

1 **Title page**

2 **Title.** Behavioural analysis of swarming mosquitoes reveals higher hearing sensitivity
3 than previously measured with electrophysiology methods

4 **Running title.** Hearing sensitivity in swarming mosquitoes

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11 **Keywords.** Auditory processing, bioacoustics, free-flying, Johnston's organ, insect
12 sensory system, sound sensitivity.

13

14 **Summary statement**

15 We measured hearing sensitivity to sound playback recordings in free-flying male
16 mosquitoes using a behavioural method for tracking flight dynamics and wingbeat
17 frequency.

18 **Abstract**

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

36

37 **MAIN TEXT**

38

39 **Introduction**

40

41 Hearing is a key sensory modality for mosquito mating; it enables males to detect females
42 at a distance through the combined sounds of their respective flapping wings (Warren et
43 al., 2009; Simões et al., 2018; Feugère et al., 2021b). The more sensitive males are to
44 flight sounds, the further away they can hear a female and the sooner they detect and close
45 in on a nearby female in the context of highly competitive mating-swarms. The male
46 antennal organs of mosquitoes are the most sensitive to sound described so far among
47 arthropods (Göpfert and Robert, 2000), however, the measurement of hearing sensitivity is
48 usually performed on tethered males, which prevents natural body movement such as
49 antennal orientation and wing flapping behaviour in response to female sound. Only a few
50 studies have measured hearing thresholds behaviourally (Menda et al., 2019; Lapshin and
51 Vorontsov, 2021; Feugère et al., 2021b). The measurement of behavioural sound-
52 sensitivity in flying male mosquitoes faces the difficulty of monitoring how much sound
53 energy actually reaches their antennae because the sound level meter is at a fixed-position,
54 whereas the position of the male mosquito is continuously changing during his flight. The
55 aim of this study was to quantify behaviourally the overall sound-level threshold at which
56 males can hear females, i.e. the limit of sensitivity of a male to locate a female in flight.
57 Accordingly, we had to determine the components of female-wingbeat sound that male
58 mosquitoes are most responsive to, so that our definition of the sound level includes only
59 the frequency bands audible to males.

60

61 Mosquitoes hear airborne sound by detecting air-particle velocity through friction between
62 air particles and the mosquito's fibrillae located on the flagellum of their antennae.
63 Unfortunately, there are no instruments that can truly measure particle-velocity on the
64 market as yet (Zhou and Miles, 2017), however, it can be estimated by using pressure-
65 gradient microphones (commonly called 'particle-velocity microphones'). Another
66 strategy to estimate particle-velocity is to use pressure microphones located in the far-field
67 of the sound source, i.e., where the sound pressure level (SPL) can be approximated to that
68 of sound particle velocity level (SVL). However, SPL hearing thresholds have sometimes
69 been measured under the near-field condition instead of far-field (Tischner, 1953; Belton,
70 1961; Dou et al., 2021), which means there is a risk that some reported hearing thresholds
71 may have been under-estimated, as elaborated in the Discussion section.

72
73 Hearing thresholds can be assessed by measuring a physiological or behavioural response
74 to a given stimulus sound level and sound frequency. Among the physiological methods,
75 laser vibrometry records the vibration of the flagellum (Göpfert et al., 1999; Pennetier et
76 al., 2010), however, it is limited when assessing hearing threshold because the recorded
77 vibration only refers to the input to the hearing chain (i.e., flagella movement) and does
78 not provide any indication as to whether or not the neurons of the mosquito have been
79 neuro-electrically activated following the sound-induced vibration of the flagella. Unlike
80 laser vibrometry, electrical responses of the JOs to airborne sound stimuli result from the
81 complete sensory chain of the auditory system (i.e., from the mechanical vibration of the
82 flagella to the electrical response of the JOs). With this method, the electrical response-
83 threshold in male *Culex pipiens pipiens* JOs showed a mean sensitivity of 32 dB SVL per
84 JO scolopidia (range of 22-44 dB SVL; n=74 JO scolopidia; criterion = 2 dB above noise
85 floor; 18-21°C) (Lapshin and Vorontsov, 2019) and a mean of 44 dB SVL per mosquito in

86 three male *Culex quinquefasciatus* JOs (range of 36-52 dB SVL; n=3 males; criterion = 10
87 dB above noise floor) (Warren et al., 2009). In *Aedes aegypti*, the male JO nerve was
88 shown to respond to a mean of 40 dB SVL (range of 31-50 dB SVL; n=11 males) (Menda
89 et al., 2019). In some species, such as *Anopheles coluzzii*, the antennal fibrillae are
90 extended only during their active phase, which improves their JO hearing sensitivity by 17
91 dB in terms of SVL (Pennetier et al., 2010). Under this antennal physiological state,
92 Pennetier *et al.* (2010) measured a JO response-threshold in two male *An. coluzzii* of only
93 10 dB SVL (range of 5-12 dB SVL, i.e., particle velocity of $1.5 \pm 0.6 \cdot 10^{-7}$ m/s; n=4
94 measurements on 2 males; criterion = 1.4 recording noise floor).

95
96 In a distortion-product based hearing system, as proposed for mosquitoes, hearing
97 sensitivity can be further enhanced (or even produced) by the mosquito beating its wings
98 (Lapshin, 2012). However, electrophysiological and laser vibrometry methods prevent
99 mosquitoes from beating their wings, so in order to simulate the effect of male flight on
100 the male auditory organ, it is possible to combine the male's flight sound-frequency with
101 the female stimulus sound. For example, male *Cx. pipiens pipiens* JO sensitivity was
102 improved by 7 dB with the addition of simulated flight sound at the main frequency
103 optimum (18-22 °C) (Lapshin, 2012).

104
105 The results of electrophysiological and laser vibrometry studies can be difficult to
106 compare against each other due to differences in methodologies used to assess threshold
107 responses (e.g. determination of statistical definitions of neural thresholds and variations
108 in the locations of electrodes). In addition, the main goal of these studies is not always
109 about measuring absolute hearing thresholds, and as a consequence the number of
110 replicates can be too few to analyse statistically.

111
112 Behavioural methods also face similar constraints, however, the assessment of
113 physiological responses to sound stimuli offer a more natural context that enables more
114 natural responses to sound. Behavioural responses provide more robust evidence of
115 auditory outcomes because the whole auditory chain plus the motor responses are
116 included. To our knowledge, there are only three published behavioural studies of
117 mosquito sensitivity to sound intensity. First, Menda *et al.* (2019) measured the
118 behavioural response of *Ae. aegypti* to 40 and 65 dB SVL by monitoring the take-off of
119 resting mosquitoes in a cage located in the far-field of the sound-source. However, the
120 behavioural methodology was not appropriate for the natural physiological context of
121 swarming behaviour in this species; in the field both male and female *Ae. aegypti* fly
122 continuously once the males detect the female's flight tones (i.e., they rarely rest and take-
123 off again). Indeed, male responsiveness to sound was found to be reduced when not flying
124 (Lapshin, 2012).

125 Second, Feugère *et al.* (2021b) measured the flight and wingbeat frequency response of
126 free-flying, swarming male *An. coluzzii* to a range of sound levels of a played-back group
127 of females and found a response at 33 ± 3 dB SPL. However, males may respond better to
128 the sound of individual females rather than a group of females that would occupy a
129 relatively wide range of wingbeat frequencies, as described for *Ae. aegypti* (Wishart and
130 Riordan, 1959).

131 Third, Lapshin and Vorontsov (Lapshin and Vorontsov, 2021) showed an increase in
132 flight speed in swarming male *Aedes communis* in response to the sound frequency of
133 females in the field, with a hearing sound-level threshold of 26 dB SVL on average (26 dB
134 SPL under far-field conditions; 12°C).

135

136 The aim of our study was to investigate the behavioural hearing threshold of *An. coluzzii*
137 males; Pennetier et al. (2010) measurements suggest that their JO may be as sensitive as
138 10 dB SVL (range of 5-12 dB SVL, n=4 measurements on 2 males, criterion = 1.4
139 recording noise floor). As suggested 70 years ago by Roth (1948), male hearing may be
140 enhanced during swarming behaviour (i.e., flying in loops over a floor marker, station-
141 keeping while they wait for females to join the swarm) when male sensitivity to the sound
142 of flying females is expected to be maximised. Therefore, we used a modified approach of
143 Lapshin *et al.* who worked in the field with *Ae. communis* (Lapshin and Vorontsov, 2021).
144 Our study was performed under the following conditions: 1) in a laboratory sound-proof
145 chamber, with controlled measurement of sound levels; 2) with a range of type of sounds
146 to be exposed to males; 3) by monitoring both the male flight-tone and the flight-dynamic
147 quantitatively; and 4) with *An. coluzzii*, a swarming species belonging to the *Anopheles*
148 *gambiae* complex. ‘Sound-level values’ depend on how sound level is defined and on the
149 type of sound stimuli, therefore, a meaningful sound-level definition should be related to
150 the sound-frequency band and temporal patterns which mosquitoes are sensitive to. For
151 this reason, our main aim of quantifying hearing threshold was inter-connected with the
152 following questions:

- 153 ○ Is the second harmonic of female flight tones necessary to stimulate a response in
154 males? We need this information to establish the frequency band(s) for which the
155 sound level is defined to be appropriate to mosquito hearing.
- 156 ○ Is temporal variation in natural female sound required for males to detect females
157 or is a single-frequency at a constant amplitude sufficient?
- 158 ○ Do the flight tones of a group of females have the same effect on male hearing as
159 those of a single female, over a range of sound levels? The main interest in the last
160 two questions is to investigate whether we can use single-frequency sounds to

161 mimic female sound, which will make the hearing threshold easier to estimate in
162 future studies.

163

164 **Materials and Methods**

165

166 **Mosquitoes**

167

168 All experiments were performed with virgin *An. coluzzii* Coetzee & Wilkerson. The
169 colony was established at the Natural Resources Institute (NRI), University of Greenwich
170 (UK) from eggs provided by the Institut de Recherche en Sciences de la Santé (IRSS),
171 Burkina Faso. Eggs were obtained from a colony established in 2017 from wild gravid
172 females collected from inhabited human dwellings in Bama, Burkina Faso (11°23'14"N,
173 4°24'42"W). Females were identified to species level by PCR (Fanello et al., 2002). The
174 NRI colonies were kept in environmentally controlled laboratory rooms with a 12h:12h
175 light:dark cycle (lights went off at 15h00), >60% relative humidity and ~24-26°C. Larvae
176 were fed Tetramin® fish-flakes and rice powder. Adult males and females were separated
177 < 12h post-emergence to ensure all females were virgin and fed a solution of 10% sucrose
178 and 1%-saline *ad libitum*. Adult mosquitoes were kept in cube cages of ~30 cm sides,
179 populated with a) ~300 virgin females and b) ~20 males.

180

181 **Experimental setup**

182

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

185

186 **Sound-proof chamber.** All experiments were conducted in a sound-proof chamber to limit
187 interference from external sounds. The chamber consisted of double-skin sound-proof
188 walls, ceiling and floor ($L \times W \times H = 2.7 \text{ m} \times 1.9 \text{ m} \times 2.3 \text{ m}$), producing a reverberation
189 time $\leq 0.07 \text{ s}$ for frequencies above 200 Hz (IAC Acoustics, manufacturers). The SPL in
190 the sound-proof room without any playback was always quieter than that with playback of
191 the sound stimuli in the third-octave frequency band of the sound stimulus (Fig. S1 A).
192 Below 176 Hz (upper limit of the 125 Hz octave band), the ambient noise level rose (Fig.
193 S1 B; 25 dB at 125 Hz), due to low-frequency vibration of the building's aeration system,
194 which may have been detected by the *An. coluzzii* auditory system (Pennetier et al., 2010)
195 as a low-frequency background noise to the sound stimulus.

196
197 **Swarming arena.** The swarming arena in the sound-proof chamber was designed to
198 include the key environmental conditions and sensory cues known to control mating and
199 swarming flight in the field. A large mosquito bed-net enclosure (NATURO, $L \times W \times H =$
200 $1.8 \text{ m} \times 1.7 \text{ m} \times 2 \text{ m}$) filling most of a sound-proof chamber (Fig. 1) enabled mosquitoes to
201 fly freely in a volume 100 times greater than that covered by the typical swarming space.
202 Lighting was provided by an artificial-sunlight system to imitate natural daylight, sunrise
203 and sunset (LEDs 5630, HMC0 FLEXIBLE dimmer, and PLeD software, custom-built).
204 Dimming the ambient light level at the appropriate circadian time elicits mosquitoes to
205 take-off, followed by swarming behaviour in response to the presence of a visually
206 conspicuous matt-black marker on the floor; both males and virgin females fly in loops
207 above the marker, but this is rarely observed if males are present because males mate with
208 females quickly and mated females cease swarming behaviour (Poda et al., 2019; Gibson,
209 1985). We used virgin female swarming behaviour to record their flight sound within a
210 relatively limited distance from the marker.

211

212 ***Sound recording and monitoring.*** The wingbeats (aka, ‘flight tones’) of mosquitoes in the
213 laboratory were recorded with a weatherproof microphone (Sennheiser MKH60; RF-
214 condenser; super-cardioid polar pattern at 0.5-1 kHz, with amplitude decrease of > 15 dB
215 beyond 90° from the microphone head; sensitivity at 1 kHz: 40 mV/Pa; A-weighting
216 equivalent noise level: 8 dB) directed toward the swarm location. The microphone was
217 located at a distance of 0.89 m from the centre of the swarm area for the experimental
218 male mosquitoes and the sound recording of the 30-female swarm stimulus (Fig. 1),
219 except for the recording of the 1-female sound-stimulus for which the microphone was
220 located at 0.75 m from the centre of the swarm area. The microphone was plugged into a
221 Scarlett 18i8 audio interface on a Windows7 computer running Pro Tools First 12.8 (Avid
222 Technology, Inc).

223

224 ***Flight track recording.*** The 3D flight trajectories of male mosquitoes were recorded at a
225 sampling rate of 50 Hz with Trackit software (SciTrackS GmbH, Switzerland (Fry et al.,
226 2004)). Two video cameras (Basler, ace A640-120gm) were fitted with wide-angle lenses
227 (Computar, T3Z3510CS, 1/3" 3.5-10.5mm f1.0 Varifocal, Manual Iris) to maximize 3D
228 volume of video-tracking. IR lights (Raytec RM25-F-120 RAYMAX 25 FUSION)
229 enabled the tracking system to detect flying mosquitoes as silhouettes against an IR-
230 illuminated white back-wall made of thin cotton cloth (Fig. 1). The 3D-flight trajectories
231 were smoothed using a cubic spline interpolation at a sampling frequency of 200 Hz on
232 Matlab (version R2017a)

233

234 ***Temperature monitoring.*** Temperature was monitored by type-T thermocouples (IEC 584
235 Class 1, Omega) associated with a temperature logger (HH506RA, Omega) totalling a

236 measurement accuracy error of $\pm 0.9^{\circ}\text{C}$. The chosen thermocouple was located on a room
237 wall at a height of 85 cm from the floor. The four recordings of the reference sound
238 stimuli (two species, two sexes) were recorded at 28.0°C . The mean temperature and
239 standard deviation of the behavioural assays were $28.0 \pm 0.3^{\circ}\text{C}$.

240

241 **Sound stimuli**

242

243 **Recording context.** Two recordings of the natural flight-sounds of 3-6 days-old swarming
244 females were recorded and used to produce the played-back stimuli for the behavioural
245 assays. These sound recordings consisted of 1) a single swarming female or 2) a group of
246 30 swarming females; in both cases mosquitoes were released into the swarming arena 2
247 days before the experiment to acclimatize. The standard environmental conditions in the
248 room were: 12h:12h light:dark cycle with a 1h artificial dawn/dusk transition in light
249 intensity and ~60-75% RH.

250

251 **Signal generation.** We generated 4 types of stimulation signals ('2-harmonic 1-female',
252 '2-harmonic 30-female', '1-harmonic 1-female' and '1-harmonic constant') (Audio S1 to
253 S4; signal spectrum in Fig. 2) over a range of sound levels, producing 10 stimuli in total.
254 First, we selected the first 7s section of the sound of a single female swarming over the
255 marker (Audio S5). Second, a 7s section of the sound of 30 swarming females was
256 selected (Audio S6), ~10 min after the first female started to swarm. Four sound levels for
257 each of the 1- and 30-female sounds were selected (10-45 dB SPL, Table 1), based on
258 results of preliminary experiments. These 8 stimuli contained the two first harmonics. A
259 high-pass filter was added to all the stimuli to remove the electrical noise below the first
260 harmonic (at the noise level, see Fig. 2 and Table S1). In addition, we generated a 33 dB

261 SPL stimulus, which has been shown in preliminary experiments to be the lowest level
262 sound stimulus that females detect in the sound-proof chamber (but see Method section
263 ‘Corrected SPLs for estimating the hearing threshold’ below). This sound stimulus
264 included only the first harmonic because it has been shown electrophysiologically that the
265 male auditory organ is more sensitive to the first harmonic than higher harmonics
266 (Pennetier et al., 2010; Warren et al., 2009). Finally, we generated a synthetic 1-harmonic
267 sound, called ‘1-harmonic constant stimulus’, with constant frequency and amplitude over
268 time (set at the same mean peak-amplitude and mean frequency as the ‘1-harmonic 1-
269 female’ sound). A gradual increase/decrease over 1 s in the level of the start and end
270 sounds were added to avoid creating sound artefacts due to the signal truncation, and to
271 make the stimulus more natural (possibly important for active antennal amplification
272 (Jackson and Robert, 2006)). The 10 stimuli were played sequentially, with a 10 s interval
273 of silence to be played-back during the behavioural assays. To avoid an effect of the order
274 in which stimuli were played, 10 different sequences were generated, each containing the
275 10 sounds in random order. All stimuli were sampled at 8 kHz / 24 bits and designed in
276 Matlab (R2017a, The Mathworks Inc, Natick, USA). Fig. 2 gives the sound spectrum and
277 amplitude along time of each type of stimulus. Table S1 gives the filter/frequency
278 parameters used to generate the stimuli. Table 1 gives the sound levels for each of them.
279 Audio S5 and Audio S6 are the original 1-female and 30-female sound recordings,
280 respectively. Audio S1 to S4 are the 4 types of stimuli; 2-harmonic 1-female, 2-harmonic
281 30-female, 1-harmonic 1-female, 1-harmonic constant, respectively.

282
283 **Sound diffusion.** Sequences of sound stimuli were played-back from a speaker (Genelec
284 8010A) plugged into a Scarlett 18i8 sound card running pro-Tools First and Audacity on
285 Windows 7. The speaker is composed of two membranes (\varnothing 76 mm and 19 mm). The
286 centre of the larger speaker’s membrane was located 57 cm above the floor, 15 cm from

287 the back wall and 0.9 m from the swarming centre (Fig. 1). The speaker's self-generated
288 noise was less than 5 dB SPL (A-weighted) and the sound card's Equivalent Input Noise
289 was -127 dBu.

290
291 **Data Subsets.** While stimuli were played-back in random order during a single
292 experiment, they can be grouped into three overlapping subsets (Fig. 3), each of which
293 corresponds to one of the questions presented at the end of the Introduction;
294 Subset A: study of the effect of the second harmonic on male hearing (1-harmonic vs 2-
295 harmonic stimuli), Subset B: investigation of the effect of 'types of sound stimulus'
296 (single-frequency vs pre-recorded played-back stimuli) and Subset C: effect of the number
297 of females (1 vs 30) in the recorded-sound stimuli and of the sound levels of the sound
298 stimuli on male hearing to estimate the hearing threshold.

299
300 **Behavioural assays**
301
302 To investigate the sensitivity of swarming males to female sounds, we played-back the
303 female sound stimuli to swarming males in the sound-proof chamber. About twenty 3-4
304 days-old males were released the day prior to experiments at ~ 18h00 in the sound
305 recording flight arena. At 15h00, after the ceiling lights had dimmed to the lowest
306 intensity, the horizon light completed a 10 min dimming period and then kept at a constant
307 dim light intensity until the experiment was finished. When at least one male started to
308 swarm robustly over the marker, the first sequence of all 10 sound stimuli (i.e. the 4 types
309 of stimuli, with 4 sound levels for 2 of them, see Method section 'Signal generation') was
310 played-back from the speaker (see Movie S1 with a male exposed to one sound stimulus;
311 see Fig. S2 for examples of responses for each type of stimulus). After 10 stimuli were

312 played and if the male(s) was still swarming, or as soon as at least one male started
313 swarming, a new sequence of 10 stimuli was immediately played and so on, until up to 10
314 sequences were played or after 50 min of constant horizon light, either of which marking
315 the end of the experiment for the day (= 1 replicate). Males were then collected and
316 removed from the flight arena. A new group of ~20 male mosquitoes were released in the
317 sound-proof chamber, to be used for a new replicate the next day (one replicate per day,
318 for 10 days in August-September 2018).

319

320 **Sound pressure level (SPL)**

321

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

328

329 **Third-octave bands.** All SPLs reported in this study included only the frequency bands
330 that are audible to male mosquitoes, i.e., mostly the first harmonic of the female (Warren
331 et al., 2009; Pennetier et al., 2010). They were calculated as follows: $10\log_{10}(10^{0.1L_{B1}} +$
332 $10^{0.1L_{B2}})$ where L_{B1} and L_{B2} are SPL measurements in frequency bands $B1$ and $B2$;
333 $B1=500$ Hz and $B2=630$ Hz are the third-octave bands nearest the female's wingbeat
334 frequency of the first harmonic (Table 1; and Fig. S1 for all third-octave values).

335

336 ***Corrected SPLs for estimating the hearing threshold.*** The sound of 1-female was
337 recorded at a distance of 0.7 ± 0.2 m, which gave a relatively low signal-to-noise ratio
338 compared to the high signal-to-noise ratio of the sound of 30-females recorded at 0.9 ± 0.2
339 m. As explained in the Method section ‘Sound stimuli’, noise was removed below the first
340 harmonic and above the second harmonic but not in-between to limit artefacts in the sound
341 stimulus. SPL was computed over the frequency-band of the first harmonics, which, for
342 the 2-harmonic 1-female sound, included a part of the noise between the first and second
343 harmonics. Results from Subset A indicated that males did not need this noise to respond
344 to sound because they reacted to the 2-harmonic 1-female sound as much as to the 1-
345 harmonic 1-female sound. Since these two stimuli had the same first-harmonic amplitude
346 but a SPL difference of 8 dB (Table 1), and because SPL was defined over a frequency
347 band below the second harmonic, we established that the noise between the first and
348 second harmonics is responsible for 8 dB in our SPL measurements. In order to estimate
349 an accurate hearing threshold, we applied a correction of 8 dB to the sound level of the 2-
350 harmonic 1-female stimuli (Subset C). All sound levels, with correction or not, are
351 summarized in Table 1.

352
353 ***Control of distance between live mosquito and playback speaker.*** Swarming mosquitoes
354 confine themselves to a limited area of the flight arena naturally, which enables us to
355 estimate the incident SPL at the mosquito’s location, because the distance between
356 swarming mosquitoes and the sound stimulus source was limited to a known range. The
357 speaker (Genelec 8010A) that reproduced the females’ flight tones was placed 0.9 m from
358 the centre of the swarm marker. Their flight positions were recorded by 3D-tracking
359 Trackit Software (Fry et al., 2004) (Figs 4 A, 4 B) which enabled us to determine the

360 distance between a mosquito and the speaker emitting mosquito sound to be 0.9 ± 0.2 m,
361 95%-CI (Fig. 4 C).

362
363 ***Estimate of SPL errors at mosquito's location.*** Two types of SPL errors were taken into
364 account. The first is related to the time variation of the sound stimulus levels which were
365 between ± 0.3 dB and ± 0.9 dB (maximum error), depending on the stimulus (see Fig. 2 for
366 an example of stimulus sound-level over time). The second type of measurement
367 uncertainty arises when the sound level should be estimated from the mosquito's position,
368 and not from the fixed microphone position. Indeed, SPLs were measured at the expected
369 centre of the station-keeping swarm-flight of the test male mosquitoes. However, the
370 distance between the male and the speaker varied as 0.9 ± 0.2 m (95%-CI, Fig. 4 C), due to
371 the males' swarming-flight pattern, which changed the sound level they were exposed to,
372 accordingly. We evaluated this error by playing-back the *An. coluzzii* female sound
373 stimulus and measured the sound level in a sphere around the expected swarming area
374 centre: the maximum error was ± 2 dB. This error is considered to be conservative (at least
375 95%-CI) and was used to interpret the results of the experiments (see Table 1).

376
377 ***Physical sound quantities produced by a speaker and sensed by mosquitoes.*** We
378 monitored the sound level of the played-back stimuli by recording the sound pressure level
379 (SPL), however, mosquito hearing organs are sensitive to particle velocity level (SVL)
380 (Fletcher, 1978). The root-mean square value (RMS) particle velocity v_{RMS} and the RMS
381 sound pressure p_{RMS} can be related as follows, assuming the speaker to be a point source
382 radiating spherically a sound frequency f at a distance r from the source (air impedance
383 $Z_{air}(28^\circ\text{C}) = 408 \text{ N}\cdot\text{s}\cdot\text{m}^{-3}$; sound speed $c(28^\circ\text{C}) = 348 \text{ m/s}$) (Beranek and Mellow, 2012):

384
$$v_{RMS}(r) = \frac{p_{RMS}(r)}{Z_{air}} \sqrt{1 + \left(\frac{c}{2\pi fr}\right)^2}, \quad (1).$$

385 The SPL $L_p \stackrel{\text{def}}{=} 20 \log_{10}(p_{RMS}/p_0)$ and the associated particle-velocity level

386 $L_v = 20 \log_{10}(v_{RMS} Z_{air}/p_0)$ (reference $p_0 = 2.0 \cdot 10^{-5}$ Pa) can be calculated as follows:

387
$$L_v(r) = L_p(r) + 10 \log_{10} \left(1 + \left(\frac{c}{2\pi f r} \right)^2 \right), \quad (2).$$

388 Considering that the female sound stimulus does not have any frequency components
389 below $f = 440$ Hz (the smallest frequency value of the group of first harmonics of the
390 swarming females at -12 dB below the peak at 536 Hz, Fig. 2), the SVL is equal to the
391 SPL at 0.9 m away from a monopole sound source of these frequencies, under a negligible
392 error of less than 0.1 dB (due to the mosquito oscillating distance of ± 0.2 m to the speaker,
393 calculated from equation (2)). As a consequence, and since mosquitoes are sensitive to
394 SVL and for easier comparison with other studies, we report the SPL as SVL. Arthur et al.
395 (2014) measured the particle-velocity attenuation rate in front or behind *Ae. aegypti* to be
396 between a monopole and a dipole. Note that our monopole assumption for mosquito wing-
397 flapping is conservative since higher orders (dipole, quadripole) produce sound levels that
398 decrease more rapidly with distance (Bennet-Clark, 1998).

399

400 **Extraction of traits used to quantify male responses**

401

402 Following the results of preliminary experiments, we used two components of male flight:

403 1) **angular-speed**, calculated from their 3D trajectories and 2) **wingbeat frequency**,

404 extracted from sound recordings (see Fig. 4 B for example and statistics of wingbeat and

405 flight dynamic characteristics before, during and after exposure to the loudest 1-female

406 sound-stimuli (44 ± 2 dB SVL)). The two components were synchronized using the same

407 techniques as in a previously published study (Feugère et al., 2021c).

408

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

$$414 \quad \Delta\theta = \arccos \frac{\mathbf{v}_n \cdot \mathbf{v}_{n+1}}{|\mathbf{v}_n| |\mathbf{v}_{n+1}|}, \quad (3)$$

415
416 where \mathbf{v}_n is the three-dimensional linear velocity vector of the mosquito at time index n
417 and $|\mathbf{v}_n|$ is its magnitude. The criteria used to include a tracked flight in the data analysis
418 were that the mosquito was swarming over the marker for at least 1 s before and after the
419 sound stimulus onset.

420
421 **Wingbeat frequency.** Only the first and/or the second harmonic of female sound stimuli
422 were played-back (~400-1200 Hz) in order to free the frequency domain of the male's
423 third harmonic from the female's sound. This allowed us to capture the male's third
424 harmonic without overlapping with the sound stimulus (example of spectrogram in Fig. 4
425 A). The peak of the third harmonic was detected every 40 ms between 2190 and 2920 Hz
426 using the Fast Fourier Transform algorithm (256-ms FFT-window, Hanning-windowed).
427 When several mosquitoes (from 1 to 6) were present over the swarming marker, the
428 detected value was the peak of the energy in the frequency band 2190-2920 Hz and not the
429 mean of the peak from individual mosquitoes (because it was not possible to track the
430 wingbeat frequencies of individual mosquitoes). Then, the male's third-harmonics (i.e., 3
431 x wingbeat frequency) were divided by 3 to get the wingbeat frequency (i.e., the first-
432 harmonic frequency). Finally, a 3-point median filter was applied over time to reduce
433 wingbeat tracking error. Fig. 4 A gives an example of detected wingbeat frequencies of

434 males while Fig. 4 B shows the distribution of the detected wingbeat frequency over time
435 for all recordings.

436

437 ***Upper-quartile difference.*** Since preliminary experiments suggested that mosquitoes
438 responded to sound by increasing their wingbeat frequency and their angular speed
439 somewhere during the first second of the sound stimuli, the upper-quartile angular-speeds
440 and the upper-quartile wingbeat frequencies were automatically detected during the first
441 1 s stimulus time interval. Indeed, ‘upper-quartile’ is 1) a more robust metric than median
442 or mean to measure the amplitude of a short peak, which the onset time cannot be
443 predictable precisely and 2) a more reliable metric than ‘maximum’ to avoid false
444 detection. Then, this value was subtracted from the upper-quartile value computed during
445 the 1 s segment just before the stimulus onset, for each individual recording to reduce noise
446 related to individual mosquito variability (Fig. 4 A shows graphically how the parameters
447 were computed).

448

449 **Statistics**

450

451 Wingbeat-frequency and angular-speed values for a given stimulus were averaged over the
452 different responses of the same day to form a replicate. The wingbeat and angular-speed
453 response-parameters were analysed using a Bayesian Linear Mixed-Effects Model (*blmer*
454 function, *lme4* package, R). Stimulus sound levels (continuous), number of females in the
455 recording (1 or 30), number of harmonics (1 or 2) and sound type (recording or synthesis)
456 and their interaction were considered as fixed effects. Days, for which replicates were
457 performed, were considered random effects. The dataset was split into the 3 subsets A, B,
458 C, as shown in Fig. 3. A total of 6 models were built (2 parameters x 3 subsets). Stepwise

459 removal of terms was used for model selection, followed by likelihood ratio tests. Term
460 removals that significantly reduced explanatory power ($p < 0.05$) were retained in the
461 minimal adequate model (Crawley, 2007). No data transformation was needed to ensure
462 variance homogeneity of variables (Fligner-Killeen test, *Fligner.test* function, R) and
463 normality of model residuals (Shapiro-Wilk test, *shapiro.test* function, R), except for
464 Subset C wingbeat-frequency which was transformed via optimality (*MLE_LambertW*
465 function, *LambertW* package, R (Goerg, 2016)); see Fig. S3 for normality *qqplots* and
466 Table S2 for normality and variance homogeneity test results.

467
468 For subsets A and B, an additional one-sample t-test (with BF-correction for multiple
469 comparisons) was performed independently for each distribution to measure the
470 significance of the mean to 0, which is the “no response” reference. For subset C, the
471 quietest 2-harmonic 30-female sound-stimulus was not included in the model because its
472 sound level was too close to the background noise level to be corrected as the three other
473 2-harmonic 30-female sound-stimuli. The hearing threshold was estimated by the crossing
474 of the $y=0$ axis (i.e., no response, including with the LambertW transformation) with the
475 prediction of the fixed-effect components of the mean and associated 95%-CI (*bootMer*
476 function with *nsim=500*, *lme4* package, R). The Lambert transformation does not change
477 the 0 value of the distribution. All analyses were performed using R (version 3.5.3).

478
479 Model Subsets resulted in a sampling size of $n=10$ for Subset A and B and $n=9$ or $n=10$
480 for Subset C (see legend of Fig. 5 for details; see Method Section ‘Behavioural assays’ for
481 how a replicate was defined).

482

483

484 **Results**

485

486 **Males mostly use the female's first harmonic to hear her flight tone (Subset A)**

487

488 Subset-A sound-stimuli with one or two harmonics were heard by males as the response
489 distributions are different from the null distribution (Fig. 5 A), for both angular-speed
490 (upper-quartile angular-speed difference: one-sample $t=5.7$, $df=9$, BH-corrected $p<0.001$,
491 mean=1.5 rad/s; one-sample $t=5.0$, $df=9$, BH-corrected $p<0.001$, mean=1.0 rad/s,
492 respectively) and wingbeat frequency (upper-quartile wingbeat-frequency difference: one-
493 sample $t=5.2$, $df=9$, BH-corrected $p<0.001$, mean=16 Hz; one-sample $t=4.6$, $df=9$, BH-
494 corrected $p=0.0013$, mean=13 Hz, respectively).

495

496 Our results show no differences in response of males exposed to the first-harmonic's
497 sound of a female flight tone or the combination of the first and second harmonic sounds
498 with noise in-between of the same female flight tone (upper-quartile angular-speed
499 difference: LRT, $\chi^2=2.6$, $df=1$, $p=0.11$; upper-quartile wingbeat-frequency difference:
500 LRT, $\chi^2=1.1$, $df=1$, $p=0.29$).

501

502 **Males react to a 'pure-sound' (1-harmonic constant sound) at least as much as to a**
503 **'natural sound' (1-harmonic 1-female sound) (Subset B)**

504

505 Subset-B stimuli, i.e. 1-harmonic 1-female sound and 1-harmonic constant sound, were
506 both heard by the males because the response distributions were different from the null
507 distribution (Fig. 5 B), for both the angular-speed (upper-quartile angular-speed
508 difference: one-sample $t=5.7$, $df=9$, BH-corrected $p<0.001$, mean=1.5 rad/s; one-sample

509 $t=5.4$, $df=38$, BH-corrected $p<0.001$, mean=1.6 rad/s, respectively) and the wingbeat
510 frequency (upper-quartile wingbeat-frequency difference: one-sample $t=5.2$, $df=9$, BH-
511 corrected $p<0.001$, mean=16 Hz; one-sample $t=5.1$, $df=38$, BH-corrected $p<0.001$,
512 mean=30 Hz, respectively).

513
514 Our results show there is little difference in the male response between the 1-harmonic 1-
515 female sound-stimulus and the 1-harmonic constant sound of the same mean
516 frequency/SVL. While males change their angular-speed with the same amplitude in
517 response to these two stimuli, they change their wingbeat frequency two times more with
518 the 1-harmonic constant sound (upper-quartile angular-speed difference: LRT $\chi^2=0.052$,
519 $df=1$, $p=0.82$; upper-quartile wingbeat-frequency difference: LRT $\chi^2=4.5$, $df=1$, $p=0.033$,
520 respectively).

521
522 **Males react to the 1-female sound more than to the 30-female sound, with a hearing**
523 **threshold less than 20 dB SVL (Subset C)**

524
525 Using data subset C (see Table 1 for sound levels), our results (Fig. 5 C) show that free-
526 flying males respond to the sound stimuli, providing the sound level was high enough, by
527 increasing both their angular-speed and their wingbeat frequency as the tested sound
528 levels increased (upper-quartile angular-speed difference: LRT $\chi^2=36.8$, $df=1$, $p<0.001$,
529 effect size=0.12 rad/s per dB SVL; and LambertW-transformed upper-quartile wingbeat-
530 frequency difference: LRT $\chi^2=23.8$, $df=1$, $p<0.001$, respectively). The number of females
531 had small effect, but this was not interpretable, because of distinct values of sound levels
532 for each number of females (upper-quartile angular-speed difference: LRT $\chi^2=3.3$, $df=1$,
533 $p<0.001$, 1.2 rad/s for 1-female vs 0.6 rad/s for 30-female stimuli; and LambertW-

534 transformed upper-quartile wingbeat-frequency difference: LRT $\chi^2=3.2$, $df=1$, $p=0.073$, 20
535 Hz for 1-female vs 10 Hz for 30-female stimuli, respectively). However, globally, the
536 males responded more to the 1-female sound than to the 30-female sound as the sound
537 level increased (i.e. interaction between the sound level and the number of female; upper-
538 quartile angular-speed difference: LRT $\chi^2=3.3$, $df=1$, $p=0.070$, effect size = additional 0.05
539 rad/s per dB SVL for 1-female sound-stimulus; and LambertW-transformed upper-quartile
540 wingbeat-frequency difference: LRT $\chi^2=10.3$, $df=1$, $p=0.0013$, respectively).

541
542 For 2-harmonic 30-female sound-stimuli (Fig. 5 C, red colour), the mean sound-level
543 threshold was 21 dB SVL with a 13-27 dB SVL 95%-CI, if considering the angular-speed
544 as response parameter. Using the wingbeat frequency parameter, the mean sound-level
545 threshold was 19 dB SVL with a 9-23 dB SVL 95%-CI. For 2-harmonic 1-female sound-
546 stimuli (Fig. 5 C, green colour), the mean sound-level threshold was 15 dB SVL with an
547 9-19 dB SVL 95%-CI, if considering the angular-speed to be a response parameter. Using
548 the wingbeat frequency parameter, the mean sound-level threshold was 17 dB SVL with a
549 13-20 dB SVL 95%-CI. Considering these latter stimuli, which are the most ecological
550 ones, a conservative estimate of the hearing threshold is then 20 dB SVL.

551

552

553 **Discussion**

554

555 **Behavioural assessment of hearing threshold in swarming mosquitoes**

556

557 Inter-mosquito acoustic communication is believed to occur at short range only (Feugère
558 et al., 2021b), during mating behaviour when mosquitoes are flying in loops near a visual

559 marker. *Anopheles coluzzii* males gather in tens to thousands over station-keeping swarm
560 sites, while virgin females join the swarm in much fewer numbers as they mate only once
561 in a life-time. Once a male detects a female's presence from her wing-flapping sound, the
562 male starts to chase the female (Pantoja-Sanchez *et al.*, 2019). Thus, there is strong
563 competition between males to detect relatively rare females (~1% male:female ratio
564 (Kaindoa *et al.*, 2017; Charlwood and Jones, 1980). Accordingly, acute hearing sensitivity
565 is highly advantageous to males, along with other factors such as their own wingbeat
566 acoustic power (Lapshin, 2012) and frequency (Somers *et al.*, 2021) in the context of
567 distortion-product hearing.

568
569 Under laboratory conditions (27-29°C), we show that male *An. coluzzii* respond strongly
570 to 1-harmonic constant sound of 26±2 dB SVL at the female's mean wingbeat frequency
571 (Fig. 5 B and Table 1) and we estimate the hearing threshold to be 20 dB SVL or less with
572 a 95%-CI using 2-harmonic 1-female sounds (13-20 dB SVL). Researchers have used
573 electrophysiological mosquito preparations to measure hearing thresholds in the
574 Johnston's organ, which does not involve free-flying, pre-mating behaviour, such as
575 swarming (but see Feugère *et al.* (2021) and Lapshin and Vorontsov (2021)). This may
576 explain why these electrophysiological studies usually found far higher sound thresholds
577 than in our study (see Introduction section). Lower hearing thresholds measured by
578 electrophysiological methods can partly be explained by the absence of flight tones in
579 males, which is known to be important to enhance the sensitivity in males to female
580 sound. This creates mixed-harmonics for which the JO is tuned to, as shown by
581 electrophysiology mosquito preparations exposed to flight sound simulation, which lowers
582 the hearing threshold by 7 dB in *Cx. pipiens pipiens* (Lapshin, 2012). However, this may
583 not be the only explanation. Mosquitoes exhibit 'active hearing', which can be triggered

584 only during specific physiological states (Göpfert and Robert, 2001; Su et al., 2018), one
585 of which may be swarming. It may be that males can enhance hearing to detect a female
586 that is approaching a male swarm before she is chased by a competitor.

587
588 The only other species to have been explored in relation to these aspects of swarming
589 flight is *Ae. communis* (Lapshin and Vorontsov, 2021); in the field, the mean hearing-
590 threshold of males at the female's wingbeat frequency was shown to be particularly low,
591 26 dB SVL. However, their method consisted in monitoring flight-speed changes in
592 natural swarms by eye, which may not have enabled them to measure the smallest
593 response amplitudes, thereby over-estimating the threshold (Lapshin and Vorontsov,
594 2021). On the contrary, we measured both flight dynamics and wingbeat frequency from
595 quantitative measurements. Also, ambient temperatures were very different (~12°C for
596 Lapshin and Vorontsov (2021) vs 27-29°C for our recording), which can change hearing
597 sensitivities.

598
599 Finally, electrophysiological measurements in the JO are usually averaged over JO
600 scolopidia, however, this could misrepresent the effective signal that triggers a
601 behavioural response. Indeed, in addition to individual sensitivity in frequency and
602 threshold, JO scolopidia are sensitive to the direction of the sound wave, and then only the
603 JO scolopidia which are aligned with the sound wave-front display a low response-
604 threshold. As a consequence, averaging all JO-scolopidium thresholds may over-estimate
605 hearing thresholds (Lapshin and Vorontsov, 2019).

606

607 **Male response to sound and the effect of number of females**

608

609 Males change their wingbeat frequency with a greater amplitude when exposed to 1-
610 female sound than to 30-female sound, however, the change in angular-speed was small
611 and its statistical significance was marginal (Subset C). This occurs despite the relatively
612 greater amount of noise in-between the 1st and 2nd harmonic in the sound stimulus of the
613 1-female; the difference may have been stronger if the prominence of the harmonics had
614 similar values in the tested stimuli. Two comments merit emphasis; the first is that a group
615 of frequencies that are attractive alone (e.g., grouped-female sounds) have a masking
616 effect on mosquito auditory perception. These results support reports published 80 years
617 ago with *Ae. aegypti* males; it was observed that these mosquitoes were not attracted to
618 two or more sounds at a time, even though each of these sounds were attractive on their
619 own (Wishart and Riordan, 1959).

620
621 Second, it is interesting that males respond more with their wingbeat frequency than with
622 their flight trajectory or dynamics. The change in wingbeat-frequency is consistent with a
623 current theory that during a chase between a male and a female, the male moves to the
624 sound source by tracking the female's wingbeat sound and adjusts his own wingbeat
625 frequency to hear her better, through an auditory mechanism based on antennal distortion
626 products (Warren et al., 2009, Simões et al., 2019). In our case, the sound wave-front is
627 almost planar at the male's position, due to the distance and membrane dimension of the
628 speaker, contrary to the sound wave of a female of the same sound level which would be
629 far more spherical. This may create contradictory signals in the mosquito auditory system,
630 i.e., the sound level suggests that the female is very close, but the sound wave-shape gives
631 poor information about her actual location.

632
633 **The question of hearing higher harmonics and the significance of background noise**

634

635 Males are known to detect mainly the female's first harmonic to hear her flight tone.

636 Indeed, *Ae. aegypti* respond (with clasping and seizing movements in flight) to low

637 frequencies under 500 Hz (i.e., 1-harmonic sounds) using tuning forks (Roth, 1948), while

638 other species, such as *Cx. pipiens pipiens*, have a narrower frequency range of response

639 (500-600 Hz) when swarming (Gibson, 1985). In *Toxorhynchites brevivalpis*, *Cx. pipiens*

640 *pipiens*, and *An. gambiae s.l.*, electrophysiology revealed that male antennae are sensitive

641 to a large frequency-band up to 2 kHz that encompasses the two first harmonics, however,

642 the electrical tuning of their JO is very narrow and centred on the difference wingbeat

643 frequency of the two sexes which is close to the female's first harmonic (Gibson et al.,

644 2010). With respect to behaviour, Wishart and Riordan (1959) trapped as many *Ae.*

645 *aegypti* males with the sound of 1-harmonic tones as with the complete flight sound.

646 Moreover, when removing the first harmonic from female flight tone recordings, *Ae.*

647 *aegypti* males did not respond anymore, but the authors reported their results without any

648 further information. This absence of a male's response if the female's first-harmonic is

649 removed from the stimulus is similar to our results with *An. gambiae*, which shows a

650 similar male response if the second harmonic of the female flight-tone is removed. On the

651 contrary, it has been reported that male *Ae. aegypti* can hear the female second-harmonic,

652 but without inferential statistics (Cator et al., 2009), and their results were also contested

653 with arguments based on auditory processing of phasic information in the JO nerves of *Cx.*

654 *quinquefasciatus* (Warren et al., 2009). However, the image channel resulting from the

655 non-linear vibration of the antennae from the sound of the two sexes was shown to

656 reinforce the hearing sensitivity of males close to/slightly above the frequency of the

657 female's second harmonic in electrophysiological measurements in *Cx. pipiens pipiens*

658 (Lapshin, 2012) and *Ae. communis* (Lapshin and Vorontsov, 2021). The results of our

659 behavioural assay suggest that this reinforcement is negligible in practice, at least in *An.*
660 *coluzzii*.

661
662 The limitation of our stimulus recording approach is to be found in the long distances
663 between the microphone and the single female (0.7 ± 0.2 m), which induced a low signal-
664 to-noise ratio of 1.7, despite noise filtering below the first harmonic and above the second
665 harmonic (against a ratio of ~ 48 for the 2-harmonic 1-female stimulus; if considering the
666 noise level as the noise floor between the 2 harmonics, using the Matlab function *snr*).
667 Indeed, because of these different signal-to-noise ratios, the 2-harmonic 1-female stimulus
668 can be seen as a frequency band of noise (ranging from the first to the second harmonic
669 frequencies) instead of a true 2-harmonic sound.

670
671 However, this noise asymmetry between the two stimuli also shows that males are not
672 fundamentally disturbed by noise; the noisiest stimulus (2-harmonic 1-female) induced as
673 much response as the least noisy stimulus (2-harmonic 30-female). Wishart and Riordan
674 (1959) found that female sound (500 Hz) is still an attractant to males, with at least up to
675 10 dB of noise above the signal sound level for *Ae. aegypti* males, but was not an
676 attractant on the next tested step of 20 dB of noise above the signal level. The noise was
677 composed of the superposition of sine waves of 100, 156, and 282 Hz plus square waves
678 of 933, 1840, and 4130 Hz, which probably did not create as much noise around the
679 female sound frequency as in our case. The hearing mechanism based on antennal
680 distortion products uses the loud wingbeat frequency of the listener to amplify the nearby,
681 but possibly quiet, wingbeat frequency of a potential mate (Lapshin 2012). By changing
682 its own wingbeat-frequency, it is possible to change the distortion product frequency
683 elicited by the nearby flying mate, which, theoretically, may help detect very faint

684 harmonics against a relatively high level of background noise, especially when this noise
685 is limited to the frequency band between the two harmonics.

686

687 **Constant sound vs ‘natural’ sound**

688

689 Constant sound and female pre-recorded flight-tones have been known to trigger a
690 response in mosquitoes for a long time (Roth, 1948; Kahn and Offenhauser, 1949).

691 However, to our knowledge, no comparisons has been formally analysed between pre-

692 recorded sounds and constant sound of the same frequency. Our results in Subset B show

693 that the 1-harmonic constant sound behaves somewhat like a supernormal stimulus (for

694 the wing-beat frequency response-parameter) compared to a 1-harmonic natural sound, at

695 least at 26 dB SVL. Furthermore, Subset A allows us to conclude that males respond as

696 much to 1-harmonic ‘natural’ sound as to 2-harmonic ‘natural’ sound. By combining

697 results from Subset A and B, we deduce that mosquitoes hear natural sound as well as

698 pure sound. This means that the information carried in the sound that elicits a male

699 response is mostly the mean wingbeat frequency. A proper study could be carried out 1)

700 with 1-harmonic constant sounds to control the sound level better than with pre-recorded

701 sounds, and 2) by using larger ranges of frequencies and sound levels than in the present

702 study, i.e., we would need to conduct a ‘behavioural audiogram’.

703

704 **Monitoring SVL from SPL measurements**

705

706 Many studies report hearing thresholds based on SPL, which is a physical quantity that

707 mosquitoes do not detect. We also monitored sound level with SPL, but we fulfilled the

708 experimental conditions to provide equivalence between SPL and SVL, which mosquitoes

709 do detect (see Methods section). Some studies have referred to SPL values as hearing
710 thresholds, even though the equivalence conditions were not fulfilled or were unknown.
711 Wishart and Riordan (1959) estimated that *Ae. aegypti* responds to a sound of
712 approximately 20 dB SPL from experiments involving 30cm-side netting cages and sound
713 stimuli presented through a diffuse speaker held against the cage netting. However,
714 mosquitoes could be located a few centimetres from the loudspeaker, where SPL and SVL
715 are not equivalent at this distance, i.e., when SPL would not be a good physical quantity to
716 describe what the mosquito auditory organs are exposed to. Another example is Belton *et*
717 *al.* (1961); a response threshold in the Johnston's organs of male *Ae. aegypti* was
718 measured to be between 0 and 10 dB SPL; SPL to SVL using a formulae that assumed far-
719 field condition (without stating so, though). Unfortunately, the study did not provide
720 enough details of the experimental setup to know the distance between the pressure
721 microphone and the loudspeaker; thus, the thresholds were probably inaccurate. More
722 recently, Dou *et al.* (2021) put their loudspeaker at 2.5 cm against their 30cm-side cage to
723 measure the response of mosquitoes and monitored the sound level with an SPL meter in
724 the middle of the cage. They measured flight response to sound in *Ae. aegypti* females for
725 the first time, from a threshold of 79 dB SPL, which could be far more in terms of SPL
726 since it was measured in the middle of the cage and mosquitoes were free to move along
727 the cage's sides, near the loudspeaker. In addition, SVL may have been far greater than
728 SPL at this distance from the speaker. Taken together, SVLs probably do not occur with
729 ecologically-relevant sounds, however, this could be used to inform the design of sound
730 traps or reveal unknown auditory mechanisms.

731

732

733 **Acknowledgments**

734 Natalie Morley (insect rearing), Stephen Young (discussion about statistics; design of the
735 synchronization electronic tool; design of high-pass frequency electronics to adjust the
736 dimming LED system).

737

738 **Competing interests**

739 The authors declare no conflict of interest.

740

741 **Funding Statement**

742 This work is supported by a Research Grant from Agence Nationale de la Recherche
743 [JCJC-15-CE35-0001-01 to O.R.] and Human Frontier Science Program [RGP0038/2019
744 to G.G].

745

746 **Data Availability**

747 Raw response files (sound and tracked flight trajectories) are available at:
748 <https://doi.org/10.5061/dryad.9cnp5hqhj> (Feugère et al., 2021a). Sound stimuli (Audio S1
749 to S4), as well as the original sound recordings (Audio S5, Audio S6) are available as
750 supplementary material. Custom audio–video code for parameter-extraction and audio–
751 video synchronization (modified Matlab files from
752 <https://doi.org/10.17632/hn3nv7wxpk.3> (Feugère, 2020)), custom statistics code for data
753 analysis and figure plot (R files) and dataset (Text files) are available at
754 <https://doi.org/10.17632/6w5jttwkj8.2> (Feugère, 2021).

755

756 **Author contributions**

757 Conceptualization LF, GG; Methodology LF, GG; Software LF; Formal Analysis LF;
758 Investigation LF; Data Curation LF; Writing – Original Draft LF; Writing – Review &

759 Editing OR, GG; Visualization LF; Supervision GG, OR; Funding Acquisition OR, GG,
760 LF.

761

762 **References**

763

764 **Arthur, B. J., Emr, K. S., Wytenbach, R. A. and Hoy, R. R.** (2014). Mosquito (*Aedes*
765 *aegypti*) flight tones: Frequency, harmonicity, spherical spreading, and phase
766 relationships. *J. Acoust. Soc. Am.* **135**(2), 933-941.

767

768 **Bennet-Clark, H. C.** (1998). Size and scale effects as constraints in insect sound
769 communication. *Philos. Trans. R. Soc., B.* **353**, 407-429.

770

771 **Beranek, L. L. and Mellow, T. J.** (2012). Chapter 5 – Microphones. In
772 *Acoustics: sound fields and transducer* (ed. L. L. Beranek and T. J. Mellow), pp. 199-240,
773 Oxford:Academic Press.

774

775 **Belton, P.** (1961). The physiology of sound reception in insects. *Proc. Ent. Soc. Ont.* **92**,
776 Entomological Society of Ontario, 20-26.

777

778 **Cator, L. J., Arthur, B. J., Harrington, L. C. and Hoy, R. R.** (2009). Harmonic
779 convergence in the love songs of the dengue vector mosquito. *Science* **323**, 1077-1079.

780

781 **Charlwood, J. D. and Jones, M. D. R.** (1980). Mating in the mosquito, *Anopheles*
782 *gambiae s.l.*. *Physiol. Entomol.* **5**, 315-320.

783

- 784 **Crawley, M. J.** (2007). Mixed-Effects Models. In *The R book*, pp. 627-660. New York:
785 John Wiley & Sons.
786
- 787 **Dou, Z., Madan, A., Carlson, J. S., Chung, J., Spoleti, T., Dimopoulos, G.,**
788 **Cammarato, A. and Mittal, R.** (2021). Acoustotactic response of mosquitoes in
789 untethered flight to incidental sound. *Sci. Rep.* **11**, 1884.
790
- 791 **Fanello, C., Santolamazza, F. and Della Torre, A.** (2002). Simultaneous identification
792 of species and molecular forms of the *Anopheles gambiae* complex by PCR-RFLP. *Med.*
793 *Vet. Entomol.* **16**, 461-464.
794
- 795 **Feugère, L.** (2020). Code for audio-video parameter extraction and statistical tests related
796 to mosquito response to opposite-sex sound-stimuli. *Mendeley Data*, V3,
797 <https://doi.org/10.17632/hn3nv7wxpk.3>
798
- 799 **Feugère, L.** (2021). Code for audio-video parameter extraction, statistical tests and figure
800 design related to characterization of male-mosquito hearing. *Mendeley Data*, V2,
801 <https://doi.org/10.17632/6w5jttwkj8.2>
802
- 803 **Feugère, L., Gibson, G. and Roux, O.** (2021a), Audio and 3D flight-track recordings of
804 mosquito responses to opposite-sex sound-stimuli, *Dryad, Dataset*,
805 <https://doi.org/10.5061/dryad.9cnp5hqhj>
806

- 807 **Feugère, L., Gibson, G., Manoukis, N. C. and Roux, O.** (2021b). Mosquito sound
808 communication: are male swarms loud enough to attract females? *J. R. Soc. Interface*
809 **18**(177), 20210121.
- 810
- 811 **Feugère, L., Gibson, G., Manoukis, N. C., Roux, O.** (2021c). Supplementary material
812 from “Mosquito sound communication: are male swarms loud enough to attract females?”.
813 *The Royal Society. Collection.* <https://doi.org/10.6084/m9.figshare.c.5361985.v1>
- 814
- 815 **Fletcher, N. H.** (1978). Acoustical response of hair receptors in insects. *J. Comp. Physiol.*
816 **127**, 185-189.
- 817
- 818 **Fry, S., Muller, P., Baumann, H.-J., Straw, A., Bichsel, M. and Robert, D.** (2004).
819 Context-dependent stimulus presentation to freely moving animals in 3D. *J. Neurosci.*
820 *Methods* **135**, 149-157.
- 821
- 822 **Gibson, G.** (1985). Swarming behavior of the mosquito *Culex pipiens quinquefasciatus*: a
823 quantitative analysis. *Physiol. Entomol.* **10**, 283-296.
- 824
- 825 **Gibson, G., Warren, B. and Russel, J. I.** (2010). Humming in tune: sex and species
826 recognition by mosquitoes on the wing. *JARO* **11**, 527-540.
- 827
- 828 **Goerg, G. M.** (2016). Lambert W random variables—a new family of generalized skewed
829 distributions with applications to risk estimation. *Ann. Appl. Stat.* **5**(3), 2197-2230.
- 830

- 831 **Göpfert, M., Briegel, H. and Robert, D.** (1999). Mosquito hearing: sound-induced
832 antennal vibrations in male and female *Aedes aegypti*. *J. Exp. Biol.* **202**(20), 2727-2738.
833
- 834 **Göpfert, M. C. and Robert, D.** (2000). Nanometre-range acoustic sensitivity in male and
835 female mosquitoes. *Proc. R. Soc. B* **267**, 453-457.
836
- 837 **Göpfert, M. C. and Robert, D.** (2001). Active auditory mechanics in mosquitoes. *Proc.*
838 *R. Soc. B* **268**(1465), 333-339.
839
- 840 **Jackson, J. C. and Robert, D.** (2006). Nonlinear auditory mechanism enhances female
841 sounds for male mosquitoes. *Proc. Natl. Acad. Sci. U. S. A.* **103**(45), 16734-16739.
842
- 843 **Kahn, M. C. and Offenhauser, W.** (1949). The first field tests of recorded mosquito
844 sounds used for mosquito destruction. *Am. J. Trop. Med.* **s1-29**(5), 811-825.
845
- 846 **Kaindoa E. W, Ngowo H. S, Limwagu A., Mkandawile G., Kihonda J., Masalu J. P.,**
847 **Bwanary H., Diabate A. and Okumu F. O.** (2017). New evidence of mating swarms of
848 the malaria vector, *Anopheles arabiensis* in Tanzania. *Wellcome Open Res.* **2**, 88.
849
- 850 **Lapshin, D. N.** (2012). Mosquito bioacoustics: Auditory processing in *Culex pipiens*
851 *pipiens l.* males (Diptera, Culicidae) during flight simulation. *Entomol. Rev.* **92**(6), 605–
852 621.
853
- 854 **Lapshin, D. N. and Vorontsov, D. D.** (2019). Directional and frequency characteristics of
855 auditory neurons in *Culex* male mosquitoes. *J. Exp. Biol.* **222**(21), jeb208785

856

857 **Lapshin, D. N. and Vorontsov, D. D.** (2021). Frequency tuning of swarming male
858 mosquitoes (*Aedes communis*, Culicidae) and its neural mechanisms. *J. Insect Physiol.* **0**,
859 104233.

860

861 **Menda, G., Nitzany, E. I., Shamble, P. S., Wells, A., Harrington, L. C., Miles, R. N.**
862 **and Hoy, R. R.** (2019). The long and short of hearing in the mosquito *Aedes aegypti*.
863 *Curr. Biol.* **29**, 709-714.

864

865 **Pantoja-Sanchez, H., Gomez, S., Velez, V., Avila, F.W., and Alfonso-Parra, C.** (2019).
866 Precopulatory acoustic interactions of the new world malaria vector *Anopheles albimanus*
867 (Diptera: Culicidae). *Parasites Vectors* **12**(1), 386.

868

869 **Pennetier, C., Warren, B., Dabiré, K. R., Russel, J. I. and Gibson, G.** (2010). “Singing
870 on the wing” as a mechanism for species recognition in the malarial mosquito *Anopheles*
871 *gambiae*. *Curr. Biol.* **20**, 131-136.

872

873 **Poda, S. B., Nignan, C., Gnankiné, O., Dabiré, R. K., Diabaté, A. and Roux, O.**
874 (2019). Sex aggregation and species segregation cues in swarming mosquitoes: role of
875 ground visual markers. *Parasites Vectors* **12**(1), 589.

876

877 **Roth, L. M.** (1948). A study of mosquito behavior. An experimental laboratory study of
878 the sexual behavior of *Aedes aegypti* (Linnaeus). *Am. Midl. Nat.* **40**(028), 265-352.

879

880 **Simões, P. M. V., Ingham, R. A., Gibson, G. and Russell, I. J.** (2018). Masking of an
881 auditory behaviour reveals how male mosquitoes use distortion to detect females. *Proc. R.*
882 *Soc. B* **285**(1871).

883
884 **Somers, J., Georgiades, M., Su, M. P., Bagi, J., Andrés, M., Mills, G., Ntabaliba, W.,**
885 **Moore, S. J., Spaccapelo, R. and Albert, J. T.** (2021). Flight-tone mediated circadian
886 control of audibility in mating swarms of *Anopheles* mosquitoes. *bioRxiv*
887 doi:2021.07.12.452033.

888
889 **Su, M. P., Andrés, M., Boyd-Gibbins, N., Somers, J. and Albert, J. T.** (2018). Sex and
890 species specific hearing mechanisms in mosquito flagellar ears. *Nat. Commun.* **9**, 3911.

891
892 **Tischner, H.** (1953). Über den gehörsinn von stechmücken. *Acustica* **3**, 335-343.

893
894 **Warren, B., Gibson, G. and Russel, J. I.** (2009). Sex recognition through midflight
895 mating duets in *Culex* Mosquitoes is mediated by acoustic distortion. *Curr. Biol.* **19**, 485-
896 491.

897
898 **Wishart, G. and Riordan, D. F.** (1959). Flight responses to various sounds by adult
899 males of *Aedes aegypti* (L.) (Diptera: Culicidae). *Can. Entomol.* **91**, 181-191.

900
901 **Zhou, J. and Miles, R. N.** (2017). Sensing fluctuating airflow with spider silk, *Proc. Natl.*
902 *Acad. Sci. U. S. A.* **114**(46), 12120-12125.

903

904

905 **Figure legends**

906

907 **Fig. 1. Sound-proof chamber setup for recording sound and video of *An. coluzzii***
908 **behaviour (modified version from (Feugère et al., 2021b)).** (A) Bird's-eye and (B) side
909 views of sound-proof chamber. Blue shaded areas indicate the 3D fields-of-view of
910 cameras recording mosquito flight paths. Two IR-sensitive cameras fitted with IR pass
911 filters recorded flying mosquitoes as black silhouettes against evenly lit IR- background.
912 A separate lighting system provided gradual semi-natural dusk visible to mosquitoes,
913 consisting of dispersed dim white lights on ceiling and 'sunset' lighting below horizon
914 (opaque wall ~40 cm tall). A microphone recorded flight sounds of mosquitoes swarming
915 directly above black swarm marker. A thermocouple (85 cm above ground level) recorded
916 temperature at ~ mean swarm height. A speaker located behind IR-illuminated thin-cotton
917 sheet, outside net enclosure played back sound stimuli.
918 (C) Bird's-eye and (D) side views of the superimposed flight tracks of the entire dataset.

919

920 **Fig. 2. Spectral and temporal properties of sound stimuli.** Spectral (first column) and
921 temporal (second column) properties of sound stimuli of one single swarming *An. coluzzii*
922 female (top row) compared to that of 30 females (bottom row). The originally recorded
923 sounds are represented with a dotted line (Audio S1 and Audio S2 for a unfiltered 1-
924 female and 30-female, respectively; not used directly as sound stimuli). The 1-harmonic 1-
925 female sound is shown as a semi-dotted-dashed red line, while the 2-harmonic sounds are
926 represented by a solid pink line. Magnitude spectra were calculated over 7 s and averaged
927 over 50-Hz windows. The root-mean-square pressure levels were computed over a 0.1 s
928 time window with 0.05 s overlap, along the 7 s duration of the stimuli. See Table S1 for
929 characteristics of filters applied to Audio S1 and Audio S2 to generate the 1-harmonic and
930 2-harmonic stimuli.

931

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

936

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

940 **(A)** Example of male response to the loudest 2-harmonic 1-female sound-stimulus over 27
941 s of recording. Stimulus was played-back 10 s from beginning of flight recording and
942 lasted 7 s (red rectangular shading). First five rows show flight parameters (relative X, Y
943 and Z positions, plus linear and angular flight speeds). ‘Z’ dimension represents relative
944 distance to the speaker (located 0.9 m from Z=0). Before-last row shows mean wingbeat
945 frequency (WBF). Periodic flight pattern, typical of swarming behaviour, is evident in X,
946 Y and Z plots. In the angular-speed and wingbeat frequency plots, the two red lines
947 correspond to the upper-quartile over 1s and the arrows represent the differences between
948 the two red lines, which are the parameters computed for monitoring the male response
949 (see Methods section ‘Extraction of traits used to quantify male responses’). Last row
950 shows the spectrogram of sound recordings before, during and after the sound stimulus;
951 the colour gradient represents the sound level given a frequency and a time (the darker the
952 colour, the louder the frequency). Movie S1 gives the associated raw image and sound
953 recording. See Fig. S2 for examples of responses to the 4 types of sound stimulus.

954 **(B)** Same as (A) but without spectrogram and for all-male responses to the loudest 2-
955 harmonic 1-female sound-stimulus. Darkest coloured lines represent running median,
956 darkest areas represent second and third quartiles and light areas represent the 90th

957 percentile of data. The sample size of the distribution of flight coordinates and velocities
958 corresponds to the number of male flight tracks (n=104), and that of the WBF distribution
959 corresponds to the number of swarms (n=61) where mean WBFs over the number of
960 mosquitoes per swarm were calculated (1 to 6 males per swarm). Linear and angular
961 speed, and wingbeat frequency clearly increased in response to the onset of this sound
962 stimulus, plus there was a slight tendency to increase in flight height (Y (m)).

963 **(C)** Probability distribution of distance between a male and the speaker during sound
964 stimulus playback for all stimuli; distances ranged between 0.9 ± 0.2 m. This distance
965 interval was used to estimate the uncertainties of the acoustic prediction in Table 1. The
966 sample size of the distribution of distances corresponds to the number of male flight tracks
967 (n=104).

968
969 **Fig. 5. Results of behavioural experiment.** Top and bottom rows show the increase in
970 upper-quartile angular-speed and wingbeat frequency, respectively, when playing-back a
971 given sound stimulus. Black dotted lines represent the absence of change in parameters
972 before and during the stimuli. Each sample is the average of several measurements on the
973 same day. Each sample corresponds to a different group of mosquitoes (consisting of 1 to
974 6 in each sample). See Method Section ‘Statistics’ and Results Sections for statistical tests.
975 **(A)** Male *An. coluzzii* responses to 1- or 2-harmonic sounds of a single female (data subset
976 A, n=10 in each boxplot). Boxplots of the parameters show the median, 2nd and 3rd
977 quartiles. Outliers shown as diamond shapes are outside the interval $[Q1 - 1.5 * IQD, Q3$
978 $+ 1.5 * IQD]$ which is represented by whiskers (Q1 = first quartile; Q3 = third quartile and
979 IQD = interquartile distance). Disk and error bars in each distribution show mean and
980 standard error.

981 **(B)** Male *An. coluzzii* responses to 1-harmonic 1-female sound or to single-frequency
 982 sound (data subset B, n=10 in each boxplot). Boxplots, disk and error bars have the same
 983 meaning as in (A).
 984 **(C)** Male *An. coluzzii* responses to 2-harmonic sounds of single or 30 females along SVLs
 985 (data subset C, n=9 for the quietest 1-female stimulus and the two loudest 30-female
 986 stimuli, n=10 for other stimuli). Continuous lines and associated coloured areas represent
 987 the mean and 95%-CI. SVL were corrected as explained in Method section ‘Corrected
 988 SPLs for estimating the hearing threshold’. The green dotted lines represent the lowest
 989 estimate of the hearing threshold from the response to 1-female 2-harmonic sound-stimuli.

990

991

992 **Table**

Subset	Single/ Group	Number of harmonics	Recording/ Synthetic	Sound level (dB SPL)				
				SPL measurement of the two 1/3-octave bands closest to the first-harmonic 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	
	Group (~30)				44.2	36.2	± 0.6	± 2
					17.1		± 0.5	± 2
					23.1		± 0.4	± 2
					32.9		± 0.5	± 2
				44.9		± 0.5	± 2	

993

994 **Table 1. Description of stimulus sound-levels.** This table gives the sound pressure levels
995 (SPL ref 20 μ Pa) and associated errors of all played-back sound stimuli at the male's mean
996 location in the frequency range of the female's first harmonic. See Methods 'Corrected
997 SPLs for estimating the hearing threshold' for the corrected SPL mean value and Methods
998 section 'Estimate of SPL errors at mosquito's location' for last two columns. SPLs are
999 equal to SVLs in our setup (see Method section 'Monitoring SVL from SPL
1000 measurements). For frequency characteristics, see Fig. 2 and Table S1.

Sound-proof room walls

Visual marker

Camera's angle of view

Mosquito net

Camera

Microphone

Speaker

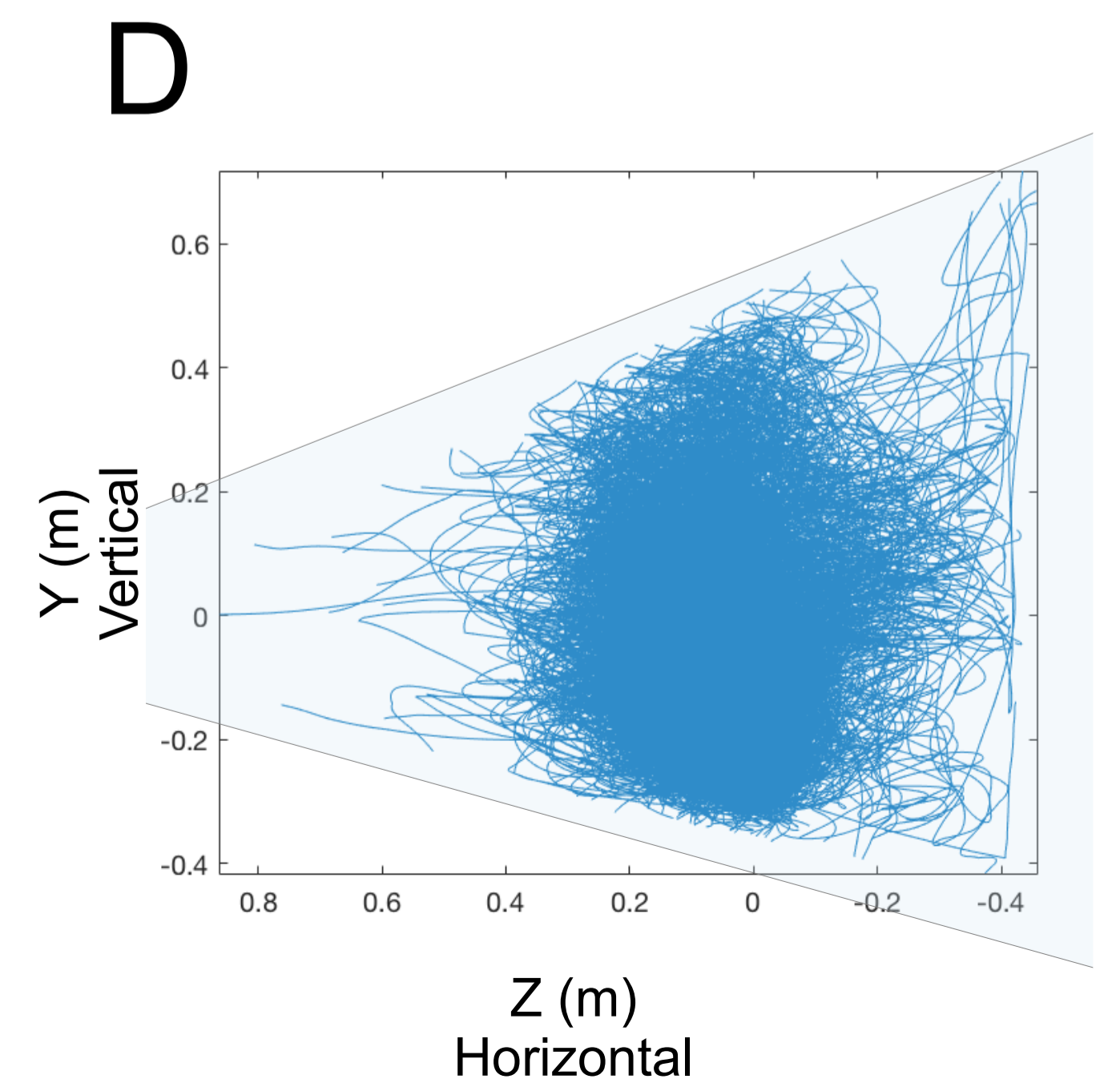
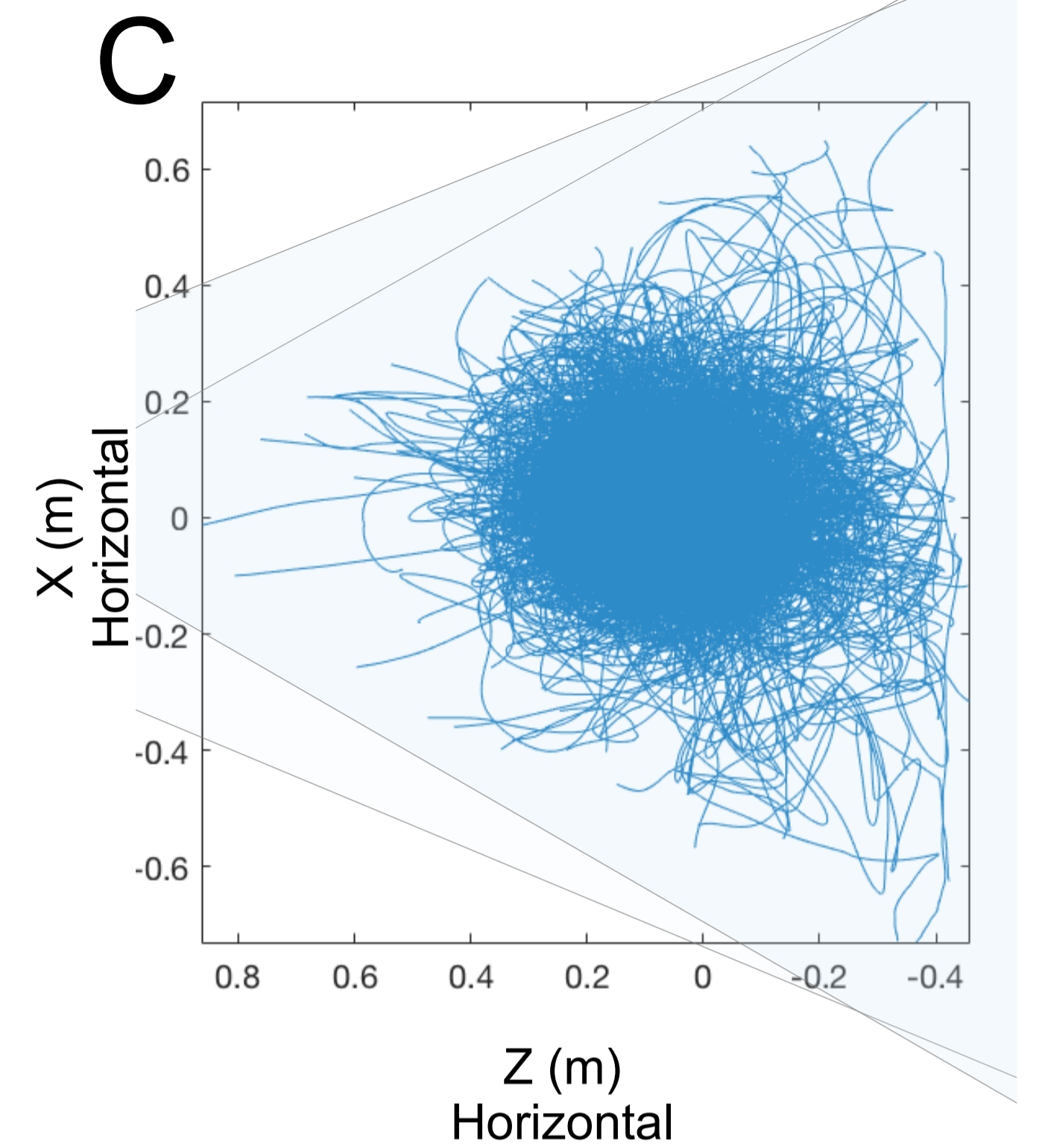
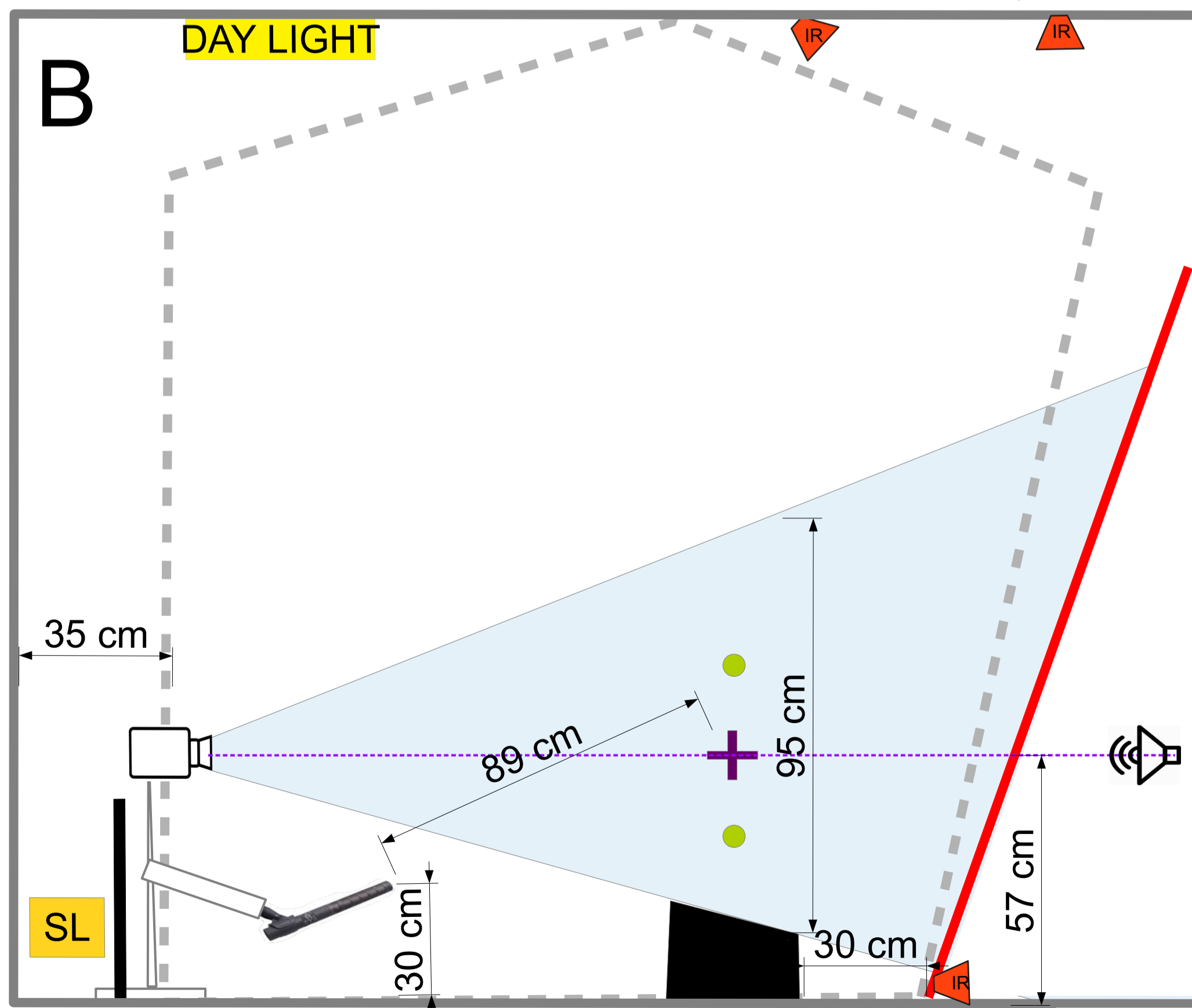
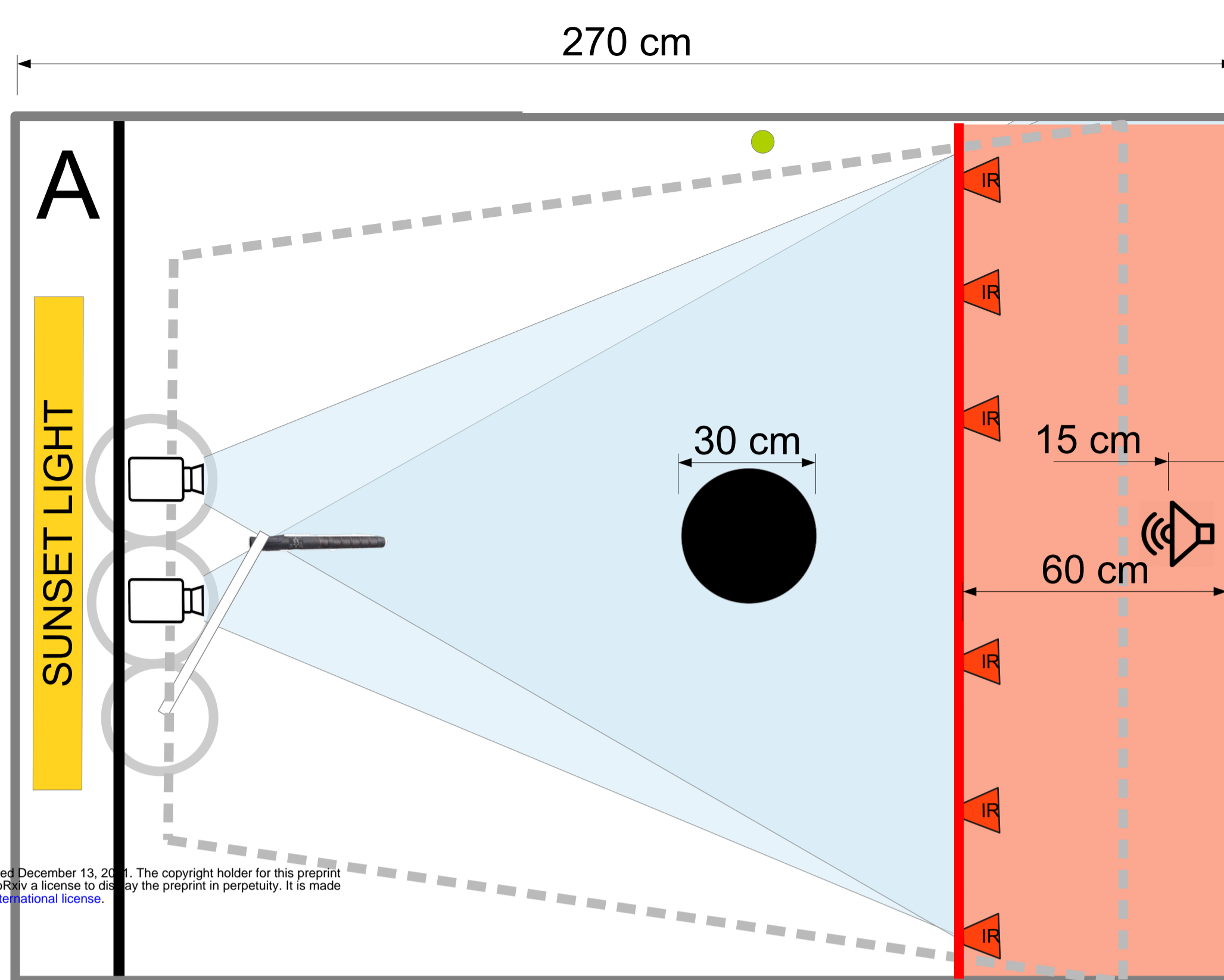
Horizon line

IR lights

IR-illuminated backwall

Expected center position of the swarm

Thermocouples



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