

## RESEARCH ARTICLE

# Behavioural analysis of swarming mosquitoes reveals high hearing sensitivity in *Anopheles coluzzii*

Lionel Feugère<sup>1,2,\*</sup>, Olivier Roux<sup>1,3</sup> and Gabriella Gibson<sup>2</sup>

## ABSTRACT

Mosquitoes of many species mate in station-keeping swarms. Mating chases ensue as soon as a male detects the flight tones of a female with his auditory organs. Previous studies of hearing thresholds have mainly used electrophysiological methods that prevent the mosquito from flying naturally. The main aim of this study was to quantify behaviourally the sound level threshold at which males can hear females. Free-flying male *Anopheles coluzzii* were released in a large arena (~2 m high×2 m×1 m) with a conspicuous object on the ground that stimulated swarming behaviour. Males were exposed to a range of natural and synthetic played-back sounds of female flight. We monitored the responses of males and their distance to the speaker by recording changes in their wingbeat frequency and angular speed. We show that the mean male behavioural threshold of particle velocity hearing lies between 13 and 20 dB sound particle velocity level (SVL) (95% confidence interval). A conservative estimate of 20 dB SVL (i.e. <math>0.5 \mu\text{m s}^{-1}</math> particle velocity) is 12–26 dB lower than most of the published electrophysiological measurements from the Johnston's organ. In addition, we suggest that (1) the first harmonic of a female's flight sound is sufficient for males to detect her presence, (2) males respond with a greater amplitude to single-female sounds than to the sound of a group of females and (3) the response of males to the playback of the flight sound of a live female is the same as to that of a recorded sound of constant frequency and amplitude.

**KEY WORDS:** Auditory processing, Bioacoustics, Free flight, Johnston's organ, Insect sensory system, Sound sensitivity

## 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 (Warren et al., 2009; Simões et al., 2018; Feugère et al., 2021b). The more sensitive males are to flight sounds, 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. The male antennal organs of mosquitoes are the most sensitive to sound described so far among arthropods (Göpfert and Robert, 2000); however, the measurement

of hearing sensitivity is usually performed on tethered males, which prevents natural body movement such as antennal orientation and wing flapping behaviour in response to female sound. Only a few studies have measured hearing thresholds behaviourally (Menda et al., 2019; Lapshin and Vorontsov, 2021; Feugère et al., 2021b). The measurement of behavioural sound sensitivity in flying male mosquitoes faces the difficulty of monitoring how much sound energy actually reaches their antennae because the sound level meter is at a fixed position, whereas the position of the male mosquito is continuously changing during his flight. The aim of this study was to quantify behaviourally the overall sound level threshold at which males can hear females, i.e. the limit of sensitivity of a male to locate a female in flight. Accordingly, we had to determine the components of female wingbeat sound that male mosquitoes are most responsive to, so that our definition of the sound level includes only the frequency bands audible to males.

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 on the market as yet that can truly measure particle velocity (Zhou and Miles, 2017); 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). However, SPL hearing thresholds have sometimes been measured under the near-field condition instead of the far-field condition (Tischner, 1953; Belton, 1961; Dou et al., 2021), which means there is a risk that some reported hearing thresholds may have been underestimated, as elaborated in the Discussion.


Hearing thresholds can be assessed by measuring a physiological or behavioural response to a given stimulus sound level and sound frequency. Among the physiological methods, laser vibrometry records the vibration of the flagellum (Göpfert et al., 1999; Penner et al., 2010); however, it is limited when assessing hearing threshold because the recorded vibration only refers to the input to the hearing chain (i.e. flagella movement) and does not provide any indication as to whether or not the neurons of the mosquito have been neuro-electrically activated following the sound-induced vibration of the flagella. Unlike laser vibrometry, electrical responses of the Johnston's organ (JO) 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 JO). With this method, the electrical response threshold in male *Culex pipiens pipiens* JOs showed a mean sensitivity of 32 dB SVL per JO scolopidia (range 22–44 dB SVL;  $n=74$  JO scolopidia; criterion of 2 dB above noise floor; 18–21°C) (Lapshin and Vorontsov, 2019) and a mean of 44 dB SVL per mosquito in male *Culex quinquefasciatus* JOs (range 36–52 dB

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SVL;  $n=3$  males; criterion of 10 dB above noise floor) (Warren et al., 2009). In *Aedes aegypti*, the male JO nerve was shown to respond to a mean of 40 dB SVL (range 31–50 dB SVL;  $n=11$  males) (Menda et al., 2019). 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 (Pennetier et al., 2010). Under this antennal physiological state, Pennetier et al. (2010) measured a JO response threshold in two male *An. coluzzii* of only 10 dB SVL [range 5–12 dB SVL, i.e. particle velocity of  $1.5(\pm 0.6) \times 10^{-7}$  m s $^{-1}$ ;  $n=4$  measurements on 2 males; criterion of 1.4 recording noise floor].

In a distortion product based hearing system, as proposed for mosquitoes, hearing sensitivity can be further enhanced (or even produced) by the mosquito beating its wings (Lapshin, 2012). However, electrophysiological and laser vibrometry methods 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 combine the male's flight sound frequency with 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 (18–22°C) (Lapshin, 2012).

The results of electrophysiological and laser vibrometry studies can be difficult to compare against each other because of differences in methodologies used to assess threshold responses (e.g. determination of statistical definitions of neural thresholds and variation in the locations of electrodes). In addition, the main goal of these studies is not always about measuring absolute hearing thresholds, and as a consequence the number of replicates can be too few to analyse statistically.

Behavioural methods face similar constraints; however, the assessment of physiological responses to sound stimuli offers a more natural context that enables more natural responses to sound. Behavioural responses provide more robust evidence of auditory outcomes because the whole auditory chain plus the motor responses are included. To our knowledge, there are only three published behavioural studies of mosquito sensitivity to sound intensity. First, Menda et al. (2019) measured the behavioural response of *Ae. aegypti* to 40 and 65 dB SVL by monitoring the take-off of resting mosquitoes in a cage located in the far-field of the sound source. However, the behavioural methodology was not appropriate for the natural physiological context of swarming behaviour in this species; in the field, both male and female *Ae. aegypti* fly continuously once the male detects the female's flight tones (i.e. they rarely rest and take-off again). Indeed, male responsiveness to sound was found to be reduced when not flying (Lapshin, 2012).

Second, Feugère et al. (2021b) 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  $33 \pm 3$  dB SPL. However, males may respond better to the sound of individual females rather than to a group of females that would produce a relatively wide range of wingbeat frequencies, as described for *Ae. aegypti* (Wishart and Riordan, 1959).

Third, Lapshin and Vorontsov (2021) 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; 12°C).

The aim of our study was to investigate the behavioural hearing threshold of *An. coluzzii* males; Pennetier et al.'s (2010) measurements suggest that their JO may be as sensitive as 10 dB

SVL (range 5–12 dB SVL,  $n=4$  measurements on 2 males, criterion of 1.4 recording noise floor). As suggested 70 years ago by Roth (1948), male hearing may be enhanced during swarming behaviour (i.e. flying in loops over a floor marker, 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. Therefore, we used a modified approach of Lapshin and Vorontsov (2021), who worked in the field with *Ae. communis*. Our study was performed under the following conditions: (1) in a laboratory sound-proof chamber, with controlled measurement of sound levels; (2) with a range of types of sound that males were exposed to; (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 that mosquitoes are sensitive to. For this reason, our main aim of quantifying hearing threshold was inter-connected with the following questions. (1) 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. (2) Is temporal variation in natural female sound required for males to detect females or is a single frequency at a constant amplitude sufficient? (3) 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 single-frequency sounds to mimic female sound, which will make the hearing threshold easier to estimate in future studies.

## MATERIALS AND METHODS

### Mosquitoes

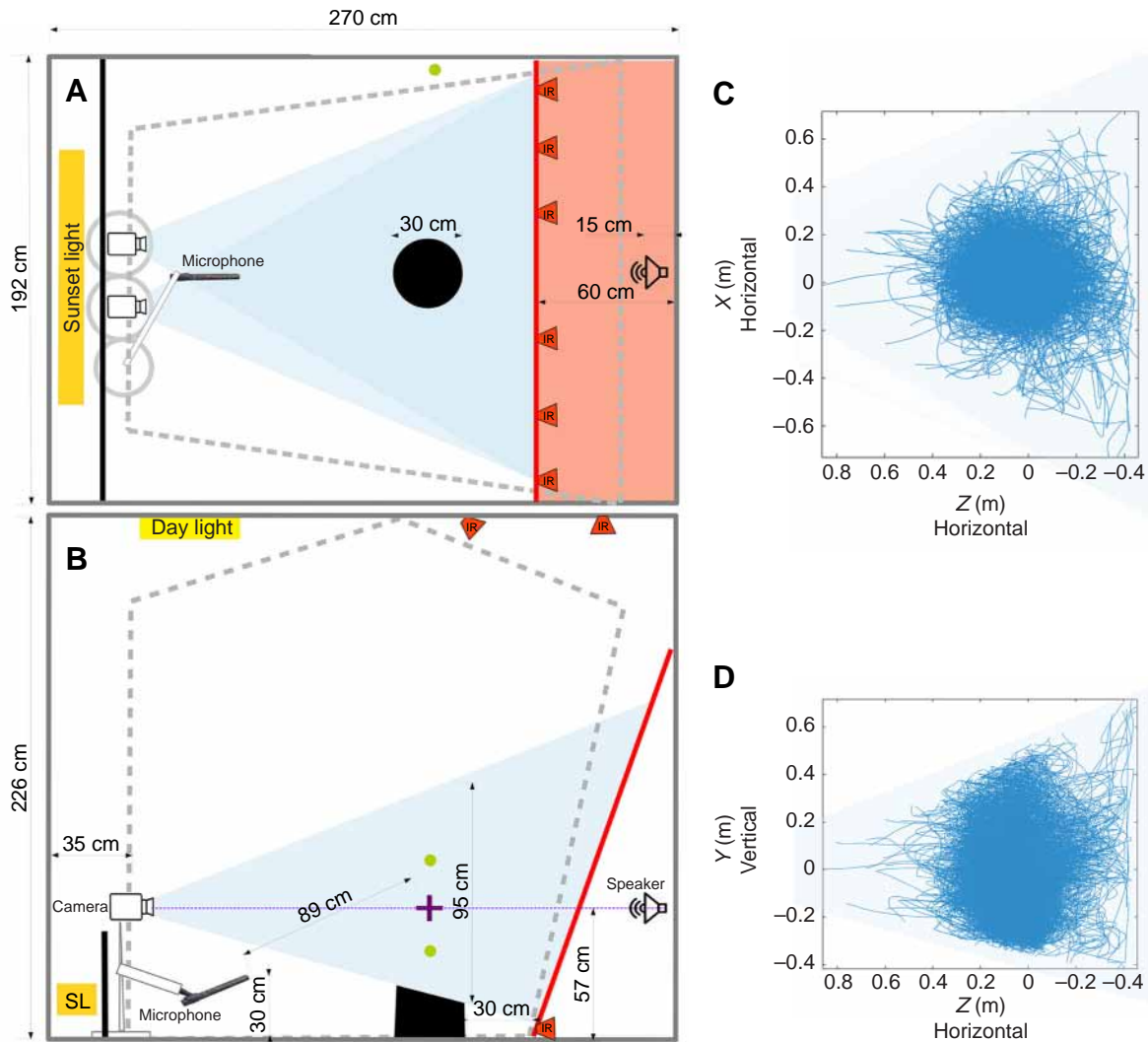
All experiments were performed with virgin *An. coluzzii* Coetzee & Wilkerson. The colony was established at the Natural Resources Institute (NRI), University of Greenwich (UK) from eggs provided by the Institut de Recherche en Sciences de la Santé (IRSS), Burkina Faso. Eggs were obtained from a colony 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 (Fanello et al., 2002). The NRI colonies were kept in environmentally controlled laboratory rooms on a 12 h:12 h light:dark cycle (lights off at 15:00 h), >60% relative humidity (RH) and ~24–26°C. Larvae were fed Tetramin® fish flakes and rice powder. Adult males and females were separated <12 h post-emergence to ensure all females were virgin and fed a solution of 10% sucrose and 1% saline *ad libitum*. Adult mosquitoes were kept in cube cages (~30 cm sides), populated with (i) ~300 virgin females and (ii) ~20 males.

### Experimental setup

The basic experimental setup (Fig. 1) is the same as for a previous study with *An. coluzzii* (Feugère et al., 2021b) as described below.

### 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×W×H 2.7 m×1.9 m×2.3 m), producing a reverberation time  $\leq 0.07$  s for frequencies above 200 Hz (IAC Acoustics). The SPL in the sound-proof room without any playback was always quieter than that with



**Fig. 1. Sound-proof chamber setup for recording sound and video of *Anopheles coluzzii* behaviour.** The setup is modified from Feugère et al. (2021b). (A) Bird's-eye and (B) side views of the sound-proof chamber (the grey outline indicates the sound-proof walls). Blue shaded areas indicate the 3D field of view of the cameras recording mosquito flight paths. Two infrared (IR)-sensitive cameras fitted with IR pass filters recorded flying mosquitoes as black silhouettes against an evenly lit IR background (red IR lights and shading). A separate lighting system provided gradual semi-natural dusk visible to mosquitoes, consisting of dispersed dim white lights on the ceiling and 'sunset' lighting (sunset light, SL) below the horizon (opaque wall ~40 cm tall: black vertical bar in B). A microphone recorded flight sounds of mosquitoes swarming directly above the black swarm marker (black circle in A; + and black trapeze in B marks the centre of the swarm in B). A thermocouple (85 cm above ground level; green circles) recorded temperature at approximately mean swarm height. A speaker located behind an IR-illuminated thin-cotton sheet, outside the mosquito net enclosure (indicated by the dashed lines) played back sound stimuli. (C) Bird's-eye and (D) side views of the superimposed flight tracks of the entire dataset with blue-shaded areas indicating the field of view of the cameras.

playback of the sound stimuli in the third-octave frequency band of the sound stimulus (Fig. S1A). Below 176 Hz (upper limit of the 125 Hz octave band), the ambient noise level rose (Fig. S1B; 25 dB at 125 Hz), as a result of low-frequency vibration of the building's aeration system, which may have been detected by the *An. coluzzii* auditory system (Pennetier et al., 2010) as a low-frequency background noise to the sound stimulus.

### 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×W×H 1.8 m×1.7 m×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 typical 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 stimulates mosquitoes to take-off, followed by swarming behaviour in response to the presence of a visually conspicuous matt-black marker on the floor; both males and virgin females fly in loops above the marker, but this is rarely observed if males are present because males mate with females quickly and mated females cease swarming behaviour (Poda et al., 2019; Gibson, 1985). We used virgin female swarming behaviour to record their flight sound within a relatively limited distance from the marker.

### 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 an amplitude decrease of >15 dB beyond 90 deg from the microphone head; sensitivity at 1 kHz: 40 mV Pa<sup>-1</sup>; A-weighting equivalent noise level: 8 dB) directed toward the swarm location. The microphone tip was located at a distance of 0.89 m from the centre of the swarm area for the experimental male mosquitoes and the sound recording of the 30-female swarm stimulus (Fig. 1); for the recording of the 1-female sound stimulus, the microphone was located 0.75 m from the centre of the swarm area. The microphone was 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; Fry et al., 2004). Two video cameras (Basler, ace A640-120gm) were fitted with wide-angle lenses (Computar, T3Z3510CS, 1/3 inch 3.5–10.5 mm f1.0 Varifocal, Manual Iris) to maximize 3D volume of video-tracking. Infrared (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 in Matlab (version R2017a).

#### Temperature monitoring

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±s.d. temperature of the behavioural assays was 28.0±0.3°C.

#### Sound stimuli

##### Recording context

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

##### Signal generation

We generated four kinds of stimulation signals ('2-harmonic 1-female', '2-harmonic 30-female', '1-harmonic 1-female' and '1-harmonic constant') (Audios 1, 2, 3, 4; signal spectrum in Fig. 2) over a range of sound levels, producing 10 stimuli in total. First, we selected the first 7 s section of the sound of a single female swarming over the marker (Audio 5). Second, a 7 s section of the sound of 30 swarming females was selected (Audio 6), ~10 min after the first female started to swarm. Third, four sound levels for each of the 1- and 30-female sounds were generated (10–45 dB SPL; Table 1), based on the results of preliminary experiments. These eight stimuli contained the two first harmonics. Fourth, a high-pass filtering was added to all the stimuli to remove the electrical noise below the first harmonic (at the noise level; see Fig. 2; Table S1). Fifth, 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 Materials and Methods, '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 to higher harmonics (Pennetier et al., 2010; Warren et al., 2009). Sixth, we generated a synthetic 1-harmonic sound, the '1-harmonic constant' stimulus, with constant frequency and amplitude over time (set at the same mean peak amplitude and mean frequency as the '1-harmonic 1-female' sound). Seventh, gradual increase/decrease over 1 s in the level of the start and end sounds was added to avoid creating sound artefacts as a result of the signal truncation, and to make the stimulus more natural [possibly important for active antennal amplification (Jackson and Robert, 2006)].

The 10 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 in which stimuli were played, 10 different sequences were generated, each containing the 10 sounds in random order. All stimuli were sampled at 8 kHz/24 bits and designed in Matlab (R2017a, The MathWorks Inc., Natick, MA, USA). Fig. 2 gives the sound spectrum and amplitude over time of each type of stimulus. Table S1 gives the filter/frequency parameters used to generate the stimuli. Table 1 gives the sound levels for each of them. Audios 5 and 6 are the original 1-female and 30-female sound recordings, respectively. Audios 1, 2, 3 and 4 are the four kinds of stimuli: 2-harmonic 1-female, 2-harmonic 30-female, 1-harmonic 1-female, 1-harmonic constant, respectively.

#### Sound diffusion

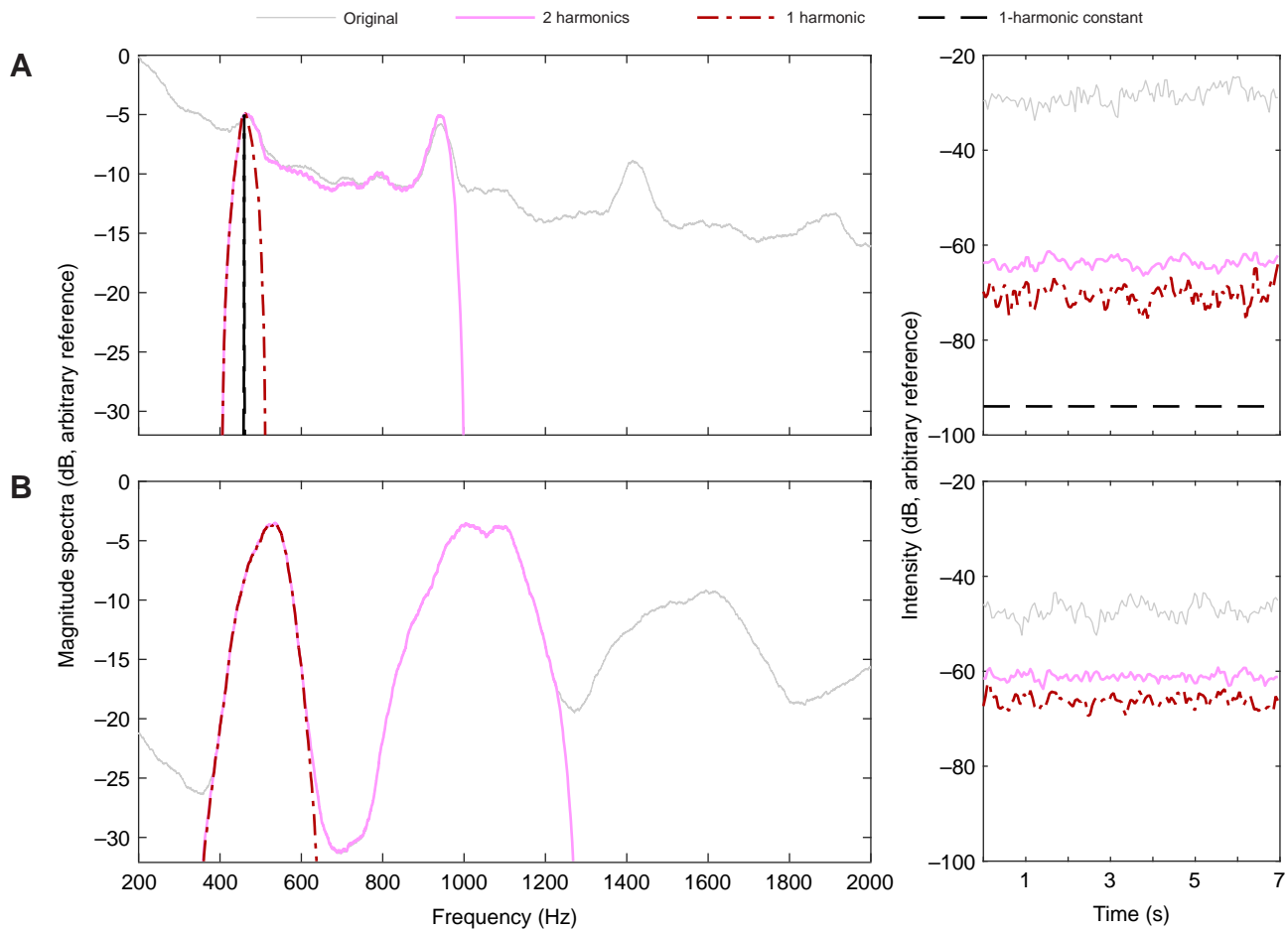
Sequences of sound stimuli were played back from a speaker (Genelec 8010A) plugged into a Scarlett 18i8 sound card running pro-Tools First and Audacity on Windows 7. The speaker is composed of two membranes (diameter 76 mm and 19 mm). The centre of the larger speaker's membrane was located 0.57 m above the floor, 0.15 m from the back wall and 0.9 m from the swarming centre (Fig. 1). The speaker's self-generated noise is less than 5 dB SPL (A-weighted) and the sound card's Equivalent Input Noise is -127 dBu.

#### Data subsets

Although stimuli were played back in random order during a single experiment, they can be grouped into three overlapping subsets (Fig. 3), each of which corresponds 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 'types of sound stimulus' (single-frequency versus pre-recorded played-back stimuli); and subset C – effect of the number of females (1 versus 30) in the recorded-sound stimuli and of the sound levels of the sound stimuli on male hearing to estimate the hearing threshold.

#### 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 20, 3–4 day old males were released the day prior to experiments at ~18:00 h in the sound recording flight arena. At 15:00 h, after the ceiling lights had dimmed to the lowest intensity, the horizon light completed a 10 min dimming period and then 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 all 10 sound stimuli (i.e. the four kinds of stimuli, with four sound levels for two of them, see Materials and Methods, 'Signal generation') was played back



**Fig. 2. Spectral and temporal properties of sound stimuli.** Spectral (left) and temporal (right) properties of sound stimuli of a single swarming *An. coluzzii* female (A) compared with that of 30 females (B). The originally recorded sounds are represented by a grey line (Audios 1 and 2 for unfiltered 1-female and 30-female sounds, respectively; not used directly as sound stimuli). The 1-harmonic 1-female sound is shown as a dash-dotted red line; while the 2-harmonic sound is represented by a solid pink line; the 1-harmonic constant sound is shown by a dashed black line (which looks like a solid line in the spectra in A because of the thinness of the sound frequency bandwidth). Magnitude spectra were calculated over 7 s and averaged over 50 Hz windows. The RMS pressure levels were computed over a 0.1 s time window with 0.05 s overlap, over the 7 s duration of the stimuli. See Table S1 for characteristics of filters applied to Audios 1 and 2 to generate the 1-harmonic and 2-harmonic stimuli.

from the speaker (see Movie 1 with a male exposed to one sound stimulus; see Fig. S2 for examples of responses to each kind of 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 10 sequences were played or after 50 min of constant horizon light, either of which marked the end of the experiment for the day (=1 replicate). Males were then collected and removed from the flight arena. A new group of ~20 male mosquitoes was released in the sound-proof chamber, to be used for a new replicate the next day (one replicate per day, for 10 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  $\mu$ Pa; 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/sound card 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 (Warren et al., 2009; Pennetier et al., 2010). They were calculated as follows:  $10\log_{10}(10^{0.1L_{B1}} + 10^{0.1L_{B2}})$ , 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 1; and Fig. S1 for all third-octave values).

### Corrected SPLs for estimating the hearing threshold

The 1-female sound was recorded at a distance of  $0.7\pm 0.2$  m, which gave a relatively low signal-to-noise ratio compared with the high signal-to-noise ratio of the 30-female sound recorded at  $0.9\pm 0.2$  m. As explained in Materials and Methods, 'Sound stimuli', noise was removed below the first harmonic and above the second harmonic but not in between to limit 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 part of the noise between the first and second harmonics. Results from subset A indicated that males did not need this noise to respond to sound because they reacted to the 2-harmonic 1-female sound as much as to the

**Table 1. Description of stimulus sound levels**

Subset	No. of females	No. of harmonics	Type	Sound level (dB SPL)			
				SPL of the 1/3-octave bands			Spatial (and total) error
				Mean	Corrected mean	Temporal error	
A	Single	1	Recording	25.0		±0.9	±2
		2		32.6		±0.3	±2
B	Single	1	Recording	25.0		±0.9	±2
			Constant	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
				17.1		±0.5	±2
				23.1		±0.4	±2
Group				32.9		±0.5	±2
				44.9		±0.5	±2

This table gives the sound pressure levels (SPL re. 20  $\mu$ Pa) and associated errors of all kinds of sound stimuli (number of swarming females, single or ~30; number of harmonics, 1 or 2; sound type, playback of a recorded female or of a sound of constant amplitude and frequency). SPLs of the two 1/3-octave bands closest to the first harmonic at fixed distances from the speaker (0.9 m, i.e. male's mean location) are given. Silence-playback SPL was 6.9 dB (background noise) with a total error of 0.3 dB. 'Temporal' error was due to change of level over time in the sound stimulus signals themselves and 'spatial' error was due to the  $\pm 0.2$  m oscillating distance between the mosquito and the speaker. SPLs are equal to SVLs in our setup. For details, see Materials and Methods, 'Sound pressure level (SPL)'. For frequency characteristics, see Fig. 2 and Table S1.

1-harmonic 1-female sound. As these two stimuli had the same first-harmonic amplitude but a SPL difference of 8 dB (Table 1), 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 1.

#### 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 estimate the incident SPL at the mosquito's location, 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 (Fry et al., 2004) (Fig. 4) which enabled us to determine the distance between a mosquito and the speaker emitting mosquito sound to be  $0.9 \pm 0.2$  m, 95% confidence interval (CI) (Fig. 4A).

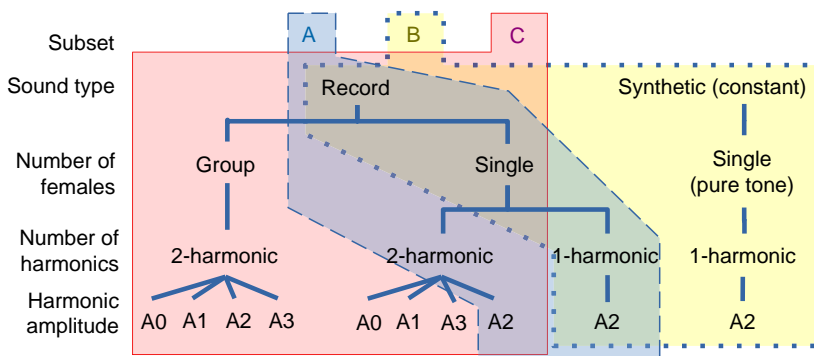
#### Estimate of SPL errors at the mosquito's location

Two types of SPL errors were taken into account. The first is related to the time variation of the sound stimulus levels, which were

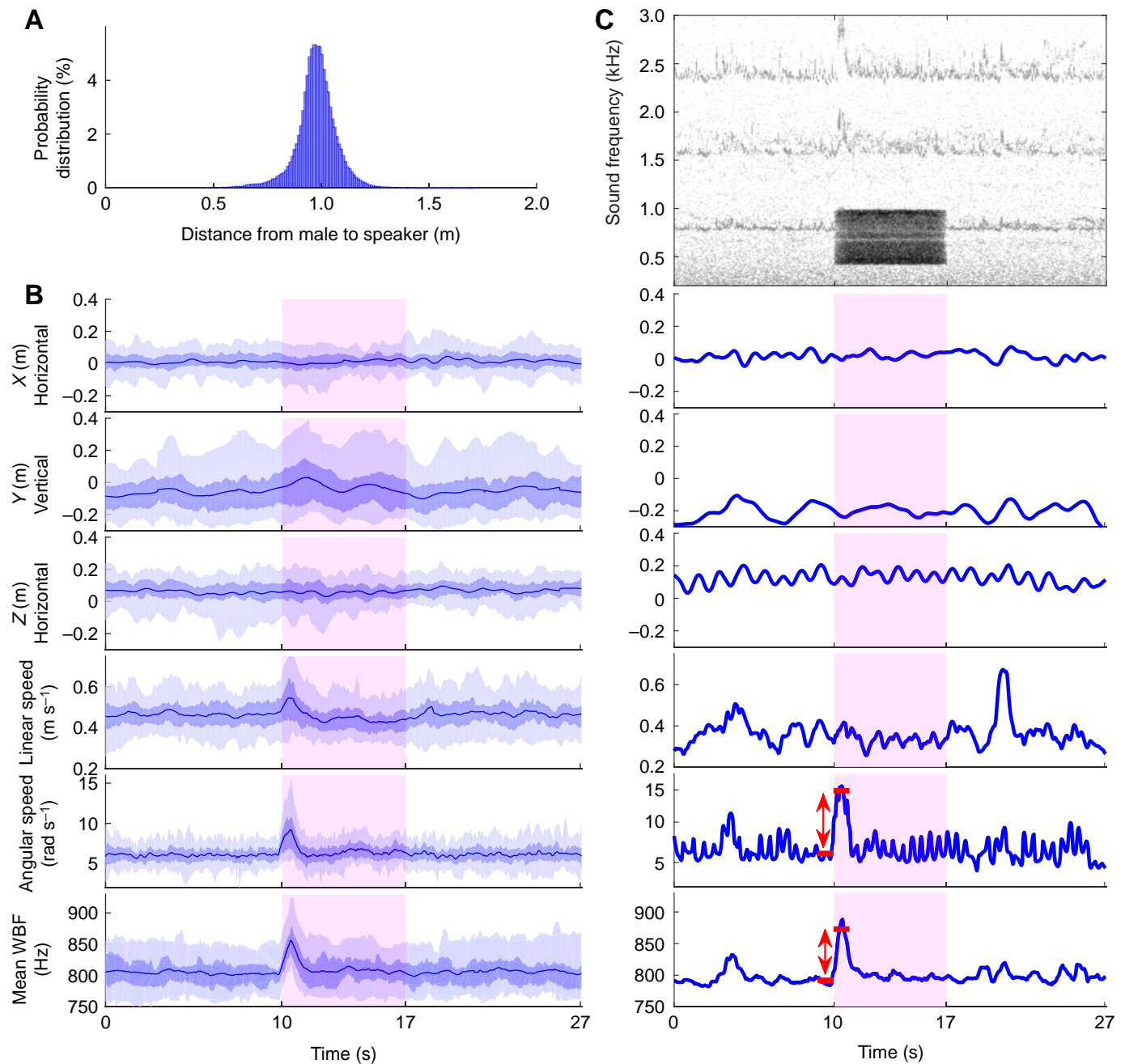
between  $\pm 0.3$  dB and  $\pm 0.9$  dB (maximum error), depending on the stimulus (see Fig. 2 for an example of stimulus sound level over time). The second type of measurement uncertainty arises when the sound level should be estimated from the mosquito's position, and not from the fixed microphone position. Indeed, SPLs were measured at the expected centre of the station-keeping swarm flight of the test male mosquitoes. However, the distance between the male and the speaker varied as  $0.9 \pm 0.2$  m (95% CI; Fig. 4A), as a result of the males' swarming-flight pattern, which changed the sound level they were 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  $\pm 2$  dB. This error is considered to be conservative (at least 95% CI) and was used to interpret the results of the experiments (see Table 1).

#### Physical sound quantities produced by a speaker and sensed by mosquitoes

We monitored the sound level of the played-back stimuli by recording the SPL; however, mosquito hearing organs are sensitive to particle velocity level (SVL) (Fletcher, 1978). The root mean square (RMS) particle velocity  $v_{\text{RMS}}$  and the RMS sound pressure  $p_{\text{RMS}}$  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 source (air impedance  $Z_{\text{air}} = 408 \text{ N s m}^{-3}$  at  $28^\circ\text{C}$ ; sound speed



**Fig. 3. Data subsets for our analysis.** 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 constant sound of the same wingbeat frequency). Subset C was used to study the effect of number of mosquitoes (1 female or 30 females).



**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 (pink shaded area) sound stimuli. (A) Probability distribution of the distance between a male and the speaker during sound stimulus playback for all stimuli; distances were in the range  $0.9 \pm 0.2$  m. This distance interval was used to estimate the uncertainties of the acoustic prediction in Table 1. The sample size of the distribution of distances corresponds to the number of male flight tracks ( $n=104$ ). (B) Distribution of all male responses to the loudest 2-harmonic 1-female sound stimulus over 27 s of recording. The stimulus was played back 10 s from the beginning of the flight recording and lasted 7 s (pink shading). The first five rows show flight parameters (relative X, Y and Z positions, plus linear and angular flight speeds). The 'Z' dimension represents relative distance to the speaker (located 0.9 m from Z=0). The last row shows the mean wingbeat frequency (WBF). The darkest coloured lines represent the running median, the darkest areas represent the second and third quartiles, and light areas represent the 90th percentile of data. The sample size of the distribution of flight coordinates and velocities corresponds to the number of male flight tracks ( $n=104$ ), and that of the WBF distribution corresponds to the number of swarms ( $n=61$ ) where mean WBFs over the number of mosquitoes per swarm were calculated (1 to 6 males per swarm). Linear and angular speed, and WBF clearly increased in response to the onset of this sound stimulus, plus there was a slight tendency to increase flight height (Y). (C) Same as in B but for a single male response, and with a spectrogram of sound recordings before, during and after the sound stimulus at the top; the spectrogram colour gradient represents the sound level given a frequency and a time (the darker the colour, the louder the frequency). Periodic flight pattern, typical of swarming behaviour, is evident in X, Y and Z plots. In the angular speed and WBF plots, the two red lines correspond to the upper quartile over 1 s and the arrows represent the differences between the two red lines, which are the parameters computed for monitoring the male response (see Materials and Methods, 'Extraction of traits used to quantify male responses'). Movie 1 gives the associated raw camera images and sound recording. See Fig. S2 for examples of responses to the four kinds of sound stimulus.

$c=348 \text{ m s}^{-1}$  at  $28^\circ\text{C}$ ) (Beranek and Mellow, 2012):

$$v_{\text{RMS}}(r) = \frac{p_{\text{RMS}}(r)}{Z_{\text{air}}} \sqrt{1 + \left(\frac{c}{2\pi fr}\right)^2}. \quad (1)$$

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

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

Considering that the female sound stimulus does not have any frequency components below  $f=440 \text{ Hz}$  (the smallest frequency value of the group of first harmonics of the swarming females at  $-12 \text{ dB}$  below the peak at  $536 \text{ Hz}$ ; Fig. 2), the SVL is equal to the SPL at  $0.9 \text{ m}$  from a monopole sound source of these frequencies, under a negligible error of less than  $0.1 \text{ dB}$  (due to the mosquito oscillating distance of  $\pm 0.2 \text{ m}$  to the speaker, calculated from Eqn 2). As a consequence, and as mosquitoes are sensitive to SVL and for easier comparison with other studies, we report the SPL as SVL. Arthur et al. (2014) measured the particle velocity attenuation rate in front of or behind *Ae. aegypti* to be between a monopole and a dipole. Note that our monopole assumption for mosquito wing flapping is conservative as higher orders (dipole, quadrupole) produce sound levels that decrease more rapidly with distance (Bennet-Clark, 1998).

### 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. 4B,C for statistics and example of wingbeat and flight dynamic characteristics before, during and after exposure to the loudest 1-female sound stimuli ( $44 \pm 2 \text{ dB SVL}$ )]. The two components were synchronized using the same techniques as in a previously published study (see supplementary information in Feugère et al., 2021b).

Angular speed refers to how much the mosquito flight direction changes per unit time. It was calculated as  $\Delta\theta/\Delta t$ , 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:

$$\Delta\theta = \cos^{-1} \frac{\mathbf{v}_n \cdot \mathbf{v}_{n+1}}{|\mathbf{v}_n| \cdot |\mathbf{v}_{n+1}|}, \quad (3)$$

where  $\mathbf{v}_n$  is the 3D linear velocity vector of the mosquito at time index  $n$  and  $|\mathbf{v}_n|$  is its magnitude, given by the Trackit software. The criteria used to include a tracked flight in the data analysis were that the mosquito was swarming over the marker for at least  $1 \text{ s}$  before and after the sound stimulus onset.

### Wingbeat frequency

Only the first or the first two harmonic(s) of female sound stimuli were played back ( $\sim 400\text{--}1200 \text{ Hz}$ ) 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 (an example of spectrogram is given in Fig. 4C). The peak of the third harmonic was detected every  $40 \text{ ms}$  between  $2190$  and  $2920 \text{ Hz}$  using the Fast Fourier Transform algorithm ( $256 \text{ ms}$  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  $2190\text{--}2920 \text{ Hz}$  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.  $3 \times$  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. 4C gives an example of detected wingbeat frequencies of males while Fig. 4B shows the distribution of the detected wingbeat frequency over time for all recordings.

### Upper-quartile difference

As preliminary experiments suggested that mosquitoes responded to sound by increasing their wingbeat frequency and their angular speed at some point 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  $1 \text{ s}$  stimulus time interval. Indeed, 'upper-quartile' (1) is a more robust metric than median or mean to measure the amplitude of a short peak, for which the onset time cannot be precisely predictable and (2) is a more reliable metric than 'maximum' to avoid false detection. This value was then subtracted from the upper-quartile value computed during the  $1 \text{ s}$  segment just before the stimulus onset, for each individual recording to reduce noise related to individual mosquito variability. Red arrows in Fig. 4C show 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). Stimulus sound levels (continuous), number of females in the recording (1 or 30), number of harmonics (1 or 2) and sound type (recording or constant sound) and their interaction were considered as fixed effects. Days, for which replicates were performed, were considered random effects. The dataset was split into the three subsets A, B, C, as shown in Fig. 3. A total of six models were built (2 parameters  $\times$  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 (Crawley, 2007). No data transformation was needed to ensure variance homogeneity of variables (Fligner–Killeen test, *Fligner.test* function, R) and normality of model residuals (Shapiro–Wilk test, *shapiro.test* function, R), except for subset C wingbeat frequency which was transformed via optimality (*MLE\_LambertW* function, *LambertW* package, R; Goerg, 2016); see Fig. S3 for normality *qqplots* and Table S2 for normality and variance homogeneity test results.

For subsets A and B, an additional one-sample *t*-test (with Benjamini–Hochberg 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 like the three other 2-harmonic 30-female sound stimuli. The hearing threshold was estimated by the crossing of the  $y=0$  axis (i.e. no response, including with the LambertW transformation) with the prediction of the fixed-effect components of the mean and associated 95% CI (*bootMer* function with *nsim=500*, *lme4* package, R). The Lambert transformation does not change the 0 value of the distribution. All



analyses were performed using R (version 3.5.3; <http://www.R-project.org/>).

Model subsets resulted in a sampling size of  $n=10$  for subset A and B and  $n=9$  or  $n=10$  for subset C (see legend of Fig. 5 for details; see Materials and Methods, 'Behavioural assays', for details on how a replicate was defined).

## RESULTS

### Males mostly use the female's first harmonic to hear her 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. 5A), for both angular speed (upper-quartile angular speed difference: one-sample  $t=5.7$ , d.f.=9, Benjamini–Hochberg corrected  $P<0.001$ , mean= $1.5 \text{ rad s}^{-1}$ ; one-sample  $t=5.0$ , d.f.=9, Benjamini–Hochberg corrected  $P<0.001$ , mean= $1.0 \text{ rad s}^{-1}$ , respectively) and wingbeat frequency (upper-quartile wingbeat frequency difference: one-sample  $t=5.2$ , d.f.=9, Benjamini–Hochberg corrected  $P<0.001$ , mean=16 Hz; one-sample  $t=4.6$ , d.f.=9, Benjamini–Hochberg corrected  $P=0.0013$ , mean=13 Hz, respectively).

Our results show no differences in the response of males exposed to the first harmonic sound of a female flight tone or to a combination of the first and second harmonic sounds (with noise in between) of the same female flight tone (upper-quartile angular speed difference: likelihood ratio test,  $\chi^2=2.6$ , d.f.=1,  $P=0.11$ ; upper-quartile wingbeat frequency difference: likelihood ratio test,  $\chi^2=1.1$ , d.f.=1,  $P=0.29$ ).

### Males react to a 'pure sound' (1-harmonic constant sound) at least as much as to a 'natural sound' (1-harmonic 1-female sound) (subset B)

Subset B stimuli, i.e. 1-harmonic 1-female sound and 1-harmonic constant sound, were both heard by the males because the response distributions were different from the null distribution (Fig. 5B), for both the angular speed (upper-quartile angular speed difference: one-sample  $t=5.7$ , d.f.=9, Benjamini–Hochberg corrected  $P<0.001$ , mean= $1.5 \text{ rad s}^{-1}$ ; one-sample  $t=5.4$ , d.f.=38, Benjamini–Hochberg corrected  $P<0.001$ , mean= $1.6 \text{ rad s}^{-1}$ , respectively) and the wingbeat frequency (upper-quartile wingbeat frequency difference: one-sample  $t=5.2$ , d.f.=9, Benjamini–Hochberg corrected  $P<0.001$ , mean=16 Hz; one-sample  $t=5.1$ , d.f.=38, Benjamini–Hochberg corrected  $P<0.001$ , mean=30 Hz, respectively).

Our results show there is little difference in the male response between the 1-harmonic 1-female sound stimulus and the 1-harmonic constant sound of the same mean frequency/SVL. While males changed their angular speed with the same amplitude in response to these two stimuli, they changed their wingbeat frequency twice as much as with the 1-harmonic constant sound (Fig. 5B) (upper-quartile angular speed difference: likelihood ratio test  $\chi^2=0.052$ , d.f.=1,  $P=0.82$ ; upper-quartile wingbeat frequency difference: likelihood ratio test  $\chi^2=4.5$ , d.f.=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 (see Table 1 for sound levels), our results (Fig. 5C) show that free-flying males responded to the sound stimuli, providing the sound level was high enough, by increasing both their angular speed and their wingbeat frequency as the tested

sound levels increased (upper-quartile angular speed difference: likelihood ratio test  $\chi^2=36.8$ , d.f.=1,  $P<0.001$ , effect size= $0.12 \text{ rad s}^{-1}$  per dB SVL; and LambertW-transformed upper-quartile wingbeat frequency difference: likelihood ratio test  $\chi^2=23.8$ , d.f.=1,  $P<0.001$ ). The number of females had a small effect, but this was not interpretable, because of distinct values of sound levels for each number of females (upper-quartile angular speed difference: likelihood ratio test  $\chi^2=3.3$ , d.f.=1,  $P<0.001$ ,  $1.2 \text{ rad s}^{-1}$  for 1-female versus  $0.6 \text{ rad s}^{-1}$  for 30-female stimuli; and LambertW-transformed upper-quartile wingbeat frequency difference: likelihood ratio test  $\chi^2=3.2$ , d.f.=1,  $P=0.073$ , 20 Hz for 1-female versus 10 Hz for 30-female stimuli). 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 females; upper-quartile angular speed difference: likelihood ratio test  $\chi^2=3.3$ , d.f.=1,  $P=0.070$ , effect size of an additional  $0.05 \text{ rad s}^{-1}$  per dB SVL for 1-female sound stimulus; and LambertW-transformed upper-quartile wingbeat frequency difference: likelihood ratio test  $\chi^2=10.3$ , d.f.=1,  $P=0.0013$ , respectively).

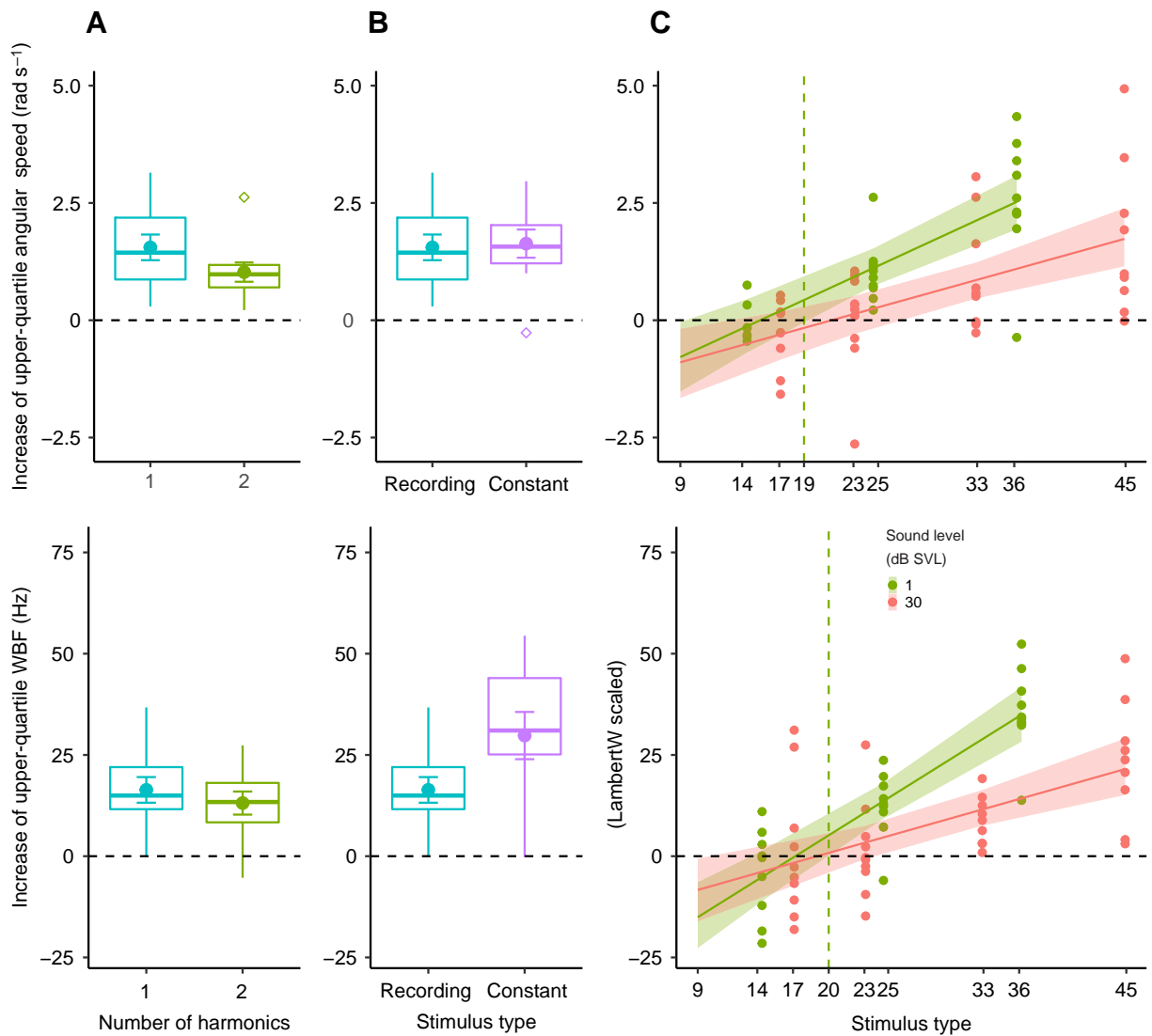
For 2-harmonic 30-female sound stimuli (Fig. 5C, red), the mean sound level threshold was 21 dB SVL with a 13–27 dB SVL 95% CI, if considering the angular speed as response parameter. Using the wingbeat frequency parameter, the mean sound level threshold was 19 dB SVL with a 9–23 dB SVL 95% CI. For 2-harmonic 1-female sound stimuli (Fig. 5C, green), the mean sound level threshold was 15 dB SVL with a 9–19 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 the 2-harmonic 1-female 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 (Feugère et al., 2021b), during mating behaviour when mosquitoes are flying in loops near a visual marker. *Anopheles 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 lifetime. Once a male detects a female's presence from her wing-flapping sound, the male starts to chase the female (Pantoja-Sanchez et al., 2019). Thus, there is strong competition between males to detect relatively rare females (~1% male:female ratio; Kaindoa et al., 2017; Charlwood and Jones, 1980). Accordingly, acute hearing sensitivity is highly advantageous to males, along with other factors such as their own wingbeat acoustic power (Lapshin, 2012) and frequency (Somers et al., 2021 preprint) in the context of distortion-product hearing.

Under laboratory conditions (27–29°C), we show that male *An. coluzzii* respond strongly to 1-harmonic constant sound of  $26 \pm 2$  dB SVL at the female's mean wingbeat frequency (Fig. 5B and Table 1) 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 thresholds in the JO, which does not involve free-flying, pre-mating behaviour, such as swarming (but see Feugère et al., 2021b; Lapshin and Vorontsov, 2021). This may explain why these electrophysiological studies usually found far higher sound thresholds than in our study (see Introduction). Lower male hearing thresholds measured by electrophysiological methods



**Fig. 5. Results of the behavioural experiment.** The two plotted parameters are the increase in upper-quartile angular speed (top) and wingbeat frequency (WBF; bottom), when playing back a given sound stimulus. Black dashed lines represent the absence of change in parameters before and during the stimulus presentation. Each sample is the average of several measurements on the same day, and corresponds to a different group of mosquitoes (with 1–6 in each sample). See Materials and Methods, ‘Statistics’, and Results for statistical tests. (A) Male *An. coluzzii* responses to 1- or 2-harmonic sounds of a single female (data subset A,  $n=10$  in each boxplot). Boxplots of the parameters show the median, and 2nd and 3rd quartiles. Outliers (diamonds) are outside the interval  $[Q1 - 1.5IQD, Q3 + 1.5IQD]$  which is represented by whiskers (where Q1 is the first quartile, Q3 is the third quartile and IQD is the interquartile distance). The circle and error bars in each distribution are the mean and s.e.m. (B) Male *An. coluzzii* responses to 1-harmonic 1-female sound or to single-frequency sound (data subset B,  $n=10$  in each boxplot). Boxplots, circles and error bars have the same meaning as in A. (C) Male *An. coluzzii* responses to 2-harmonic sounds of a single female or 30 females over sound particle velocity level (SVL) (data subset C,  $n=9$  for the quietest 1-female stimulus and the two loudest 30-female stimuli,  $n=10$  for other stimuli). Continuous lines and associated coloured areas represent the mean and 95% confidence interval. SVL was corrected as explained in Materials and Methods, ‘Corrected SPLs for estimating the hearing threshold’. The green dashed lines represent the lowest estimate of the hearing threshold from the response to 1-female 2-harmonic sound stimuli.

can partly be explained by the presence of male flight tones, which is known to be important to enhance the sensitivity in males to female sound. This creates mixed harmonics 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* (Lapshin, 2012). However, this may not be the only explanation. Mosquitoes exhibit ‘active hearing’, which can be triggered only during specific physiological states (Göpfert and Robert, 2001; Su et al., 2018), one of which may be swarming. It may be that males can enhance hearing to detect a female that is approaching a male swarm before she is chased by a competitor.

The only other species to have been explored in relation to these aspects of swarming flight is *Ae. communis* (Lapshin and Vorontsov, 2021); in the field, the mean hearing threshold of males at the female’s wingbeat frequency was shown to be particularly low, at 26 dB SVL. However, their method consisted in monitoring flight speed changes in natural swarms by eye, which may not have enabled them to measure the smallest response amplitudes, thereby overestimating the threshold (Lapshin and Vorontsov, 2021). In contrast, we measured both flight dynamics and wingbeat frequency from quantitative measurements. Also, ambient temperatures were very different [ $\sim 12^{\circ}\text{C}$  for Lapshin and Vorontsov (2021) versus  $27\text{--}29^{\circ}\text{C}$  for our recordings], which can change hearing sensitivity.

Another reason for finding lower hearing thresholds with our behavioral methods than with some previously published studies with electrophysiological measurements in the JO could be related to the averaging over JO scolopidia. If so, this could misrepresent the effective signal that triggers a behavioural response. Indeed, in addition to individual sensitivity in frequency and threshold, JO scolopidia are sensitive to the direction of the sound wave, and then only the JO scolopidia which are aligned with the sound wave front display a low response threshold. As a consequence, averaging all JO scolopidium thresholds may lead to an over-estimate of hearing thresholds (Lapshin and Vorontsov, 2019).

#### Male response to sound and 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). This occurred despite the relatively greater amount of noise in between the 1st and 2nd harmonic in the 1-female sound stimulus than in the 30-female sound; the difference may have been stronger if the prominence of the harmonics had had similar values in the two tested stimuli. Two comments merit emphasis. First, a group of frequencies that are attractive alone (e.g. grouped-female sounds) have a masking effect on mosquito auditory perception. These results support reports published 80 years ago with *Ae. aegypti* males; it was observed that these mosquitoes were not attracted to two or more sounds at a time, even though each of these sounds were attractive on their own (Wishart and Riordan, 1959). Second, it is interesting that males respond more with their wingbeat frequency than with their flight trajectory or dynamics. The change in wingbeat frequency is consistent with a current theory that during a chase between a male and a female, the male moves to the sound source by tracking the female's wingbeat sound and adjusts his own wingbeat frequency to hear her better, through an auditory mechanism based on antennal distortion products (Warren et al., 2009; Simões et al., 2018). In our case, the sound wavefront is almost planar at the male's position, as a result of the distance and membrane dimension of the speaker, contrary to the sound wave of a female of the 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 and the significance of background noise

Males are known to detect mainly the female's first harmonic to hear her flight tone. Indeed, *Ae. aegypti* respond (with clapping and seizing movements in flight) to low frequencies under 500 Hz (i.e. 1-harmonic sounds) produced using tuning forks (Roth, 1948), while other species, such as *Cx. pipiens pipiens*, have a narrower frequency range of response (500–600 Hz) when swarming (Gibson, 1985). 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 in wingbeat frequency of the two sexes, which is close to the female's first harmonic (Gibson et al., 2010). With respect to behaviour, Wishart and Riordan (1959) trapped as many *Ae. aegypti* males with the sound of 1-harmonic tones as with the complete flight sound. Moreover, when removing the first harmonic from female flight tone recordings, *Ae. aegypti* males did not respond anymore, but the

authors reported their results without any further information. This absence of a male's response if the female's first harmonic is removed from the stimulus is similar to our results with *An. gambiae*, which showed a similar male response if the second harmonic of the female flight tone was removed. In contrast, it has been reported that male *Ae. aegypti* can hear the female's second harmonic, but without inferential statistics (Cator et al., 2009), and these results were also contested with arguments based on auditory processing of phasic information in the JO nerves of *Cx. quinquefasciatus* (Warren et al., 2009). 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 close to/slightly above the frequency of the female's second harmonic in electrophysiological measurements in *Cx. pipiens pipiens* (Lapshin, 2012) and *Ae. communis* (Lapshin and Vorontsov, 2021). The results of our behavioural assay suggest that this reinforcement is negligible in practice, at least in *An. coluzzii*.

The limitation of our stimulus recording approach is to be found in the long distance between the microphone and the single female (0.7±0.2 m), which induced a low signal-to-noise ratio of 1.7, despite noise filtering below the first harmonic and above the second harmonic (against a ratio of ~48 for the 2-harmonic 1-female stimulus; if considering the noise level as the noise floor between the two harmonics, using the Matlab function *snr*). Indeed, because of these different signal-to-noise ratios, the 2-harmonic 1-female stimulus can be seen as a frequency band of noise (ranging from the first to the second harmonic frequencies) instead of a true 2-harmonic sound.

However, this noise asymmetry between the two stimuli also shows that males are not fundamentally disturbed by noise; the noisiest stimulus (2-harmonic 1-female) induced as much response as the least noisy stimulus (2-harmonic 30-female). Wishart and Riordan (1959) found that female sound (500 Hz) is still an attractant to *Ae. aegypti* males with 10 dB of noise above the signal sound level, but was not an attractant on the next tested step of 20 dB of noise above the signal level. The noise was composed of the superposition of sine waves of 100, 156 and 282 Hz plus square waves of 933, 1840 and 4130 Hz, which probably did not create as much noise around the female sound frequency as in our case. The hearing mechanism based on antennal distortion products uses the loud wingbeat frequency of the listener to amplify the nearby, but possibly quiet, wingbeat frequency of a potential mate (Lapshin, 2012). By changing its own wingbeat frequency, it is possible for a mosquito to change the distortion product frequency elicited by the nearby flying mate, which, theoretically, may help it to detect very faint harmonics against a relatively high level of background noise, especially when this noise is limited to the frequency band between the two harmonics.

#### Constant sound versus 'natural' sound

Constant sound and female pre-recorded flight tones have been known to trigger a response in mosquitoes for a long time (Roth, 1948; Kahn and Offenhauser, 1949). However, to our knowledge, no comparisons have been formally made between pre-recorded sound and constant sound of the same frequency. Our results in subset B show that the 1-harmonic constant sound behaves somewhat like a supernormal stimulus (for the wingbeat frequency response parameter) compared with a 1-harmonic natural sound, at least at 26 dB SVL. Furthermore, subset A allows us to conclude that males respond as much to 1-harmonic 'natural' sound as to 2-harmonic 'natural' sound. By combining

results from subsets A and B, we deduce that mosquitoes hear natural sound as well as pure sound. This means that the information carried in the sound that elicits a male response is mostly the mean wingbeat frequency. A proper study could be carried out (1) with 1-harmonic constant sounds to control the sound level better than with pre-recorded sounds, and (2) by using larger ranges of frequencies and sound levels than in the present study; i.e. a ‘behavioural audiogram’.

### Monitoring SVL from SPL measurements

Many studies report hearing thresholds based on SPL, which is a physical quantity that mosquitoes do not detect. We also monitored sound level with SPL, but we fulfilled the experimental conditions to provide equivalence between SPL and SVL, which mosquitoes do detect (see Materials and Methods). Some studies have referred to SPL values as hearing thresholds, even though the equivalence conditions were not fulfilled or were unknown. Wishart and Riordan (1959) estimated that *Ae. aegypti* responds to a sound of approximately 20 dB SPL from experiments involving 30 cm-side netting cages and sound stimuli presented through a diffuse speaker held against the cage netting. However, mosquitoes could be located a few centimetres from the loudspeaker, where SPL and SVL are not equivalent at this distance, i.e. when SPL would not be a good physical quantity to describe what the mosquito auditory organs are exposed to. Another example is a study by Belton (1961): a response threshold in the JOs of male *Ae. aegypti* was measured to be between 0 and 10 dB SPL; he related SPL to SVL using a formulae that assumed far-field condition (but without stating so). Unfortunately, the study 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 inaccurate. More recently, Dou et al. (2021) put their loudspeaker at 2.5 cm against their 30 cm-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 as it was measured in the middle of the cage and mosquitoes were free to move along the cage sides, near the loudspeaker. In addition, SVL may have been far greater than SPL at this distance from the speaker. Taken together, SVLs probably do not occur with ecologically relevant sounds; however, this could be used to inform the design of sound traps or reveal unknown auditory mechanisms.

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### Competing interests

The authors declare no competing or financial interests.

### Author contributions

Conceptualization: L.F., G.G.; Methodology: L.F., G.G.; Software: L.F.; Formal analysis: L.F.; Investigation: L.F.; Data curation: L.F.; Writing - original draft: L.F.; Writing - review & editing: O.R., G.G.; Visualization: L.F.; Supervision: G.G., O.R.; Funding acquisition: O.R., G.G., L.F.

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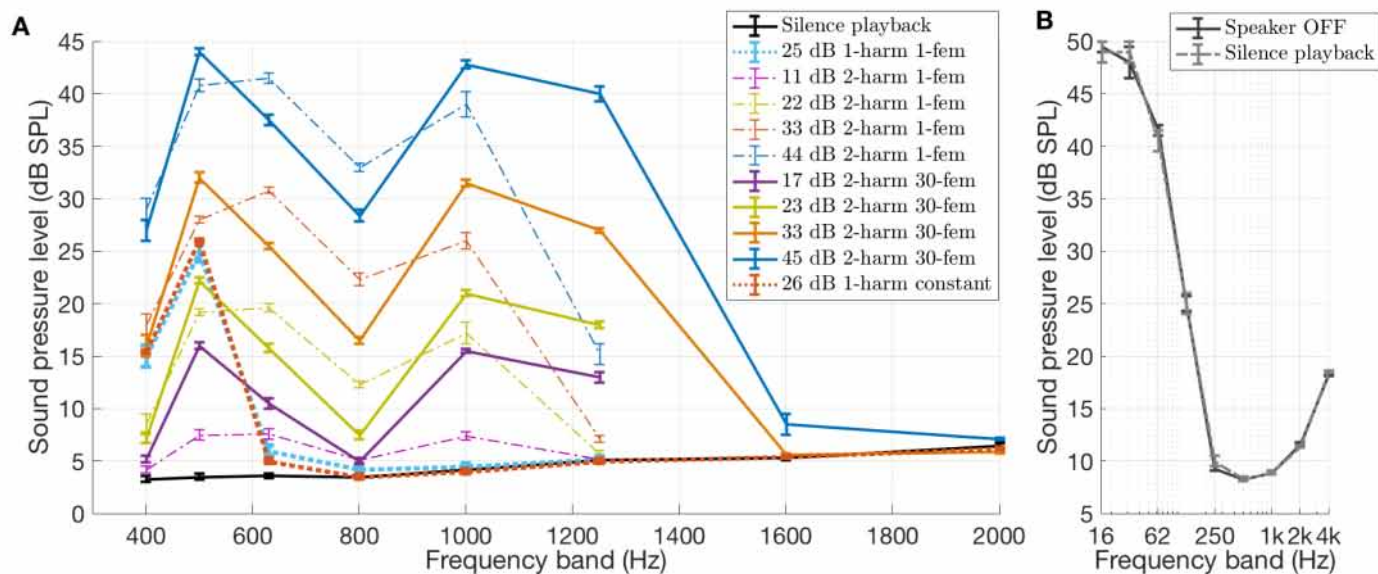
### Data availability

Raw response files (sound and tracked flight trajectories) are available from the Dryad digital repository (Feugère et al., 2021a): <https://doi.org/10.5061/dryad.9cnp5hghj>. Custom audio–video code for parameter extraction and audio–video synchronization (modified Matlab files; <https://doi.org/10.17632/hn3nv7wpxk.3>) and custom statistics code for data analysis and figure plot (R files) and dataset (text files) (<https://doi.org/10.17632/6w5jtwkj8.2>) are available from Mendeley.

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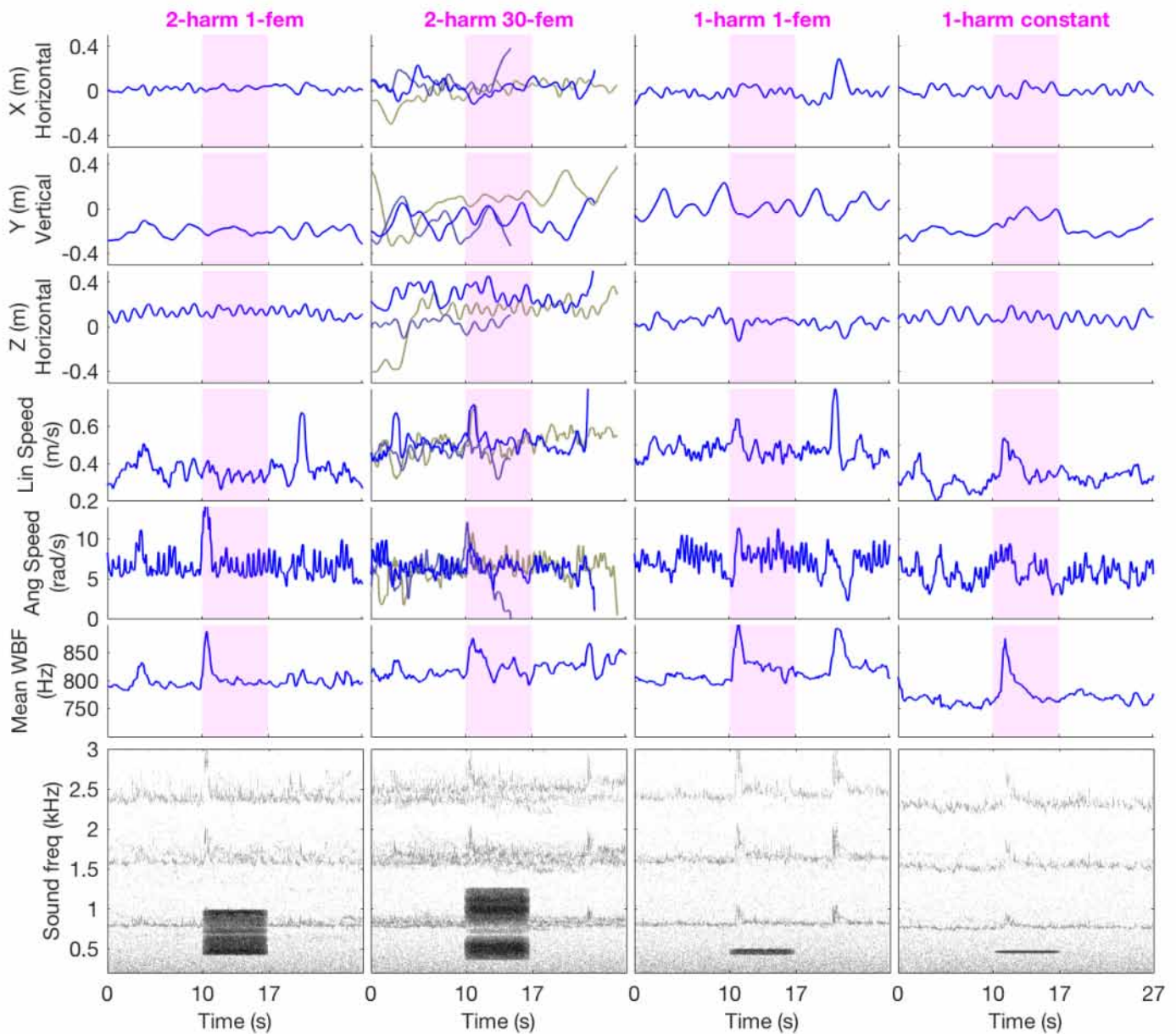
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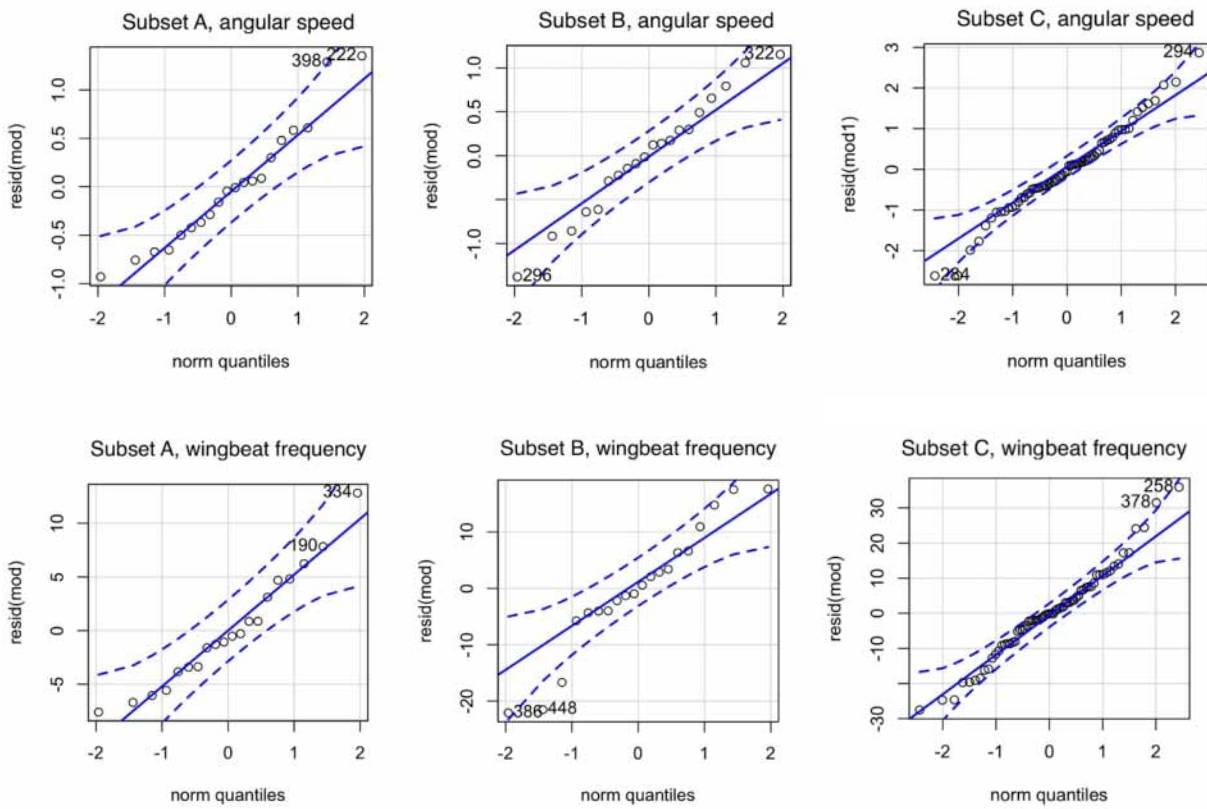
**Fig. S1. Calibrated sound-level measurements of played-back sounds and background noise.** SPLs (ref 20  $\mu$ Pa) were measured at the *Anopheles coluzzii* swarming position 1) to estimate the sound level received by the tested mosquito and 2) to know the background noise level of the sound-proof 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 (Warren et al., 2009), 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.



**Fig. S2. Examples of *An. coluzzii* male response to the loudest four kinds of sound stimuli.** Male flight-characteristics and wingbeat-frequencies (blue) before, during and after playback of female sound stimuli (red rectangle). Each column corresponds to a different type of sound stimulus (from left to right): 2-harmonic 1-female (Audio 1), 2-harmonic 30-female (Audio 2), 1-harmonic 1-female (Audio 3), 1-harmonic constant (Audio 4). 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). Row before last shows mean wingbeat frequency over all present males (WBF), while the flight positions and dynamics corresponds to those of each present mosquito, hence the multiple lines on second column where 3 mosquitoes were present in this recording. 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). See Fig. 2 for the spectrum of each sound-stimulus type.



**Fig. S3. Quantile-quantile plots on reduced model residuals.** Qqplots were computed with the R *qqplot* function. See Table S2 for associated statistical tests.

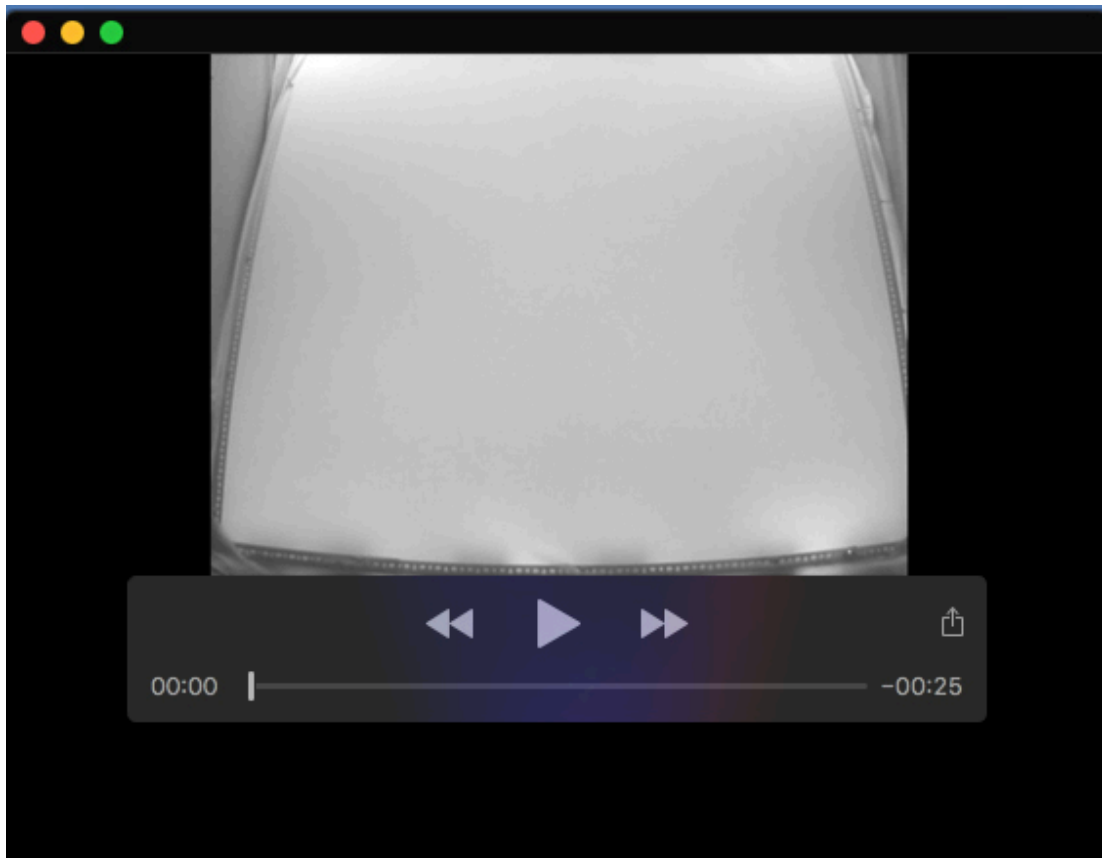


**Table S1. Filter characteristics applied to female sound recordings.** Filters were applied on sound recordings using the Matlab function *designfilt* with the parameters shown in the table, at a sampling rate of 8 kHz. The 1-harmonic constant sound was a single frequency/amplitude sound. For sound levels, see Table 1.

Stimulus	FIR Filter type	Cut-off frequency (Hz)	Bandpass frequency (Hz)	Stop-band attenuation (dB)	Frequency (Hz)
2-harmonic 30-female (Audio 3)	Highpass	356	366	100	/
	Lowpass	1271	1261	100	/
2-harmonic 1-female (Audio 4)	Highpass	422	432	100	/
	Lowpass	994	984	100	/
1-harmonic 1-female (Audio 5)	Highpass	422	432	100	/
	Lowpass	497	487	100	/
1-harmonic constant (Audio 6)	/	/	/	/	459

**Table S2. Statistical tests on model null-hypothesis.** Tests were performed on reduced models for each subset and each extracted parameter (WBF: upper-quartile wingbeat frequency difference; AngSpeed: upper-quartile angular-speed difference) in R.

Subset	Variable	Fligner-Killeen test (absence of variance homogeneity)			Shapiro-Wilk tests (absence of model residual normality)	
		$\chi^2$	df	p-value	$\chi^2$	p-value
A	AngSpeed	2.5	1	0.11	0.92	0.098
	WBF	0.0098	1	0.92	0.95	0.45
B	AngSpeed	0.0067	1	0.95	0.98	0.98
	WBF	1.2	1	0.27	0.94	0.26
C	AngSpeed	9.4	6	0.15	0.98	0.48
	LambertW-transformed WBF	8.8	6	0.19	0.98	0.30



**Movie 1.** Audio-video recording of a *An. coluzzii* male exposed to the loudest *An. coluzzii* 2-harmonic 1-female sound (10-s silence + 7-s sound exposition + 10-s silence). Related to Fig. 4 A.

**Audio 1.** 2-harmonic 1-female sound stimulus (7 s).

[Click here to download Audio 1](#)

**Audio 2.** 2-harmonic 30-female sound stimulus (7 s).

[Click here to download Audio 2](#)

**Audio 3.** 1-harmonic 1-female sound stimulus (7 s).

[Click here to download Audio 3](#)

**Audio 4.** 1-harmonic constant sound stimulus (7 s).

[Click here to download Audio 4](#)

**Audio 5.** Original sound recording of the 1-female *An. coluzzii* (7 s) before any filtering and level adjustment. Related to Fig. 2.

[Click here to download Audio 5](#)

**Audio 6.** Original sound recording of the 30-female *An. coluzzii* (7 s) before any filtering and level adjustment. Related to Fig. 2.

[Click here to download Audio 6](#)