thermocouples (IEC 584 Class 1, Omega) associated with a temperature logger (HH506RA, Omega) totalling a measurement accuracy error of ±0.9 °C. The chosen thermocouple was located on a room wall at a height of 85 cm from the floor. The four recordings of the reference sound stimuli (two species, two sexes) were recorded at 28.0 °C. The mean temperature and standard deviation of the behavioural assays were 28.0±0.3 °C.