Mosquito 'mate-seeking' at long-range: are male swarms loud enough to be located by females?

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SUMMARY

The high-pitched whine of mosquitoes in flight is produced by their wingbeats, and is heard by conspecifics, who have unsurpassed sound sensitivity among arthropods. We investigated whether female mosquitoes might use the sound of a mating swarm at long-range (several meters) to identify species-specific cues. In the laboratory we exposed free-flying *An. coluzzii* females to pre-recorded male *An. coluzzii* and *An. gambiae s.s.* swarms to assess female response to male flight sounds over a range of ecologically-relevant sound-levels, based on our reference recording (70-male swarm producing 20 dB SPL 0.9m away). Sound-levels tested were related to equivalent distances between the female and the male swarm for a given number of males, enabling us to infer distances over

which females can hear large male swarms. Females did not respond to swarm sounds at 36±3 dB, but their flight speed increased significantly at 48±3 dB, equivalent to a distance of 0.6±0.2 m from a point-source swarm-sound produced by 1,000 males. However, this distance is less than the 1,000-male swarm radius. We show that even for the loudest swarms of 10,000 males, a female will hear an individual male at the edge of the swarm sooner or more loudly than the swarm as a whole, due to the exponential increase of sound at close-range. Therefore, females highly unlikely cannot use swarm sound to locate swarms at long-range. We conclude that mosquito acoustic communication is restricted to close-range dyad interactions.

KEYWORDS

Anopheles, bioacoustics, free-flying mosquitoes, insect hearing, long-range hearing, mating swarm, mosquito hearing, mosquito sound, speciation

INTRODUCTION

Mosquito hearing

There is strong evidence that mosquitoes detect individuals of the opposite sex in one-on-one acoustic interactions. The hearing organs of males and females are tuned to ‘difference tones’ derived from the combined wingbeat frequencies of both sexes, discernible indirectly by convergence patterns in the wingbeat harmonics of male and uninseminated female mosq,uitoes. This acoustic behaviour has been documented in four species of medical importance (*Anopheles gambiae s.l.*, *Anopheles albimanus*, *Aedes aegypti* and *Culex quinquefasciatus*), plus *Toxorhynchites brevipalpis* and *Culex pipiens* [1-10] as well as in other flies [11].

Mosquito antennae are sound particle-velocity sensors [12-14]. Particle-velocity amplitude increases faster than pressure amplitude during the approach to a sound source [15]. Accordingly, particle-velocity sensors have an advantage over pressure sensors at close range, and, therefore, mosquito hearing has been considered to be a relatively short-range sensory system. It is assumed that the hearing distance between a male and a female is limited to a range of a few cm to ~ 10 cm [16,17]. However, there is no physical reason to consider a particle-velocity sensor to be limited to a given hearing distance, since particle velocity is an intrinsic property of any sound, irrespective of the distance between the receiver and the sound source [15,18,19]. Indeed, males have been shown to respond to artificially loud sound levels of played-back female flight tones metres away from the sound source [19]. Thus, to test mosquito hearing at long-range, the following question was addressed: is there a natural sound source in the field loud enough to be heard by mosquitoes from significant distances?

Are male swarms loud enough to be heard by females at long-range?

In mosquitoes, ecologically-relevant sound-sources can be classified by their potential function: 1) predator avoidance, 2) detection of moving prey, and 3) communication [20]. At long-range, only detection of moving prey has been investigated in the case of frog-biting flies, such as the mosquito *Culex territans* and the midge subgenus *Corethrella Coquillett* females which fly toward sound sources of played-back frog calls [21,22].

Research on mosquito acoustic communication has been done mostly with pairs of tethered mosquitoes exposed to the sound of each other over distances of a few cm [1-6,8,9,23-25]. An individual mosquito is a relatively weak sound source. These studies do not address the question of whether mosquitoes can locate conspecific males in a mating swarm from a distance. Indeed, swarms are the only ecologically-relevant sound-sources that are significantly louder than a single mosquito and could be related to acoustic

communication. Males aggregate over swarm markers at species-specific times of day [26], and uninseminated females also display swarming behaviour [25,27-29]. However, the number of males typically increases quickly during the formation of a swarm, which raises the hypothesis that a female can be attracted from a distance to species-specific swarm sounds produced by males in established swarms. If this were the case, we might expect a single female to hear the significantly louder sound of many males swarming from a greater distance than from a single male, i.e. the larger the male swarm, the further away the female might detect the swarm. Males are not expected to hear male swarm as well as females because mosquito hearing organs are designed to detect opposite-sex sounds [3].

A swarm can consist of thousands of individuals [31-33], establishing relatively dense station-keeping aggregations [34]. Attraction of females to the sound of distant male swarms has been mentioned in the literature [35] and studies have explored the potential for using distant mosquito sound as a tool for detection and surveillance of mosquito populations [36], but to our knowledge this hypothesis has not been tested quantitatively in the context of intra-mosquito comunication. When exposed to opposite-sex sound, electrophysiological studies show that females are less sensitive to sound than males [1,17,19], however, female hearing sensitivity is similar to the male's one when their own wingbeat are simulated in addition to the oposite-sex sound, revealing that free-flying females may be as sensitive to sound as males [20]. Although females have not been shown to move toward a source of male sound (phonotaxis), they do alter their wingbeat frequency when exposed to male sound [1,3,38-40].

Potential importance of distant swarm sound in on-going speciation?

Subtle differences in swarming behaviour between closely related species minimize hybridisation within the *An. gambiae s.l.* species complex (e.g., *An. coluzzii* and *An. gambiae s.s.)* [41,42]. Female auditory detection of a con-specific swarm of males at long

range could increase the female's likelihood of locating and being inseminated by a male of the same species. A female might recognize a species-specific sound signature at long-range before males of any other species could hear, chase and mate with her. Inconsistent results on species-specific acoustic cues in *An. coluzzii* and *An. gambiae s.s*. have been reported based on studies of single male or dyad interactions.

Laboratory-based research characterising the flight tones of single males flying in small mosquito cages found no significant differences between the fundamental frequencies of *An. coluzzii* and *An. gambiae s.s.*; however, significant differences were found in the second harmonic amplitude [43]. Another study of the patterns of flight tone interactions between a tethered male and a tethered female of closely related species of *An. gambiae s.l.* found frequency-matching occurred more consistently within pairs of the same molecular form than in hetero-specific pairs [4]. However, in a separate study, a type of acoustic interaction associated with mating (rapid wingbeat frequency modulations) was elicited by males when they were close to a female [6,8,9], but rapid-frequency modulations in males of both *An coluzzii* and *An. gambiae s.s.* were similar when exposed to pure tones mimicking the female's fundamental wingbeat frequency [44].

An important lacuna in the literature remains regarding the more realistic scenario of a single female detecting an entire swarm of males. Thus, in the present study we investigated the possibility that females hear male swarms from a distance by presenting sound recordings of swarming males to free-flying uninseminated females. Results directly address the question; do females detect male aggregations in the field?

Experiment design

Our hypothesis is that *An. coluzzii* female mosquitoes can detect distant sounds of swarming males at natural sound levels. We chose to work with the mosquito species *An. coluzzii* because a) the male swarming behaviour is well known to be confined to stereo-

typical looping flight within a limited area over a stationary visual marker, b) males naturally gather in the thousands, forming relatively dense station-keeping swarms [31,33], and c) less well-known, uninseminated females also swarm [25,27-29], which is rarely observed in the field for several reasons: the ratio of females:males is extremely low (females generally mate once in a lifetime), swarming males are quick to chase a female and mate, and once insemination begins females leave the swarm [45]. Thus the mating behavior of this species is relatively better-known, and the potential for positive phonotaxis from females is high.

We recorded ambient sound in the field near naturally swarming *An. coluzzii* males to determine whether any other animal or environmental sounds were present that could hide/mask swarm sounds. Next, we conducted behavioural experiments in an environmentally controlled laboratory fitted with a soundproof chamber to isolate the behavioural set-up from extraneous sounds. Experiments were conducted with mosquitoes reared at the University of Greenwich from colonies of *An. coluzzii* and *An. gambiae s.s.* provided by IRSS.

Audio-video recording instruments were used for two purposes (Figure 1): first, to record sound of large station-keeping swarms of males of the two closely related species (Sound S1, Sound S2) and second to record the behaviour of females exposed to these playback swarm sounds (Video S1). Free-flying uninseminated females were released in a swarming arena (L x W x H = 1.8 m x 1.7 m x 2 m) that provided the visual cues to initiate swarming (figure-of-eight loops) over a visual marker, effectively confining them to a volume of 0.06 m^3 and within a fixed distance of 0.9±0.2 m from the source of male sound (Figure 2A). The physical distance between a female and the male sound-source image was simulated by adjusting the sound level of each of the sound stimuli played-back on the speaker (Figure 3). The measure of a ‘response’ in the female’s behaviour was defined as a change in flight (including phono-taxis) or wingbeat characteristics when

exposed to each of the four levels of intensity of sound stimuli playbacks, including a reference sound recording corresponding to a 70-male swarm at an equivalent distance of 0.9 m.

We used two criteria to determine whether or not a female can hear the sound stimulus: a change in wingbeat frequency and/or a change in flight speed. In principle, the significant change in her wingbeat frequency increases the strength of the input to the nervous system, thereby increasing her ability to hear and locate the male [3,11]. A change in flight speed which indicates a change in flight trajectory (uninseminated females are observed to fly toward male swarms [9,24,45], but we don't know whether it is due to the swarm sound). Finally, the measured results were extrapolated to estimate how far away a female mosquito can hear a swarm of a given number of males. Figure 3 summarises the experimental protocol and the raw results.

Our hypothesis, that female *Anopheles* mosquitoes can hear male swarms from large distances, was not supported. We show that although females do respond to the sound of a male swarm, the sound levels of swarms over distances of metres are too low to be heard by females. Some uncertainties are still present for the largest swarms, but our results indicate that it is unlikely a female can hear a swarm before coming into close proximity of a male located on the swarm's periphery.

RESULTS

Field recordings show salient swarm-sounds at least up to 3 m from the swarm

Relative flight-sound intensities of wild male *An. coluzzii* swarms were measured to characterise the sound profile of typical male swarms in relation to the background sounds of other twilight-active organisms, including humans, near rice fields in village VK5, Burkina Faso. We recorded ambient sound at ~ 1 m from a swarm consisting of several

thousand male *An. coluzzii* around sunset. The recording included background noises from insects, birds, mammals, human speech, children crying, sunset call to prayer, and motor vehicles. The loudest sounds were produced by insects and mammals, but at frequency bandwidths that did not coincide with the swarm's first harmonic. The sound of mosquito swarms was the only continuous sound in the 100-1000 Hz frequency band (see spectrograms in Figure S1).

In addition to these preliminary field recordings at 1 m from the swarm, we found that the first harmonic amplitude of the sound pressure was 10% higher than the background noise (50-Hz smoothed magnitude spectrum), irrespective of which side of the swarm was recorded, i.e. from ground level to the top of a ~3 m-high swarm, and horizontally, on two opposing sides of the swarm at ~3 m from the centre of the swarm. This indicates that the signal-to-noise ratio of the swarm sound can potentially be loud enough to be heard by females at least ~3 m away from the centre of the swarm.

Typical sound level of a 70-male swarm and species-specific cues

In the soundproof chamber with semi-absorbent walls (reverberation time of 0.05 s in the first-harmonic frequency band), the first-harmonic sound pressure level ('SPL': root-mean-square SPL ref 20 μPa) of a station-keeping swarm of ~70 male *An. coluzzii* was 20±3 dB at a distance of 0.9 m from the microphone to the swarm centre, which was 0.6 m high (see Figure 1).

The sound of a swarm is composed of the flight sound of individual males. As they probably cannot synchronize the phase of their wingbeats and since the sound of a swarm from a distance is relatively steady over time, the only species-specific sound cues of a swarm, if any, would come from the frequency content (i.e. not from specific sound phases or time-changing patterns). Sound S1 and Sound S2 are the male sound stimuli used for playback for each of the two species, respectively (before any filtering, see Figure S2).

Figure S2C shows the great similarity between the sound spectra of the swarm stimuli of the two species, *An. coluzzii* and *An. gambiae s.s*: the relative second and third harmonic amplitudes were the same; the fourth-harmonic amplitudes differed, but their respective frequencies were both far above mosquito audibility [3]; the mean swarm wingbeat frequencies differed slightly by 21 Hz (857 Hz for *An. coluzzii* and 836 Hz for *An. gambiae s.s.)*, but with a large overlap of 47 Hz of the harmonic peak bandwidth at -3 dB. Note that the 30-male *An. gambiae* swarm sound-level was increased to be the same as that of 70-male *An. coluzzii* swarm, as shown in Table 1, by using the *An. coluzzii* first-harmonic amplitude as a normalisation factor (see STAR*Methods section 'Sound stimuli').

How loud must a swarm be for a female to hear it and is is species-specific?

We played-back the sound of male swarms to a group of 1-5 swarming *An. coluzzii* females (Figure S3) at four different sound levels (Table 1) and we tested whether the females responded to the sound stimulus by changing their wingbeat frequency or flight trajectory dynamics (n=10 to 12 replicates per sound level, depending on the sound stimulus). The playback speaker was placed at a constant distance of 0.9 m from the female(s), which swarmed at an oscillating distance of ±0.2 m to the speaker (Figure 2A). The reciprocal was done with 1-6 swarming males exposed to the sound of swarming females, as a control (n=9 to 10 replicates, depending on the sound stimulus). Sound S3 and Sound S4 are the female-swarm sounds of the two species, respectively (before any filtering, see Figure S2).

Figure 2B shows the distribution of positions (in three dimensions), linear speed, angular speed and mean wingbeat frequencies produced by groups of 1-5 females or 1-6 males, before, during and after exposure to the loudest opposite-sex sound stimuli (48±3 dB SPL). For each replicate and for each stimulus sound level, we measured the

difference between the maximum wingbeat frequency reached during the 7 s sound stimulus and during the 7 s before the sound stimulus. We did the same for linear speed.

Our results (Figure 4A) show that free-flying females respond to the sound stimuli by changing their linear flight speed with respect to SPL (LRT, χ_1^2=4.3, p=0.037), and that the 48 dB SPL distribution was significantly different from the intercept (one-sample $t(22)$=3.58, BH-corrected p=0.0067, mean=4 cm/s) showing a 4 cm/s increase in the maximum linear speed reached during the sound stimulus (mean speed without sound stimulus: 44 cm/s). There was no significant effect on the wingbeat frequency parameter (LRT, χ_1^2=0.46, p=0.50) and there was no effect of species or an interaction effect between species and SPL for females exposed to male sound stimuli, as expected by the absence of significant differences in the swarm sound of the two species (see previous Results section).

Males were exposed to swarming female sounds as a control, because males are known to be more responsive to sound than females [37]. Our experimental protocol demonstrates that the reciprocal test of male response to female sound stimuli resulted in a highly significant response (Figure 4B). Indeed, for males, the effect of SPL on the maximum WBF difference was highly significant (LRT, χ_1^2=18.8, p<0.001), and the 45dB distribution was highly significantly-different from the intercept (one-sample $t(17)$=5.45, p<0.001, mean=36 Hz for a mean wingbeat frequency of 803 Hz before the sound stimulus). However, there was no overall effect of the SPL on maximum linear speed (non-significant LRT χ^2, but the 36 dB distribution shows a significant difference with the intercept ($t(17)$=3.64, BH-corrected p=0.008, mean=11 cm/s)).

Given the weak statistical significance in the female response to male sound (LRT χ^2 p=0.037 and t-test p=0.0067, see paragraph above), we decided to increase the number of tested parameters to be certain we did not miss any meaningful variables. Table S1 gives extra eight parameters tested (acoustic & flight parameters). Holm

correction of *p*-values for multiple comparisons led to no statistically significant predictors of female response.

Overall, the results support the proposition that a female can hear male-swarm sound stimuli at 48 dB SPL, but not at 36 dB. At 48 dB SPL, the effect is statistically significant but small. This suggests that the hearing threshold for females is closer to 48 dB SPL than to 36 dB SPL.

How far away can a female hear a 70-male swarm?

Neither the sound level of the 20±3 dB SPL stimulus, corresponding to the natural sound level of a 70-male swarm located 0.9 m away from the female, nor the second highest (36±3 dB SPL) level elicited a behavioural response from females in the laboratory assay. However, at the loudest sound level of 48±3 dB SPL, females heard the male swarm sounds. To test how far away a female can hear, we calculated the equivalent distance corresponding to the sound of a 70-male swarm at 36±3 or 48±3 dB SPL (see STAR*METHODS, sections ‘Experiment paradigm’ and ‘Formulae between sound level and distance’).

Accordingly, for a 70-male swarm stimulus modelled as a point source, the female’s hearing distance threshold ranged between 4±1 cm and 15±3 cm (Table 2; see STAR*METHODS, section ‘RMS particle-velocity estimation’ for discussion related to reproducing a sound-source outside the far-field range; at a distance of 15 cm from this sound-source, particle-velocity level and SPL are equal within 1 dB). If we consider the swarm radius of a 70-male swarm to be 0.2 m, as in the laboratory swarm we recorded, then a single female flying close to such a swarm will hear the male nearest the female before she would be able to hear the swarm as a whole. Indeed, the short distance between the female and the first male encountered at the edge of the swarm will produce sound that is louder than that of the rest of the swarm as a whole, because of the rapid

increase of particle velocity in the vicinity of a mosquito. Therefore, we conclude that a female cannot hear a 70-male swarm until she is within its boundary.

How far away can a female hear a male swarm of a given number of males?

From the conclusions above, it follows that a female can hear a 70-male swarm operating as a point sound-source (see STAR*METHODS, section 'Far-field critical distance for particle velocity') from a distance of 4±1 cm away. Based on this result, we estimated how far a swarm composed of more males can be heard by a female, based on acoustic prediction formulae (see STAR*METHODS, section 'Formula between hearing distance and number of individuals in the swarm'). Figure 5 shows the female hearing ranges as a function of distance to the swarm and number of males in the swarm. The 36 dB SPL (no-response) and the 48 dB SPL (response) allows us to split the 2-D plot into three areas: the 'no-response' area (red); the 'response' area (green); and the 'hearing threshold' area (white). The hearing distance threshold stands somewhere in the white area, but as we saw in a previous section, the hearing distance threshold is expected to be closer to the green area than to the red area.

For illustration, a swarm of 1,000 males can be expected to be heard by a female at a distance of 0.15±0.07 m, and would certainly not be able to be heard at a distance of 0.60±0.25 m. Based on the same acoustic prediction, we can extrapolate that a 6,000-male swarm would be heard at a maximum distance ranging from 0.4±0.1 m to 1.4±0.5 m, and from 0.5±0.2 m to 1.8±0.7 m for a 10,000-male swarm. Table 2 incorporates all the acoustic values related to the hearing or non-hearing of five orders of magnitude in the number of males.

DISCUSSION

311 **Hearing sensitivity of *An. coluzzii* females and males**

312 Previous studies estimated the hearing threshold of tethered *An. gambiae s.l.* females was
313 in the range 44-52 dB (particle velocity of 14±6 μm.s^{-1}, *n*=5) and tethered *Aedes aegypti*
314 females around 55 dB SPL (*n*=10) by monitoring the activity of the Johnston's organ nerve
315 [4,19]. In the present study, the sound level eliciting a behavioural response in free-flying
316 *An. coluzzii* females was 48±3 dB, with no response at 36±3 dB SPL. For free-flying *An.*
317 *coluzzii* males, we found a significant response to 45±3 dB SPL, and a non-significant
318 tendency at 33±3 dB, indicating that their hearing thresholds are likely to be < 45±3 dB for
319 males. This is similar to reported values for tethered male *An. gambiae s.l.* (18±6 μm.s^{-1},
320 i.e. 38-39 dB SPL for the SD range in the far-field, *n*=5) from recording the Johnston's
321 organ nerve with the antenna fibrillae extended [4], and for tethered male *Culex pipiens*
322 *pipiens* (32.0±4.4 dB sound particle-velocity level, n=74, equivalent to 32.0±4.4 dB SPL in
323 the far-field) [46]. Overall, the results are in general agreement with previous
324 electrophysiological studies.

325 Our study is the first report of sound sensitivity through behavioural responses in
326 free-flying mosquitoes. We expected higher sensitivity compared with the
327 electophysiological studies referred to above, since mosquitoes exhibit active hearing
328 [7,47] which could be triggered only by using appropriate behaviours (e.g. not tethered,
329 looking for males in the case of females). A possible reason in the case of males is that
330 the sound stimuli were not strictly natural; we played-back the sound of a large group of
331 swarming females (i.e. wide band tone) to test male sensitivity, which does not occur in the
332 field. Accordingly, we still expect a greater sensitivity for free-flying males exposed to
333 single-female sound (i.e. sharp-band tone corresponding to the sound of a single female),
334 as noted previously [16].

335

Number of males in swarms

In order to predict the sound level of swarms that have more males than the ones that we established under laboratory conditions, we need to know the range of number of males in field swarms. Few studies have investigated the range in numbers of males in mosquito swarms; in Benin, *An. coluzzii* male swarms were typically composed of tens to thousands of males, with a median of ~ 300 males [31], and in the area of our field study, single sweep-net samples of *An. coluzzii* swarms caught a median of 200 males and a quarter of the samples contained 500–2,500 males [48], indicating the likelihood that there are far more males in the swarm than these estimates, as many as 10,000 males in a swarm (pers. com. Diabaté). We have observed that larger swarms occur in areas and times of year with higher population density; i.e. in an irrigated rice growing area during the wet season swarms are numerically and spatially large, while in non-irrigated areas during drier periods, swarms are regularly composed of 20-30 individuals at their peak [34].

The 70-male swarm used for the laboratory assay is, therefore, realistic, but relatively small compared to the variation observed in the field, and the hearing range prediction based on a 300-male swarm may be considered a typical case. Figure 5 shows that a 300-male swarm cannot be heard by females even at a distance of 1 m. The same is true for a 1,500-male swarm; we predicted no response from females at 0.7±0.2 m and likely up to 0.2±0.1 m.

For the very largest swarms, the hearing distance threshold is greater than the radius of the swarm elicited under laboratory condition. Since thousands of males in a swarm is possible, it is useful to consider the relationship between the number of males in a swarm and its dimension. For these large swarms, are their dimension altered by size? and does their radius exceed the maximum hearing distance?

Swarm radius as a function of number of males

The acoustic prediction results (Figure 5) show hearing range as a function of the number of males in the swarm. A swarm composed of more mosquitoes will produce a higher sound level, and so the distance at which it is audible will increase, accordingly. However, this relationship only has a meaningful real-world impact on swarm localization if the audible distance increases faster than the swarm radius. If radius increases faster than the distance at which the aggregation is detectable, a female is likely to hear an individual male swarming at the edge of the swarm sooner or more loudly than the swarm as a whole, because particle velocity increases rapidly at close-range of an individual mosquito. For this reason, information on how a swarm radius changes with the number of males is important for the interpretation of our results.

Several studies have investigated qualities of mosquito swarms e.g., the relationship between the marker size and swarm dimension [25,29,45], between the number of males and the marker size [29] or the marker type [30]. In one of our previous studies the relationship between the number of males and the swarm dimension, given a visual marker, was quantitatively measured [34]. *Anopheles gambiae s.l.* swarms composed of 10 to 50 males in Mali were observed to conform to a bell-shaped distribution of male density over the swarm centre, with a rapid decrease in the number of individuals with distance to the swarm centroid (20% of the swarm's individuals were within a radius of 20 cm of the centre, ~70-90% within 40 cm, 98% within 1 m). Thus, the first effect of increasing the number of males in the swarm was to increase male density in the swarm centre and not throughout the entire volume of the swarm.

Figure S4 uses the data of five swarms of *An. coluzzii* and seven swarms of *An. gambiae s.s.* from [34] to predict swarm radius as a function of the total number of males and of two 'layers' of a swarm (50% most centred or 95% most centred males), with a random intercept and slope model to predict the swarm radius of bigger swarms up to the

order of thousands. We consider here the swarm radius as defined by the sphere which centre is the swarm centroid and which encompasses 95% of the males nearest this point. The results on *An. coluzzii* are consistent with observations of swarms with thousands of males as being usually < 1 m in radius [30]. For *An. coluzzii*, the predicted mean radius is 0.5±0.1 m for 95% of 1,000 swarming males (0.20±0.05 m for 50% of them) and 0.6±0.1 m for 95% of 10,000 males (0.21±0.05 m for 50% of them), representing a steep increase in density of swarming males, especially in the swarm centre (Figure S4).The swarm radius of an *An. gambiae s.s.* swarm is slightly larger for small swarms, but the predicted radius for large swarms is much larger (Figure S4).

In Figure 5, the 95%-male swarm radius of both species are superimposed over the hearing ranges of females as a function of the number of males in a swarm. To be heard at long-range, by definition, a female should be outside the swarm, i.e. the white area above the two swarm-radius lines is the only possible 'hearing-area', which is relatively small for *An. coluzzii* and absent for *An. gambiae*. However, since the hearing threshold is expected to be closer to the green area than to the red area (see Result section), it is unlikely that a female can hear a swarm before she hears a male located on the swarm edge, even for dense swarms with high numbers of males. The prediction has to be taken with caution for the greatest number of males.

Long-range hearing does not contribute to conspecific mating

First, species-specific cues of swarm sound were found to be very weak (Figure S2). Second, our behavioural assay did not show any species-specific responses in *An. coluzzii* females to the swarming sound of *An. coluzzii* or *An. gambiae s.s.* males. Third, following the conclusion of the previous section, we can reject the idea that females use the sound emanating from a swarm to determine whether to avoid entering the swarm of the wrong species, or to join the swarm of the same species, because the female will not hear the

swarm before she comes into close proximity of numerous males at the periphery of the swarm.

Swarm localization by females is much more likely to be due to responding in the same way to environmental cues as their male counterparts, thereby enhancing the likelihood of encountering con-specific males. It is also possible that encounters are partly or entirely random, especially when swarms are numerous [26]. On the other hand, females may use the close-range sound of a chasing male to avoid being inseminated by the wrong species [4], however, we can eliminate the possibility that long-range hearing cues ensure assortative mating in *An. gambiae s.l.* and focus on other cues such as vision [29] or olfaction [49,50] in future research.

Long-range hearing in mosquito communication

This study presents data and analyses that reject the hypothesis that long range interspecific acoustic communication in both sexes of *Anopheles* mosquitoes occurs before mating and insemination. Indeed, to our knowledge, male swarms are the only serious candidate source of sound, for inter-mosquito acoustic communication at long-range, which is loud enough and which can fit the tuning of the mosquito organs. Although males are more sensitive to sound than females [17], they are less likely to respond to male swarm sound because their hearing organ is not tuned to male wingbeat frequencies, unlike females.

This study does not eliminate the hypothesis that long-range hearing can be used for host location [22,10] or for predator avoidance [20], providing the prey/predator sound is loud enough and tuned to mosquito hearing.

Limitations of the experimental design

The main limit of our experimental paradigm is that we used swarming females to test their response to male-swarm sound (see STAR*Methods, section 'Experiment paradigm'). This means that when females were exposed to the swarm sound, they were above a swarm marker, while in the field they would have been on their way to the marker where the males swarm. This may have induced females to continue swarming over the marker without altering their behaviour when male sound was played-back, effectively waiting for males to approach the marker. However, we monitored a large number of variables (flight velocities, positions and wingbeat frequency changes), so it is unlikely that we overlooked any female reaction to sound and unlikely that females would not respond if they could hear a male sound.

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AUTHOR CONTRIBUTIONS

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DECLARATION OF INTERESTS

The authors declare no conflict of interest.

FIGURE LEGENDS

Figure 1. Soundproof chamber setup for recording sound and video of mosquito behaviour

Bird's-eye and side views of soundproof chamber. Two IR-sensitive cameras fitted with IR pass filters tracked flying mosquitoes as black silhouettes against evenly lit IR-background. Separate lighting system provided gradual semi-natural dusk visible to mosquitoes, consisting of dispersed dim white lights on ceiling and 'sunset' lighting below horizon (opaque wall ~40 cm tall). A microphone recorded flight sounds of mosquitoes swarming directly above black swarm marker. A thermocouple (85 cm above ground level) recorded temperature at ~ mean swarm height. Differences between setups for the two species was necessary to accommodate species-specific differences in positioning of swarming flight in relation to swarm marker [29].

(A) Setup to record sound and flight of *Anopheles coluzzii,* for sound stimulus recording and behavioural experiment. A speaker located behind IR-illuminated thin-cotton sheet, outside net enclosure played back sound stimuli.

(B) Setup to record sound of *Anopheles gambiae s.s.,* for sound stimulus recording only.

Figure 2. Flight and sound responses of females and males to sound stimuli

Female (red) and male (blue) flight-characteristics and wingbeat-frequencies before, during and after playback of male (blue rectangle) or female (red rectangle) sound stimuli.

(A) Probability distribution of distance between a female and the speaker during sound stimulus playback; 95% of distances were between 72 cm and 113 cm, with a mean and median of 94 cm. This distance interval was used to estimate the uncertainties of the acoustic prediction in Table 2 and Figure 5.

(B) *An. coluzzii* response to highest sound-level *An. coluzzii* and *An. gambiae* sound-stimulus over 27 s of experiment. Stimulus was played-back 10 s from beginning of flight recording and lasted 7 s (red or blue rectangular shading). First five rows show flight parameters (relative 'XYZ' position, plus linear and angular flight speeds). 'Z' dimension represents relative distance to the speaker (located 0.9 m from Z=0). Last row shows mean wingbeat frequency (WBF) of 1st harmonic. Darkest coloured lines represent running median, darkest areas represent second and third quartiles and light areas represent the 90th percentile of data. Distribution of flight coordinates and velocities were recorded for 149 female tracks and 104 male tracks, and the WBF distribution plot is based on mean WBFs over the number of mosquitoes per fly group (100 female-groups and 61 male-groups). No clear apparent response was observed in females, whereas for males, linear and angular speed and wingbeat frequency clearly increased in response to the sound stimulus onset, plus a slight tendency to increase the flight height was evident.

(C) Same as B (with the exception of the spectrogram), but with a single example per plot. First row shows spectrograms 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). Spectrogram in the first column displays a live *An. coluzzii* female exposed to *An. coluzzii* male sound between 10th and 17th s (Video S1), while the spectrogram in the second column displays a live *An. coluzzii* male exposed to the two first-harmonics of the *An. gambiae* female sound (Video S2). Periodic flight pattern, typical of swarming behaviour, is evident for males and females in 'XYZ' plots.

514 **Figure 3. Steps to evaluate the distance a female mosquito can detect the sound of**
515 **an *An. coluzzii* male swarm of a given number of individuals.**
516 This schematic explanation shows how methodologies from behavioural assay
517 ('measurements') and acoustic theory ('predictions') were employed in this study (case of
518 *An. coluzzii* sound stimuli). The same procedure was repeated with sound stimuli of *An.*
519 *gambiae s.s.* and the reciprocal experiment was performed with males exposed to sound
520 stimuli of a female-swarm for both species.
521 (A) First, the reference stimulus (sound of 70 males swarming) was recorded at 0.9 m from
522 the male swarm, producing a sound pressure level of 20 dB SPL.
523 (B) Second, this stimulus was played-back to 1-5 station-keeping free-flying female(s) at
524 four different sound levels (20, 25, 36 and 48 dB SPL) as measured at the mean females'
525 distance to the speaker (see Figure S3). Only the loudest stimulus produced a response in
526 females.
527 (C) Third, assuming the swarm sound emitted from the speaker to be a point source, and
528 given the natural sound level of a 70-male swarm (L_M) at a distance of 0.9 m (r_{ref}), we can
529 compute the natural distance to a similar swarm corresponding to the other three sound
530 levels (see STAR*Methods section 'Formulae between sound level and distance').
531 (D) Fouth, the effect of multiplying the number of swarming males per N over the female
532 hearing distance is predicted (see STAR*Methods section 'Formula relating hearing
533 distance and number of individuals in the swarm').

534

535 **Figure 4. Results of behavioural experiment**
536 One flight parameter (maximum linear speed difference, 1st subplot row) and one acoustic
537 parameter (maximum wingbeat frequency difference, 2nd subplot row) were extracted from
538 flight tracking and from wing-flapping sound for statistical analyses of female data (left

subplot column) and male data (right subplot column). 'Zero' (green dashed lline) means there was no difference in the metric before and during the sound stimulus.

Boxplots of the parameters show the median, 2nd and 3rd quartiles. Outliers shown as diamond shapes are outside the interval [Q1 – 1.5 * IQD, Q3 +1.5 * IQD] which shows as whiskers (Q1 = first quartile; Q3 = third quartile and IQD = interquartile distance). The black disk in each distribution shows the mean and standard error.

Two independent types of statistical tests were performed. Stepwise removal of terms was used for model selection, followed by likelihood ratio tests (orange text) [54]. An additional one-sample t-test with BF-correction for multiple comparisons was performed independently for each distribution to measure significance of the mean to zero value (dashed green lines).

(A) Female *An. coluzzii* responses to *An. coluzzii* male-swarm sounds at four SPLs. For the parameter related to linear speed, there was an effect of SPL (LRT χ_1^2=4.34, p=0.037) with a significant BH-corrected one-sample t-test (t(22)=3.6, p=0.0067, mean=0.04 m/s).

(B) Male *An. coluzzii* responses to *An. gambiae* female-swarm sounds at four SPLs. For the maximum wingbeat frequency, there was a strong effect of the SPL (LRT χ_1^2=18.87, p < 0.001), with a highly significant one-sample t-test for the 45 dB SPL distribution (one-sample t(17)=5.45, BH-corrected p<0.001, mean=36 Hz). Before the sound stimulus the mean male wingbeat frequency was 803 Hz.

Figure 5. Estimated hearing-distance and swarm radius as a function of the number of males in the swarm

Green area covers the minimal response range, while red area indicates the minimal non-response range of a female to male swarm sound from both species, as a function of the number of males in a given swarm (X-axis) and the distance to the swarm centre (Y-axis).

These areas are deducted from our behavioural results showing a response to 48 dB SPL stimulus (green-to-white boundary) and the no-response 37 dB SPL stimulus (red-to-white boundary), with their 95% confidence interval (dashed lines). The swarm is assumed to be a point source in the model and only the far-field component of the particle velocity is considered (see STAR*Methods section ‘Acoustic assumptions and formulae’): above 0.15 cm (black dotted line), the near-field component of the particle velocity is negligible (< 1 dB); below 15 cm the smaller the distance, the less linear the relationship between distance and number of males is (i.e. the hearing distance should be higher than shown on this graph). The light and dark blue lines, along with their 95% CI, represent the estimated mean swarm radius of 95% of swarming males (see Figure S4, data from [34]).

TABLES

Subjects exposed to sound stimuli	**Sound stimuli**					
Species and sex	**Sex**	**Number of swarming individuals**	**Species**	**Number of harmonics**	**Sound level**	
					Played-back gain of 50Hz-smooth 1st-harmonics	**SPL measurement of the two 1/3-octave bands closest to the 1st harmonic (dB SPL) at fixed distances from the speaker (0.9 m)**
NA	Silence playback				NA	6.9±0.3
An. coluzzii female	Male	Group (~70)	*An. coluzzii*	all	L_M, related to natural SPL 90cm away from the speaker	20±3
					L_M+6dB	26±3
					L_M+16dB	36±3
					L_M+28dB	48±3
		Group (~30)	*An. gambiae*		L_M	20±3
					L_M+6dB	25±3
					L_M+16dB	36±3
					L_M+28dB	48±3
An. coluzzii male	Female	Group (~30)	*An. coluzzii*	2	L_F, related to natural SPL 90cm away from the speaker	17±3
					L_F+6dB	23±3
					L_F+16dB	33±3
					L_F+28dB	45±3
		Group (~4)	*An. gambiae*		L_F	16±3
					L_F+6dB	22±3
					L_F+16dB	32±3
					L_F+28dB	44±3

Table 1. Description of stimulus loudness at fixed distances from the speaker

This table gives the sound level of all played-back sound stimuli at fixed distances to the speaker, according to two different approaches. The first is the relative signal gain added to the played-back sound, ranging from +0 dB to +28 dB, with 0 dB relative to the natural sound level 0.9 m away from either a 70-male *An. coluzzii* swarm (L_M) or a 30-female *An. coluzzii* swarm (L_F) (see STAR*Methods section 'Generation of sound stimuli' for the

calculation of L_M and L_F). The gain of played-back sound of the *An. gambiae* swarm sound stimuli were corrected to be the same as that of the *An. coluzzii swarm,* to balance the different number of mosquitoes in the swarms of each species. The second approach to describe the sound level is to measure a calibrated sound pressure level (SPL ref 20 μPa) of the played-back sound stimulus at the mosquito's mean location in the frequency range of the opposite-sex's harmonics audible by the mosquito (see STAR*Methods section 'Wingbeat parameter extraction from flight sound'). SPL errors were estimated using minimum and maximum sound pressure levels over time.

Index	SPL at oscillatory distances from female(s) to speaker due to swarming behaviour (room mode effect included)	Estimated distances between the female(s) and the sound-source image of a male swarm				
		r_i (calculated from L_i)	$r_{i,j}$(calculated from r_i) j number of swarming males			
i	SPL (measured) L_i (dB)	70-male swarm r_i	300-male swarm $r_{i,300}$	1500-male swarm $r_{i,1500}$	6,000-male swarm $r_{i,6000}$	10,000-male swarm $r_{i,10k}$
ref	20±3	0.9±0.2 m (recorded case)	1.9±0.4 m	4.3±1.0 m	8±2 m	11±5 m
1	26±3	0.5±0.1 m	1.0±0.2 m	2.3±0.5 m	4.6±1.0 m	6±3 m
2	36±3	15±3 cm * (37 dB SVL)	0.3±0.1 m	0.7±0.2 m	1.4±0.5 m	1.8±0.7 m
3	48±3	4±1 cm * (54 dB SVL)	8±2cm * (51 dB SVL)	18±4 cm * (49 dB SVL)	0.4±0.1 m	0.5±0.2 m

Table 2. Schematic relationship between 'sound level' and 'distance' for *An. coluzzii* sound stimuli

Table shows estimated distances from the female(s) to the sound-source image of male-swarm sound, played-back 0.9 m from the centre of the females' swarming area. SPLs were computed from the calibrated SPL measurements (ref 20 μPa) of the two nearest third-octave bands to the wingbeat frequency's first-harmonic. SPL errors were computed by taking into account the oscillating distance between the female(s) and the speaker due to their swarming behaviour above the visual marker (see Figure 2 and STAR*Methods

section ‘Sound pressure level’). The distances r_i from the female to the sound-source image of the 70-male swarm sound-stimuli were computed from an acoustic-propagation formulae using L_i and r_{ref} (see STAR*Methods equation 6) and the errors were directly derived from SPL errors. The equivalent distance $r_{i,j}$ for a j-male swarm, to result in the same sound pressure level L_i, was extrapolated from r_i using another acoustic formula (see STAR*Methods equation 7). The asterisk (*) means that the distance should be greater than indicated or the sound particle-velocity level (SVL) should be greater than the SPL as indicated (see STAR*Methods section ‘Relationship between particle-velocity and pressure levels). The SPL measurements of *An. gambiae s.s.* sound stimuli are reported in Figure S3; they were close to values for *An. coluzzii,* resulting in similar estimated distances between the female(s) and the sound-source image of a male swarm.

STAR*METHODS

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Prof. Gabriella Gibson (g.gibson@gre.ac.uk).

KEY RESOURCES TABLE

REAGENT or RESOURCE.	SOURCE	IDENTIFIER
Experimental Models: Organisms/Strains		
Anopheles coluzzii	Institut de Recherche des Sciences de la Santé, Bobo Dioulasso, Burkina Faso. The larvae were collected in Bama, Burkina Faso in 2017	G. Gibson

Anopheles gambiae s.s.	Institut de Recherche des Sciences de la Santé, Bobo Dioulasso, Burkina Faso. The larvae were collected in Soumosso, Burkina Faso in 2015	G. Gibson
Software and Algorithms		
MATLAB	Mathworks	R2017a (maci64)
Audacity	audacityteam.org	2.2.1 (Windows), 2.1.1 (Mac OS)
Pro Tools First	Avid Technology, Inc	12.8
Trackit (mosquito flight tracking)	SciTrackS GmbH, Bertschikon, Switzerland	Trackit 3D v. 2.0
Custom audio-video code for parameter-extraction (Matlab)	This study	http://dx.doi.org/10.17632/hn3nv7wxpk.1
Custom statistics code	This study	http://dx.doi.org/10.17632/hn3nv7wxpk.1
Temperature logger software	Omega Engineering, Inc	HH506RA
R	The R Foundation	3.5.3
Dim light programmer	Dimmer-leds.fr	PLeD
Windows (for lab recording)	Microsoft Corporation, Redmond, WA, USA	Windows 7
MacOS (field recording)	Apple, Inc.	10.12.6
Other		
Sound stimuli of swarming mosquito *Anopheles coluzzii*	This paper	Multimedia files included in the submission, modified as described in STAR*Methods section 'Generation of sound stimuli'
Speaker for sound stimuli	Genelec	8010A
Sound card (lab)	Scarlett	18i8
Sound card (field)	Scarlett	Solo
Microphone	Sennheiser	MKH 60-48
Sound pressure level meter	Casella	CEL633C1, Class 1
2 Cameras, wavelength peak sensitivity 840 nm	Basler	ace A640-120gm
2 camera lenses	Computar	T3Z3510CS
2 Infra-red filters (below ~840nm)	Instrument Plastics Ltd UK	G. Gibson
10 Infra-red lights	Raytec	RM25-F-120 RAYMAX 25 FUSION
'Clapper-board' to synchronise video and audio signals.	Bespoke unit produced in-house.	G. Gibson

Mosquito net	NATURO	Outdoor Double Bed Mosquito Net Canopy
Custom Day light	Dimmer-LEDs.fr	~400 LEDs 5630 (2400 lumen/m) at 10cm from and directed to the white ceiling. Plugged to HMCO FLEXIBLE DIMMER.
Custom Sunset/Sunrise light	Dimmer-LEDs.fr	~200 LEDs 5630 (2400 lumen/m) directed to the ceiling, behind a 50cm-high black wall (horizon), the rest being covered by a white thin cotton bedsheet (sunset sky). Plugged to HMCO FLEXIBLE DIMMER.
Soundproof chamber covered with white cotton bedsheets on walls and ceiling	IAC Acoustics Division	Natural Resources Institute, Univ. of Greenwich
Thermocouple (temperature sensor)	Omega Engineering, Inc	Type T, IEC 584 Class 1
Temperature logger	Omega Engineering, Inc	HH506RA

EXPERIMENTAL MODEL AND SUBJECT DETAILS

All experiments were performed with two sibling species in the *Anopheles gambiae s.l.* Giles species complex: *An. gambiae s.s.* Giles and *An. coluzzii* Coetzee & Wilkerson. Colonies of the two species were 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. *Anopheles coluzzii* 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). *Anopheles gambiae s.s.* eggs were obtained from a colony established at IRSS in 2008 and renewed with wild material in 2015 from Soumousso, Burkina Faso (11°00’46”N, 4°02’45”W). Females were identified to species level by PCR [51]. The NRI colonies were kept in environmentally controlled laboratory rooms with a 12h:12h light:dark cycle, >60% relative humidity and ~24-26°C. Adults were kept in wire cube cages (~40 cm sides) covered with cotton netting and fed a solution of 10% sucrose in an isotonic saline *ad libitum*. Females were blood-fed every 4 weeks by a human volunteer (GG). Approximately 30 females per generation laid eggs on disks of damp filter paper, and the eggs were then distributed between two larval plastic

637 breeding trays filled with ~1 L of isotonic saline. Four days after egg hatching, four groups
638 of 90 larvae of all sizes were transferred to four fresh breeding trays filled with isotonic
639 saline. Larvae were fed Tetramin® fish-flakes and rice powder. Pupae were distributed
640 between two netting cages for emergence. Adult males and females were separated < 12h
641 post-emergence to ensure females were not inseminated. Adult mosquitoes used for
642 experiments were fed 10%-sucrose in isotonic saline *ad libitum*.
643

644 **METHOD DETAILS**

645 **Experimental paradigm**

646 ***Principle***

647 It is known that male mosquitoes are attracted to a source of female flight tones, either the
648 sound of a live female or a speaker emitting female flight tones. Males and females are
649 both tuned to the 'difference-tone' between their respective wingbeat frequencies, which
650 provides a relatively robust means of locating each other in mating swarms [1-4,6,8,9,23-
651 25]. This observation raises the hypothesis that females may be attracted to the sound of
652 male swarms, and if so, might they hear larger swarms from a long distance (> 1m)? [34].
653 To test this possibility, instead of changing the distance between the test female
654 and the male swarm, we used a range of sound levels to mimic a range of distances
655 between a female and swarming males; we altered the apparent distance between the
656 female and the sound-source 'image' of the played-back swarm by changing the sound
657 level produced by the speaker. STAR*Methods section 'Formulae between sound level
658 and distance' explains how to predict apparent distances between the receiver and the
659 sound source 'image' based on sound pressure levels. By 'image', we mean that, while the
660 distance from the female to the speaker was always the same (±0.2 m), the sound levels
661 were adjusted to mimic the loudness of the sound pressure of a male swarm at specific

distances between the female and a played-back recording of a male swarm of a given number of individuals.

Control of distance between live mosquito and playback speaker

To establish fixed distances between the sound source and free-flying females, we exploited female swarming behaviour; in the absence of male mosquitoes uninseminated females swarm over a floor marker in flight patterns similar to those of conspecific males. Accordingly, we constructed a flight arena that provided visual cues that stimulated females to fly in elliptical loops over a stationary swarm marker, effectively confining them within a limited area of the flight arena [25,27-29], which enabled us to assess whether or not a female responded to the sound stimulus of the playback of swarming males at a controlled sound level.

The speaker that reproduced the males' swarming flight tones was placed 0.9 m from the centre of the swarm marker. A few females (< 15) at a time were released in the flight arena, and periodically 1 to 5 females were stimulated by the visual characteristics of the marker to switch from random flight to swarming flight. Their flight positions were recorded by 3D-tracking Trackit Software (Figure 2B, Figure 2C) which enabled us to determine the distance between a mosquito and the speaker emitting mosquito sound (0.9±0.2 m ,95%-CI, Figure 2A).

Choice of species of test subjects and for sound stimuli

We had no difficulty in triggering robust swarming behaviour in *An. coluzzii* males and females and in *An. gambiae s.s.* males, but it was difficult to obtain consistent results with *An. gambiae s.s.* females. For this reason and others given above, we focused on the response of *An. coluzzii* to sound stimuli. Overall, female responses to male flight sound were generally small, therefore, we conducted the reciprocal experiment with *An. coluzzii* males exposed to female-swarm sound, to confirm that the experimental protocol was valid (male responsiveness to female-swarm sound was robust), even if it was more

difficult to induce *An. gambiae s.s.* females to swarm (we recorded the sound of a swarm composed of 4 females a a time for *An. gambiae s.s.*, versus 30 females at a time for the *An. coluzzii*).

Experimental design

For each replicate (one per day, August-September 2018), about fifteen 3-6 days-old uninseminated females were released the day prior to experiments at ~ 18h00 in the sound recording flight arena and left to fly freely until the end of the experiment.

At 15h00, after the ceiling lights had dimmed to the lowest intensity, the horizon light completed a 10 min dimming period and then was kept at a constant dim light intensity until the experiment was finished. Some females started flying in the soundproof chamber but did not swarm over the marker immediately. When at least one female started to swarm robustly over the marker, a first sequence of sound stimuli was played (see STAR*Methods section 'Generating the different sound levels'). Each of the subsequent sequences were played immediately following the last if the previous female(s) was still swarming or as soon as at least one female started swarming. The experiment was ended when the maximum number of sequences (10) was reached or after 50 min of constant horizon light. Females were then collected and removed from the flight arena. A new group of ~15 mosquitoes were released in the soundproof chamber, to be used for a new replicate the next day.

Recording environment

Soundproof chamber

Due to the low decibel level of mosquito flight tones, all experiments were conducted in a soundproof chamber to limit interference from external sounds. The chamber consisted of double-skin soundproof walls, ceiling and floor (L x W x H = 2.7 m x 1.9 m x 2.3 m), with carpet on the floor, semi-absorbent internal walls/ceiling and a layer of white cotton cloth

714 covering all surfaces, producing a reverberation time ≤ 0.07 s for frequencies above 200
715 Hz (measurements conducted in empty room by IAC Acoustics, manufacturers).
716 Figure S5C displays the sound level per octave band when the soundproof
717 chamber was silent (dashed lines). At low frequencies (<176 Hz), the sound pressure level
718 (SPL) was ≥ 25 dB (ref 20μPa). Between 176 Hz (lower limit of the 250-Hz octave band)
719 and 1.4 kHz (upper limit of the 1-kHz octave band), i.e. the frequency range within the *An.*
720 *coluzzii* mosquito's response is the highest [3], the SPL was < 14 dB, which is 8 dB less
721 than the quietest sound stimulus used in the study.

722 ***Sound monitoring***

723 The wingbeats (aka, 'flight tones') of mosquitoes in the laboratory were recorded with a
724 weatherproof microphone (Sennheiser MKH60; RF-condenser; super-cardioid polar
725 pattern at 0.5-1 kHz, with amplitude decrease of > 15 dB beyond 90° from the microphone
726 head) directed toward the swarm location. The microphone was located at a distance of
727 0.9 m from the centre of the swarm area (Figure 1).

728 ***Flight track recording***

729 The 3D flight trajectories of mosquitoes were captured at a sampling rate of 50 Hz with
730 Trackit software (SciTrackS GmbH, Switzerland, [52]) running on a Windows7 computer.
731 Two video cameras (Basler, ace A640-120gm) were fitted with wide-angle lenses
732 (Computar, T3Z3510CS, 1/3" 3.5-10.5mm f1.0 Varifocal, Manual Iris) to maximise 3D
733 volume of video-tracking. IR lighting enabled tracking system to detect flying mosquitoes
734 as silhouettes against an IR-illuminated white back-wall made of a thin cotton sheet
735 (Figure 1). The dual IR/white lighting system enabled constant bright IR light (invisible to
736 mosquitoes) for video-tracking flying mosquitoes, while an independent lighting system
737 controlled ambient light detected by mosquitoes to provide a smoothly controlled dusk. All
738 immobile mosquitoes (i.e. at rest on surfaces in the field-of-view) were automatically

deleted. The 3D-flight trajectories were smoothed using a cubic spline interpolation at a sampling frequency of 200 Hz.

Field recording

Preliminary recordings of the flight sound of wild male *An. coluzzii* swarms in the area where our colony originated from (village VK5, Bama, Burkina Faso, 11°23'17.5"N 4°24'27.0"W, October 2017) were used to study the signal-to-noise ratio of swarm sound in the field against local background noise. The swarm was spherical (~1 m diameter), centred ~3 m above the ground and was not apparently disturbed by our presence and produced sound at acceptable levels for recording. The swarm consisted of several thousands of *An. coluzzii* (estimated by eye, by LF, OR and experienced technical staff from the IRSS). The swarm's sound was recorded from various positions and distances; from tens of cm to 3 m away. The recordings were produced with an RF-condenser microphone (MKH 60 P48) plugged into a Scarlett Solo sound card, run by Audacity software on a Mac OSX.

Environmental conditions in soundproof chamber

The swarming arena 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 (L x W x H = 1.8 m x 1.7 m x 2 m) filling most of the soundproof chamber (Figure 1) enabled mosquitoes to fly freely in a volume 100 times greater than the volume covered by the swarming space.

Light and visual cues

Lighting was provided by an artificial-sunlight system to imitate natural daylight, sunrise and sunset (LED 5630, custom-built). Daylight lamps were arranged to mimic sunset lighting; a sharp horizon ~ 40 cm above the floor on one side of the room provided a

764 ’sunset’ feature and a gradually decreasing light intensity with increasing height above the
765 floor (Figure 1).
766 The visually conspicuous matt-black swarm marker triggered swarming behaviour.
767 The marker consisted of a circle of matt-black paper (Ø=30 cm), placed > 30 cm away
768 from the closest netting. The location and height of the swarm marker was arranged
769 according to the swarming behaviour of each species in order to induce swarming flight at
770 the same location in the room for the two species: therefore, the swarm marker for *An.*
771 *gambiae s.s.* was raised by 6 cm and moved 0.8 m horizontally in the opposite direction of
772 the dusk light, compared to the position of the *An. coluzzii* swarm marker (Figure 1), as
773 previously reported [29]. The lighting system provided an artificial dusk; ceiling lights were
774 dimmed over 30 min, while the horizon lights started to dim 10 min before the ceiling light
775 turned off, whereupon the light intensity decreased gradually over 10 min and finally
776 remained constant for 1 h to provide a constant very dim light intensity that favoured
777 prolonged swarming flight during the experiments.

778 ***Temperature monitoring***

779 The temperature was monitored by type-T thermocouples associated with an Omega
780 HH506RA temperature logger (total measurement accuracy error of ±0.9 °C). The chosen
781 thermocouple was located on a room wall at a height of 85 cm from the floor. The 4
782 recordings of the reference sound stimuli (two species, two sexes) were recorded at 28.0
783 °C. The temperature mean and standard deviation of the behavioural assays were
784 28.0±0.3 °C.
785

786 **Generation of sound stimuli**

787 ***Recording of the reference sound-stimuli***

788 Swarms of *An. coluzzii* females or males, and *An. gambiae s.s.* females or males were
789 recorded in the soundproof chamber (Figure 1 and Figure S2). About 300 x 4-7 days-old

790 males or 1-4 x 2-6 days-old females were released in the swarming arena two days before
791 the experiment to acclimatise before their flight sounds were recorded. The standard
792 environmental conditions in the room were: 12h:12h light:dark cycle with a 1h artificial
793 dawn/dusk transition in light intensity, 21-28°C and ~60-75% RH.

794 One recorded 7s-sequence was selected for each sex/species, which began ~10
795 min after the first male/female started to swarm (Sound S1: *An. coluzzii* male swarm;
796 Sound S2: *An. gambiae s.s.* male swarm; Sound S3: *An. coluzzii* female swarm; Sound
797 S3: *An. gambiae s.s.* female swarm). The swarms were composed of 30-70 individuals
798 (except for the *An. gambiae s.s.* female swarm: 4 individuals) flying in loops 0.3 m above
799 the floor level with a horizontal diameter of 0.2 m. The sound amplitude was controlled by
800 fading in at the sound start and fade out at the sound end, both over 1 s to avoid creating
801 noise due to the signal truncation, and to make the stimulus more natural, i.e. mimicking
802 the male swarm sound amplitude which continuously increases when the female gets
803 closer to the swarm.

804 ***Reference sound-stimulus gain***

805 For each sex, the *An. coluzzii* swarm was the reference, and to balance the different
806 number of individuals in the swarms of the two species, the *An. gambiae s.s.* swarm sound
807 level was adjusted to that of *An. coluzzii* (based on the 50Hz-smoothed spectrum peak of
808 the first harmonic, which is known to be important in mosquito hearing [3]). We took
809 advantage of the high numbers of *An. coluzzii* mosquitoes that swarmed (70 males and 30
810 females), which we did not achieve with *An. gambiae s.s.* (30 males and 4 females), even
811 though it meant adjusting the sound level of *An. gambiae s.s.* sounds stimuli (see Figure
812 S2).

813 To playback the stimuli at natural sound levels, we first played them back in the
814 same room and at a distance to a speaker (Genelec 8010A) identical to the distance
815 between the swarm and the microphone. Second, the gain was set to ensure the same

relative sound pressure level was used as during the reference swarm recording (based on the first harmonic amplitude peak from a 50Hz-smoothed spectrum, Figure S2). The same software and hardware settings were used (Audacity on Windows7, Soundcard Scarlett 18i8, microphone MKH 60) to monitor the sound as during its initial recording.

For each sex, the reference sound stimulus was used to generate the full range of stimuli, which only differed between species by their sound level. The gain settings, applied to have a natural sound level of a 70-male swarm or 30-female swarm 0.9 m away, served as the reference (see Table 1 for sound level values for each stimulus).

Figure S2 gives the sound spectrum of the swarm sounds used in the assays. They are harmonic sounds with a large frequency bandwidth. The female harmonics (from three times the fundamental frequency) were filtered out in order to free some spectral space for male wing beat tracking, which does not change the response to the sound stimuli since these higher harmonics are unlikely to be heard by these mosquitoes [3].

Generating the different sound levels

In addition to the natural sound level of the reference sound stimulus (i.e., 70-male swarm or a 30-female swarm 0.9 m away), we generated three more stimuli for each species and each sex, to test the efficacy of sound levels over the range of the response possibilities, using Matlab (R2017a, The Mathworks Inc, Natick, USA) at a sample rate of 8 kHz / 24 bits.

The additional gains applied to the natural-sound-level reference stimuli were computed using a criterion based on the maximum value of the first harmonic on a 50-Hz-smoothed sound spectrum: +6.0 dB, +16 dB and +28 dB compared to the reference sound stimuli (see Table 1 for measured SPL of each stimulus).

A high-pass filter was added to remove the electrical noise below the first harmonic (without removing any frequency component of the swarm sound). The eight stimulus sounds (two species x four sound levels) were combined sequentially with a 10 s

842 silence interval. Ten sequences were generated, each containing the four sounds ordered
843 randomly.

844 ***Sound stimulus playback***

845 Recorded mosquito sounds were played-back from a speaker (Genelec 8010A) with its
846 membrane located 57 cm above the floor, 15 cm from the back wall, and 0.9 m from the
847 swarm marker (Figure 1). Both microphone and speaker were plugged into a Scarlett 18i8
848 sound card running pro Tools First & Audacity on Windows 7.

849

850 **QUANTIFICATION AND STATISTICAL ANALYSIS**

851 **Response Parameters**

852 ***Wingbeat parameter extraction from flight sound***

853 Wingbeat frequency was tracked every 40 ms using a Fast Fourier Transform algorithm
854 (256-ms FFT, Hanning-windowed). Since females and males do not have the same
855 wingbeat frequency and we always played-back opposite-sex sound stimuli to individuals,
856 we had to operate differently for each sex. For females, their fundamental wingbeat
857 frequencies were tracked between 370 Hz and 660 Hz (given that the mean female
858 wingbeat frequency without an added sound stimulus was 487 Hz) to avoid overlap with
859 played-back wingbeat harmonics of swarming males (female wingbeat frequencies were
860 always lower). For males, only the two first harmonics of female sound stimuli were
861 played-back and then the male's third harmonic (3 x fundamental frequency) was tracked
862 between 2190 and 2920 Hz (given that the mean male wingbeat frequency without sound
863 stimulus was 803 Hz), since it is the lowest harmonic that does not overlap with the sound
864 stimulus (example of spectrogram in Figure 2C). When several wingbeat frequencies were
865 tracked due to the presence of several mosquitoes over the swarming marker, their
866 wingbeat frequencies were averaged. Male harmonics were divided by three to get the
867 fundamental frequency. Finally, a 3-point median filter was applied over time to reduce

wingbeat tracking error. Figure 2C gives an example of detected wingbeat frequencies of females and males while Figure 2B shows the distribution of the detected wingbeat frequency over time for all recordings.

The maximum wingbeat frequencies were automatically detected during the 7 s stimulus time interval, as well as during the 7 s segment just before stimulus onset and were subtracted since we are interested in the response difference between the 'with sound' stimulus and the 'without sound' stimulus.

Position and speed parameter extraction from tracked flight trajectory

The criteria used to include a tracked flight in the data analysis were: the mosquito was swarming over the marker for at least 1 s before and after the duration of the sound stimulus onset. Linear speed at time index *n* was calculated as the square root of the sum of the three square velocity components provided by the Trackit software, and the angular speed was computed as $avel = \frac{\Delta\theta}{\Delta t}$, 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 (equivalent to the definition in [53]):

$$avel = arccos \frac{v_n . v_{n+1}}{|v_n| . |v_{n+1}|} \quad (1)$$

where v_n is the three-dimensional linear velocity vector of the mosquito at time index n and $|v_n|$ is its magnitude.

Sound and video synchronization

To synchronize sound and video data, a custom-made 'clapper-board' simultaneously switched off an IR led and a 3900-Hz bip sound (which cannot be heard by this species complex [3]). The IR light was located on the edge of the field of view where no mosquito was expected to swarm. The IR light was automatically tracked every 2 ms when the light was switched off (i.e. creating a dark silhouette) simultaneously with the sound. The 10-s bip sound was played-back before and after each stimulus sequence and manually switched off along with the IR light. The bip 'offsets' were detected manually on an 8 ms-

window spectrogram. Cumulative errors over time were controlled by using the ‘offset’ time before and after the stimulus sequence. Overall, the synchronization uncertainty was ±8 ms.

Statistics

We were not able to discriminate between mosquitoes from their wingbeat frequencies when swarming in a group, so for each sound parameter values were computed for the whole tested group of 1-5 females or of 1-6 males swarming at a time (distribution of tested-mosquito number in Figure S3). In contrast, flight location and velocities were first computed for each mosquito in the group, and then averaged over each group to form a replicate. For females exposed to male sound, a total of 10 to 12 replicates per sound level and species were tested, against a total of 9 to 10 replicates per sound level and species for males exposed to female sound. Each replicate was performed on a different day.

The sound and video response parameters were analysed using a Bayesian Linear-Mixed Model (*blmer* function, lme4 package, R). Stimulus sound levels and species were considered fixed effects and days,—for which replicates were performed— were considered random effects. Sex was considered separately. 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 [54]. An additional one-sample t-test (with BF-correction for multiple comparisons) was performed independently for each distribution to measure the significance of the mean to 0, which is the “no response” reference. All analyses were performed using R (version 3.5.1).

Sound pressure level (SPL)

Measurement

To compare the sound stimulus with previous studies on hearing sensitivity, SPLs were measured at the females' swarming position with a sound meter (Casella, CEL633C1, Class 1) set as follows: 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 reference pressure value was 20 μPa. The minimum and maximum sound level values within each stimulus duration were used to compute the mean and error of each measurement (Table 1). The speaker and the software/soundcard gains were set to be the same as during the behavioural experiment.

All SPLs reported in the paper take into account only the frequency bands that are audible by mosquitoes, i.e. mostly the first-harmonic of the opposite sex [3]. They were calculated as follows: $10log_{10}(10^{0.1L_{B1}} + 10^{0.1L_{B2}})$ where L_{B1} and L_{B2} are SPL measurements in frequency bands B1 and B2; B1 and B2 are the third-octave bands nearest the wingbeat frequency of the first-harmonics, i.e. 800 Hz and 1000 Hz for males and 500 Hz and 630 Hz for females (see full third-octave level in Table 1 and Figure S5). This method enabled us to compare our sound stimulus levels to pure sounds used in previous studies and is closer to what mosquitoes actually hear.

Estimate of SPL errors at mosquito's location

Three types of SPL errors were taken into account. The first is related to the time variation of the sound stimulus levels and were between ±0.3 dB and ±1 dB, depending on the stimulus considering maximum error (see Figure S2 for an example of stimulus RMS-pressure-level along time).

The second source of error is related to acoustical interferences caused by room boundaries. Up to this point, we have considered a free-field acoustic-propagation

943 hypothesis to simplify the problem. In a room, however, sound level can decrease
944 (destructive interference) or increase (constructive interference) independently of the
945 distance to the speaker. This effect was reduced by the semi-absorbent walls of the room,
946 but was still present because the room was not an anechoic chamber. Boundary-induced
947 'comb filtering' was reduced by locating the speaker close to the wall, but acoustic room
948 modes were still present. We played-back the *An. coluzzii* male and female swarm
949 stimulus and measured the sound level in a 0.2 m diameter sphere around the expected
950 swarm centre. The maximum error was about ±1 dB for the female sound stimulus and ±2
951 dB for male swarm stimulus. We ignored any reverberation effect, as we estimated its
952 effect to < 0.4 dB at 800 Hz, using an acoustic room model of the ratio of direct and
953 reverberant sound, given the reverberation times of the room provided by the soundproof
954 chamber designer (IAC Acoustics Ltd).

955 The last type of measurement uncertainty arises when the estimated sound level
956 should be estimated from the mosquito's point of view. SPLs were measured at the
957 expected centre of the station-keeping swarm-flight of the mosquito. However, the
958 distance between the female and the speaker varies between 72 and 113 cm (95%-CI,
959 Figure 2A) due to the females' swarming-flight pattern and sound level changes,
960 accordingly. We computed this error by considering the fluctuating distance between the
961 female mosquito and the speaker using equation 6.

962 Finally, using standard uncertainty-propagation theory, we calculated the total
963 error of sound pressure level L_i at the location of the female exposed to male sound,
964 resulting in a total error of ±3 dB SPL for the SPL. This error is considered to be
965 conservative (at least 95%) and were used to interpret the results of the experiments. For
966 errors related to the difference between what we measured (sound pressure) and what
967 mosquitoes detect (particle velocity), see 'Quantification and Statistical Analysis section',
968 subsection 'Physical sound quantities produced by a speaker and sensed by mosquitoes'.

Acoustic assumptions and formulae

Acoustic assumptions for a swarm

The density of a swarm is far greater in the centre than at the periphery [34] (Figure S4). Therefore, for the purposes of this analysis, we considered the swarm to be a point source that radiates spherically in all directions (neglecting the sound reflection on the ground or any nearby object). This approximation can be used if the swarm radius remains relatively small compared to the distance between the female and the swarm centre. Swarms can be ovoid [29,34], but this is not an issue for our point-source assumption, because the oval dimension was perpendicular to the female-to-swarm spatial axis, so each swarming male equally contributed to the radiated swarm sound toward the female at long range.

Relationship between particle-velocity and pressure levels

We monitored the sound level of swarms by recording the sound pressure level (SPL), while mosquito hearing organs are sensitive to particle velocity levels [52,13,14]. These two quantities are equal only far from the sound source, so it is important to understand how they are related to estimate the error when we are dealing with sources close to the receiver.

For any distance r from the sound source (i.e. near-field, far-field and in-between) at time t, the particle velocity $v(r,t)$ can be expressed mathematically by two additive terms; one for which the amplitude decreases with the inverse of distance (far-field component), and another, for which its amplitude decreases with the inverse of the distance squared (near-field component), while pressure $p(r,t)$ is expressed by a unique term for which the amplitude decreases with the inverse of distance [55]:

$$v(r,t) = \frac{1}{Z_{air}}\frac{1}{r}s\left(t-\frac{r}{c}\right) + \frac{c}{Z_{air}}\frac{1}{r^2}\int\left(t-\frac{r}{c}\right)dt \qquad (2)$$

$$p(r,t) = \frac{1}{r}s\left(t-\frac{r}{c}\right) \qquad (3)$$

$s\left(t-\frac{r}{c}\right)$ a progressive wave solution of the wave equation, bounded, moving at a speed $c(28°C) = 348$ m.s^{-1}, at time t and position r from the sound source. $Z_{air}(28°C) = 408$ N•s•m^{-3} is the air impedance.

Considering a particular frequency (i.e. by choosing $s(t-r/c) = cos\left(2\pi f\left(t-\frac{r}{c}\right)\right)$ and averaging over a sound period by taking the root-mean square value (RMS), the RMS particle velocity and the RMS sound pressure can be related as follows for a point source radiating spherically [56]:

$$v_{RMS}(r) = \frac{p_{RMS}(r)}{Z_{air}}\sqrt{1+\left(\frac{c}{2\pi fr}\right)^2} \quad (4)$$

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

$$L_v(r) = L_p(r) + \frac{1}{2}log_{10}\left(1+\left(\frac{c}{2\pi fr}\right)^2\right) \quad (5)$$

Therefore, particle-velocity level and SPL are equal when *r* is great. In our case, considering the male swarm sound stimulus does not have any frequency components below $f = 745Hz$ (the smallest frequency value of the group of first harmonics of the swarming males at -12dB below the peak at 857 Hz, Figure S2), then we can calculate that for $r > 15cm$, $L_v(r) = L_p(r)$ with an error less than 1 dB.

Table 2 gives the SPL of each stimulus, which is equal to the particle-velocity level for distances from the sound-source < 15 cm. Below 15 cm, the smaller the distance to the sound-source, the greater the particle-velocity level is, compared to the SPL. At 4 cm from the sound source, the particle-velocity level is 8 dB higher. When the difference between the SPL and the particle-velocity is greater than 1 dB, the particle-velocity level is added along the distance to the sound-source in Table 2.

Physical sound quantities produced by a speaker and sensed by mosquitoes

Like any sound-source, a speaker creates both a pressure field and a particle-velocity field. At 0.9-m away from the speaker, the near-field component of the particle velocity is negligible and then the particle-velocity level is equal to the SPL. As a consequence, monitoring the sound pressure level of a male-swarm sound played-back on a speaker 0.9-m away from the exposed mosquito is enough to reproduce natural soundscapes of swarms (i.e. sound-source image) located > 15 cm away from the mosquito (<1dB error). When the distance from the sound-source image to the mosquito is expected to be modelled as < 15 cm, the particle velocity created by the speaker becomes different to the one created by a natural swarm: its level is underestimated, and its phase is modified.

Formula between sound level and distance

In order to estimate the distance over which a female could hear a given-size swarm with a given number of swarming males, we are interested in determining the equivalent distance r_i (i being the sound pressure level label) to the virtual sound source (i.e. the played-back male swarm, or sound-source image) knowing the sound pressure level L_i at the female's position at a distance r_i from the virtual swarm, and the sound level L_{ref} at position r_{ref} ($r_{ref} = 0.9m$ known to be the distance to the reference sound stimulus source). The physical sound source is the speaker, at fixed distance R from the swarming marker (i.e. from the female ± its movement above the marker). The sound level is set to reproduce a natural swarm sound where the presence is virtually located at a distance r_i from the female (see Figure 3 for a visual illustration).

As a single monopole point spherically radiates in all directions (no sound reflection), the root-mean-square sound pressure $p_{RMS,i}$ is inversely proportional to the distance r_i (i.e. $p_{RMS,i} \propto \frac{1}{r_i}$). Then the sound pressure level difference ΔL_i can also be expressed as follows:

$$\Delta L_i \stackrel{\text{def}}{=} L_i - L_{ref} = 20log_{10}\left(\frac{r_{ref}}{r_i}\right) \quad (6)$$

Then from equation 6 we get the distance r_i to the sound-source image as a function of the difference level ΔL_i and the known distance r_{ref} from the female's position in relation to the sound-source image of the swarm of the reference stimulus recording:

$$r_i = r_{ref} 10^{\frac{-\Delta L_i}{20}} \quad (7)$$

Sound pressure level label *ref* corresponds to the natural sound level of an *An. coluzzii* 70-male swarm at a distance of 0.9 m. The equivalent distances r_i associated with the other sound levels L_i, (i belonging to 1, 2, 3) can be calculated from equation 7: they correspond to the SPLs 20 dB, 26 dB, 36 dB, 48 dB of a point-source 70-male swarm at a distance of 0.9, 0.5 m, 15 cm and 4 cm, respectively (Table 2). This calculus assumes that the female is far enough from the swarm so that the swarm dimensions are small enough compared to its distance to the swarm (i.e. 'point-source'). Even if it is unrealistic, it helps as a step for modelling larger distances where this issue does not occur anymore (see below).

Formula relating hearing distance and number of individuals in the swarm

Acoustic prediction was needed to cope with large swarms because of a limitation in the number of swarming males to be recorded under controlled conditions. In our experimental space, about 20% of the released *An. coluzzii* males and 10% of the released *An. gambiae s.s.* males swarmed over the swarming spot. A small number of the non-swarming males were flying without station-keeping behaviour in our experimental room space (most of the remaining males were resting). However, the chance of a flying non-swarming mosquito passing in the field of sound of the directional microphone increased with the number of released mosquitoes. Thus, above ~70 swarming males, the number of flying non-swarming males was too high and our sound recording could have been altered by flying males for which the distance to the microphone and its behaviour (i.e. non-

swarming flight) could not have been controlled. As a consequence, we decided to use the 70-male swarm in the behavioural experiments, which is the biggest station-keeping swarm we could reliably produce and record in the laboratory.

In order to estimate the results which could have been found with a bigger swarm, we predicted the behavioural assay results performed with a 70-male swarm sound stimulus using an acoustic model of the swarm sound level as a function of its number of individuals and its distance to the female.

Multiplying by *N* a number of acoustically incoherent sources, such as swarming mosquitoes, increases the SPL by $10log_{10}(N)$ [57]. Let's assume a $N \times 70$-male swarm can be modelled as a single point (see STAR*Methods section 'Acoustic assumptions for a swarm'), then the SPL at a fixed distance will be increased by $10log_{10}(N)$ (e.g. 7 dB if *N*=5 or 20 dB if *N*=100) compared to the 70-male swarm.

Then we can compute the virtual distances $r_{i,N\times70}$ of a $N \times 70$-male swarm with same SPL L_i as a 70-male swarm at distance r_i, knowing that the $N \times 70$-male swarm has a SPL $L_i + 10log_{10}(N)dB$ at distance r_i, by the following formulae derived from equation 6 (values are presented in Table 2 for a 300, 1500, 6,000 and 10,000-male swarm):

$$r_{i,N\times70} = r_i 10^{\frac{-\left(L_i-\left(L_i+10log_{10}(N)\right)\right)}{20}} = \sqrt{N}r_i \qquad (8)$$

DATA AND SOFTWARE AVAILABILITY

Software/codes used audio/video parameter extractions and statistical analysis are listed in the Key Resources Table. Raw sound files, tracked flight dataset and dataset for the statistical tests are available on request.

SUPPLEMENTAL INFORMATION

See the Supplemental Information PDF for five supplemental figures and one supplemental table.

MULTIMEDIA FILE LEGENDS

Sound S1 (Sound-S1.mp3)

Sound stimulus recording of the 70-male *An. coluzzii* (7 s) before any filtering and level adjustment. Related to Figure S2B (dotted clear blue line).

Sound S2 (Sound-S2.mp3)

Sound stimulus recording of the 30-male *An. gambiae s.s.* (7 s) before any filtering and level adjustment. Related to Figure S2B (dotted dark blue line).

Sound S3 (Sound-S3.mp3)

Sound stimulus recording of the 30-female *An. coluzzii* (7 s) before any filtering and level adjustment. Related to Figure S2B (dotted clear red line).

Sound S4 (Sound-S4.mp3)

Sound stimulus recording of the 4-female *An. gambiae s.s.* (7 s) before any filtering and level adjustment. Related to Figure S2B (dotted dark red line).

Video S1 (Video-S3.mp4)

Audio-video recording of the *An. coluzzii* female exposed to the loudest *An. coluzzii* male sound (10-s silence + 7-s sound exposition + 10-s silence). Related to Figure 2.

Video S2 (Video-S6.mp4)

Audio-video recording of the *An. coluzzii* males exposed to the loudest *An. gambiae s.s.* female sound (10-s silence + 7-s sound exposition + 10-s silence). Related to Figure 2.

REFERENCES

[1] Gibson, G. and Russel, J. I. (2006). Flying in tune: sexual recognition in mosquitoes. Current Biology *16*, 1311–1316.

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

[3] Warren, B., Gibson, G. and Russel, J. I. (2009). Sex recognition through midflight mating duets in *Culex* Mosquitoes is mediated by acoustic distortion. Current Biology *19*, 485–491.

[4] Pennetier, C., Warren, B., Dabire, K. R., Russel, J. I. and Gibson, G. (2010). “Singing on the wing” as a mechanism for species recognition in the malarial mosquito *Anopheles gambiae*. Current Biology *20*, 131–136.

[5] Warren, B. and Russell, I. (2011). Mosquitoes on the wing ”tune in” to acoustic distortion. AIP Conference Proceedings *1403*, 479–480.

[6] Simoes, P. M. V., Ingham, R. A., Gibson, G. and Russell, I. J. (2016). A role for acoustic distortion in novel rapid frequency modulation behaviour in free-flying male mosquitoes. Journal of Experimental Biology *219*, 2039– 2047.

[7] Su, M. P., Andrés, M., Boyd-Gibbins, N., Somers, J. and Albert, J. T. (2018). Sex and species specific hearing mechanisms in mosquito flagellar ears. Nature Communications *9*, 3911.

[8] Aldersley, A. and Cator, L. J. (2019). Female resistance and harmonic convergence influence male mating success in *Aedes aegypti*. Scientific Reports *9*, 2145.

[9] Pantoja-Sanchez, H., Gomez, S., Velez, V., Avila, F. W. and Alfonso-Parra, C. (2019). Precopulatory acoustic interactions of the New World malaria vector *Anopheles albimanus* (Diptera: Culicidae). Parasites & Vectors *12*, 386.

[10] Belton, P. (1974). An analysis of direction finding in male mosquitoes. In Experimental Analysis of Insect Behaviour, pp. 139–148. Berlin, Heidelberg: Springer Berlin Heidelberg.

[11] de Silva, P., Nutter, B. and Bernal, Ximena E. (2015). Use of acoustic signals in mating in an eavesdropping frog-biting midge. Animal Behaviour 103, 45-51.

[12] Stokes, G. (1851). On the effect of the internal friction of fluids on the motion of pendulums. Trans. Cambridge Phil. Soc. *IX*, 8.

[13] Fletcher, N. H. (1978) Acoustical response of hair receptors in insects. Journal of Comparative Physiology 127, 185—189

[14] Tautz, J. (1979). Reception of particle oscillation in a medium — an unorthodox sensory capacity. Naturwissenschaften 66, 452-461.

[15] Bennet-Clark, H. C. (1998). Size and scale effects as constraints in insect sound communication. Philosophical Transactions of The Royal Society Biological Sciences *353*, 407–419.

[16] Wishart, G. and Riordan, D. F. (1959). Flight responses to various sounds by adult males of *Aedes aegypti* (L.) (Diptera: Culicidae). The Canadian Entomologist *91*, 181–191.

[17] Göpfert, M. C. and Robert, D. (2000). Nanometre-range acoustic sensitivity in male and female mosquitoes. Proceedings of the Royal Society of London. Series B: Biological Sciences *267*, 453–457.

[18] Windmill, J. F. C. and Jackson, J. C. (2016). Mechanical specializations of insect ears. In Insect Hearing, Pollack, G. S., Mason, A. C., Popper, A. N and Fay, R. R. (Springer International Publishing), pp. 125–157.

[19] Menda, G., Nitzany, E. I., Shamble, P. S., Wells, A., Harrington, L. C., Miles, R. N. and Hoy, R. R. (2019). The long and short of hearing in the mosquito *Aedes aegypti*. Current Biology *29*, 709–714.

[20] Lapshin, D. N. (2013), The auditory system of blood-sucking mosquito females (Diptera, Culicidae): Acoustic perception during flight simulation. Entomological Review *93*, pp. 135-149.

[21] Bartlett-Healy, K., Crans, W., Gaugler, R. (2008). Phonotaxis to amphibian vocalizations in *Culex territans* (Diptera: Culicidae). Annals of the Entomological Society of America *101*, pp. 95-103.

[22] Bernal, X. E., Rand, A. S. and Ryan, M. J. (2006). Acoustic preferences and localization performance of blood-sucking flies (*Corethrella Coquillett*) to túngara frog calls. Behavioral Ecology *17,* 709–715.

[23] Downes, J. A. (1969). The swarming and mating flight of diptera. Annual Review of Entomology *14*, 271–298.

[24] Charlwood, J. D. and Jones, M. D. R. (1979). Mating behaviour in the mosquito, *Anopheles gambiae s.l.* I. Close range and contact behaviour. Physiological Entomology *4*, 111–120.

[25] Gibson, G. (1985). Swarming behavior of the mosquito *Culex pipiens quinquefasciatus*: a quantitative analysis. Physiological Entomology *10*, 283– 296.

[26] Sawadogo, P., Namountougou, M., Toé, K., Rouamba, J., Maïga, H., Ouédraogo, K., Baldet, T., Gouagna, L., Kengne, P., Simard, F., Costantini, C., Gibson, G., Diabaté, A., Lees, R., Gilles, J. and Dabiré, K. (2014). Swarming behaviour in natural populations of *Anopheles gambiae* and *An. coluzzii*: Review of 4 years survey in rural areas of sympatry, Burkina Faso (West Africa). Acta Tropica *132*, S42 – S52.

[27] Savolainen, E. and Syrjämaki, J. (1971). Swarming and mating of *Erioptera gemina* Tjeder (Dip., Limoniidae). Annales entomologici Fennici *37*, 79–85.

[28] Charlwood, J. D. (1976). The mating behaviour of mosquitoes. (University of Sussex).

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

[30] Diabaté, A., Yaro, A. S., Dao, A., Diallo, M., Huestis, D. L. and Lehmann, T. (2011). Spatial distribution and male mating success of *Anopheles gambiae* swarms. BMC Evolutionary Biology *11*, 184.

[31] Assogba, B. S., Djogbénou, L., Saizonou, J., Diabaté, A., Dabiré, R. K., Moiroux, N., Gilles, J. R., Makoutodé, M. and Baldet, T. (2014). Characterization of swarming and mating behaviour between *Anopheles coluzzii* and *Anopheles melas* in a sympatry area of Benin. Acta Tropica *132*, S53 – S63.

[32] Sawadogo, S. P., Costantini, C., Pennetier, C., Diabaté, A., Gibson, G. and Dabiré, R. K. (2013). Differences in timing of mating swarms in sympatric populations of *Anopheles coluzzii* and *Anopheles gambiae s.s.* (formerly An. gambiae M and S molecular forms) in Burkina Faso, West Africa. Parasites & Vectors *6*, 275.

[33] Diabate, A. and Tripet, F. (2015). Targeting male mosquito mating behaviour for malaria control. Parasites & Vectors *8*, 347.

[34] Manoukis, N. C., Diabaté, A., Abdoulaye, A., Diallo, M., Dao, A., Yaro, A. S., Ribeiro, J. M. C. and Lehmann, T. (2009). Structure and dynamics of male swarms of *Anopheles gambiae*. Journal of Medical Entomology *46*, 227–235.

[35] Wesenberg-Lund, C. (1920). Contributions to the biology of the Danish Culicidae. Kobenhavn (A.F. Host & Son)

[36] Mankin, R. W. (1994). Acoustical detection of *Aedes taeniorhynchus* swarms and emergence exoduses in remote salt marshes. Journal of the American Mosquito Control Association *10*, 302–308.

[37] Belton, P. (1994). Attraction of male mosquitoes to sound. Journal of American Mosquito Control Association 10, pp 297-301.

[38] Gibson, G., Warren, B. and Russel, J. I. (2010). Humming in tune: sex and species recognition by mosquitoes on the wing. JARO *11*, 527–540.

[39] Cator, L. J., Ng'Habi, K. R., Hoy, R. R. and Harrington, L. C. (2010). Sizing up a mate: variation in production and response to acoustic signals in *Anopheles gambiae*. Behavioral Ecology *21*, 1033–1039.

[40] Aldersley, A., Champneys, A., Homer, M. and Robert, D. (2016). Quantitative analysis of harmonic convergence in mosquito auditory interactions. Journal of The Royal Society Interface *13*.

[41] Costantini, C., Ayala, D., Guelbeogo, W. M., Pombi, M., Some, C. Y., Bassole, I. H., Ose, K., Fotsing, J.-M., Sagnon, N., Fontenille, D., Besansky, N. J. and Simard, F. (2009). Living at the edge: biogeographic patterns of habitat segregation conform to speciation by niche expansion in *Anopheles gambiae*. BMC Ecology *9*, 16.

[42] Simard, F., Ayala, D., Kamdem, G. C., Pombi, M., Etouna, J., Ose, K., Fotsing, J.-M., Fontenille, D., Besansky, N. J. and Costantini, C. (2009). Ecological niche partitioning between *Anopheles gambiae* molecular forms in Cameroon: the ecological side of speciation. BMC Ecology *9*, 17.

[43] Tripet, F., Dolo, G., Traoré, S. and Lanzaro, G. C. (2004). The ”wingbeat hypothesis” of reproductive isolation between members of the *Anopheles gambiae* complex (Diptera: Culicidae) does not fly. Journal of Medical Entomology *41*, 375–384.

[44] Simoes, P. M. V., Gibson, G. and Russel, J. I. (2017). Pre-copula acoustic behaviour of males in the malarial mosquitoes *Anopheles coluzzii* and *Anopheles gambiae s.s*. does not contribute to reproductive isolation. Journal of Experimental Biology *220*, 379–385.

[45] Charlwood, J. D. and Jones, M. D. R. (1980). Mating in the mosquito, *Anopheles gambiae s.l.* II. Swarming behaviour. Physiological Entomology *5,* 315–320.

[46] Lapshin, D. N. and Vorontsov, D. D. (2019). Directional and frequency characteristics of auditory neurons in *Culex* male mosquitoes. Journal of Experimental Biology *222*.

[47] Warren, B., Lukashkin, A. N. and Russell, I. J. (2010). The dynein-tubulin motor powers active oscillations and amplification in the hearing organ of the mosquito. Proceedings of the Royal Society B: Biological Sciences *277*, 1761–1769.

[48] Dabiré, R. K., Sawadogo, S. P., Diabaté, A., Toé, K. H., Kengne, P., Ouari, A., Costantini, C., Gouagna, L. C., Simard, F., Baldet, T., Lehmann, T. and Gibson, G. (2013). Assortative mating in mixed swarms of the mosquito *Anopheles gambiae s.s.* M and S

molecular forms, in Burkina Faso, West Africa. Medical and Veterinary Entomology *27*, 298–312.

[49] Mozūraitis, R., Hajkazemian, M. Zawada, J. W., Szymczak, J., Pålsson, K., Sekar, V., Biryukova, I., Friedländer, M. R., Koekemoer, L. L., Baird, J. K., Borg-Karlson, A.-K., Emami, S. N. (2020). Male swarming aggregation pheromones increase female attraction and mating success among multiple African malaria vector mosquito species. Nature Ecology & Evolution

[50] Poda, S. B., Buatois, B., Lapeyre, B., Dormont, L., Diabaté, A., Gnankiné, O.,Dabiré , R. K. (2020). No evidence for long-range male sex pheromones in two malaria mosquitoes, bioRxiv, doi: https://doi.org/10.1101/2020.07.05.187542

[51] Fanello, C., Santolamazza, F. and Della Torre, A. (2002). Simultaneous identification of species and molecular forms of the *Anopheles gambiae* complex by PCR-RFLP. Medical and Veterinary Entomology *16*, 461–464.

[52] Fry, S., Muller, P., Baumann, H.-J., Straw, A., Bichsel, M. and Robert, D. (2004). Context-dependent stimulus presentation to freely moving animals in 3D. Journal of Neuroscience Methods *135*, 149–157.

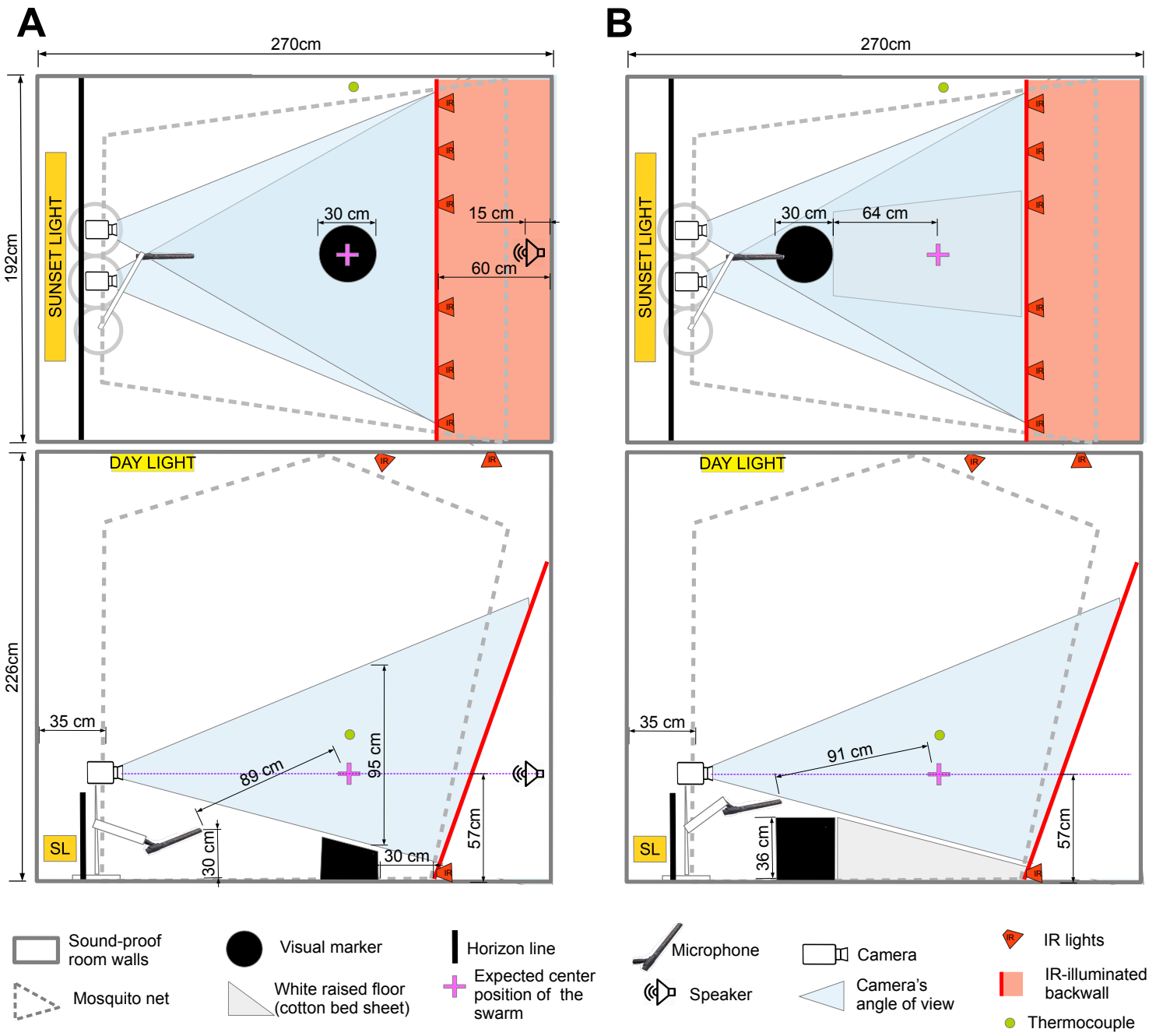
[53] Cribellier, A., van Erp, J. A., Hiscox, A., Lankheet, M. J., van Leeuwen, J. L., Spitzen, J. and Muijres, F. T. (2018). Flight behaviour of malaria mosquitoes around odour-baited traps: capture and escape dynamics. Royal Society Open Science *5*, 180246.

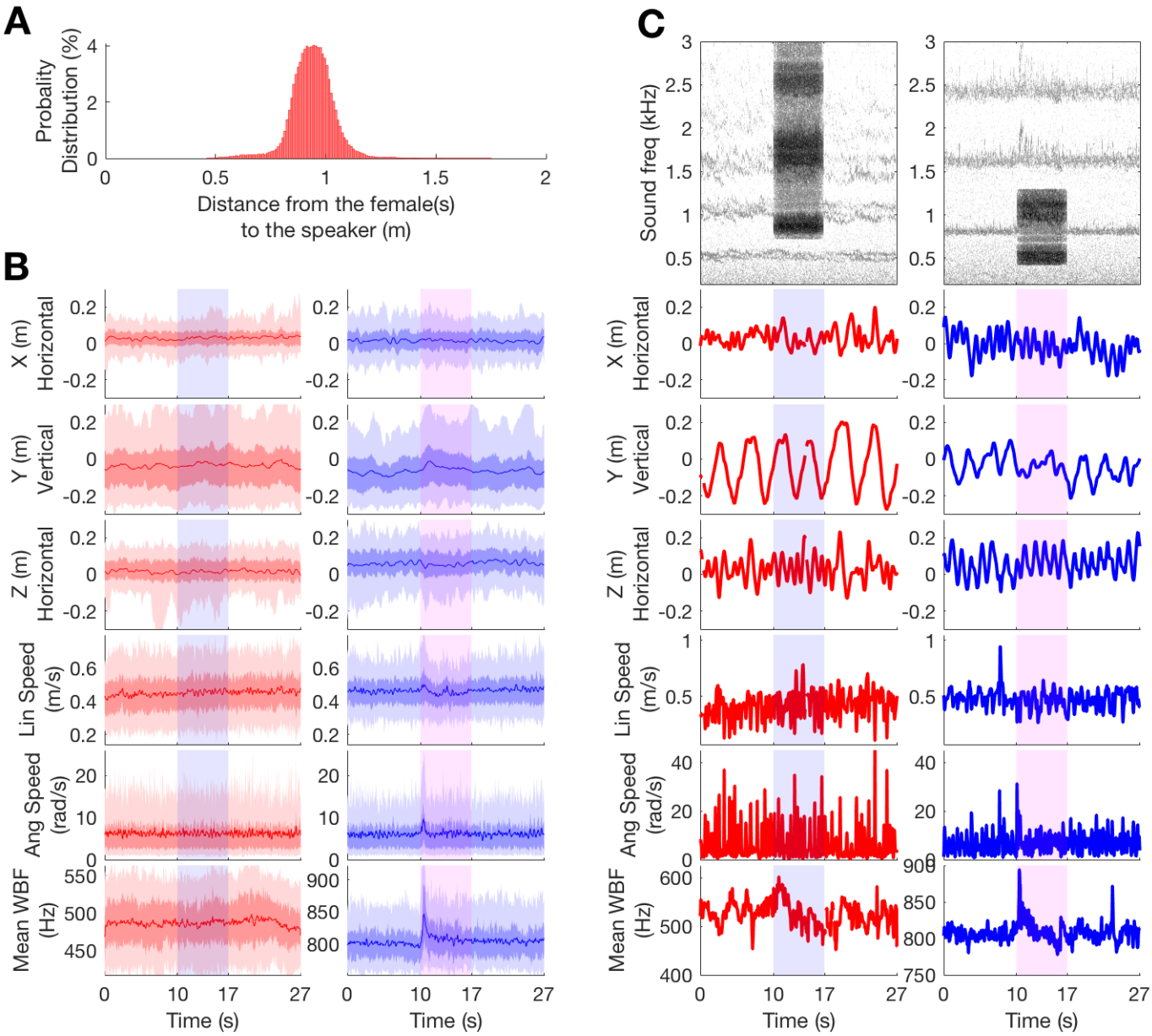
[54] Crawley, M. J. (2007). Mixed-Effects Models. In The R book (John Wiley & Sons), pp. 627–660.

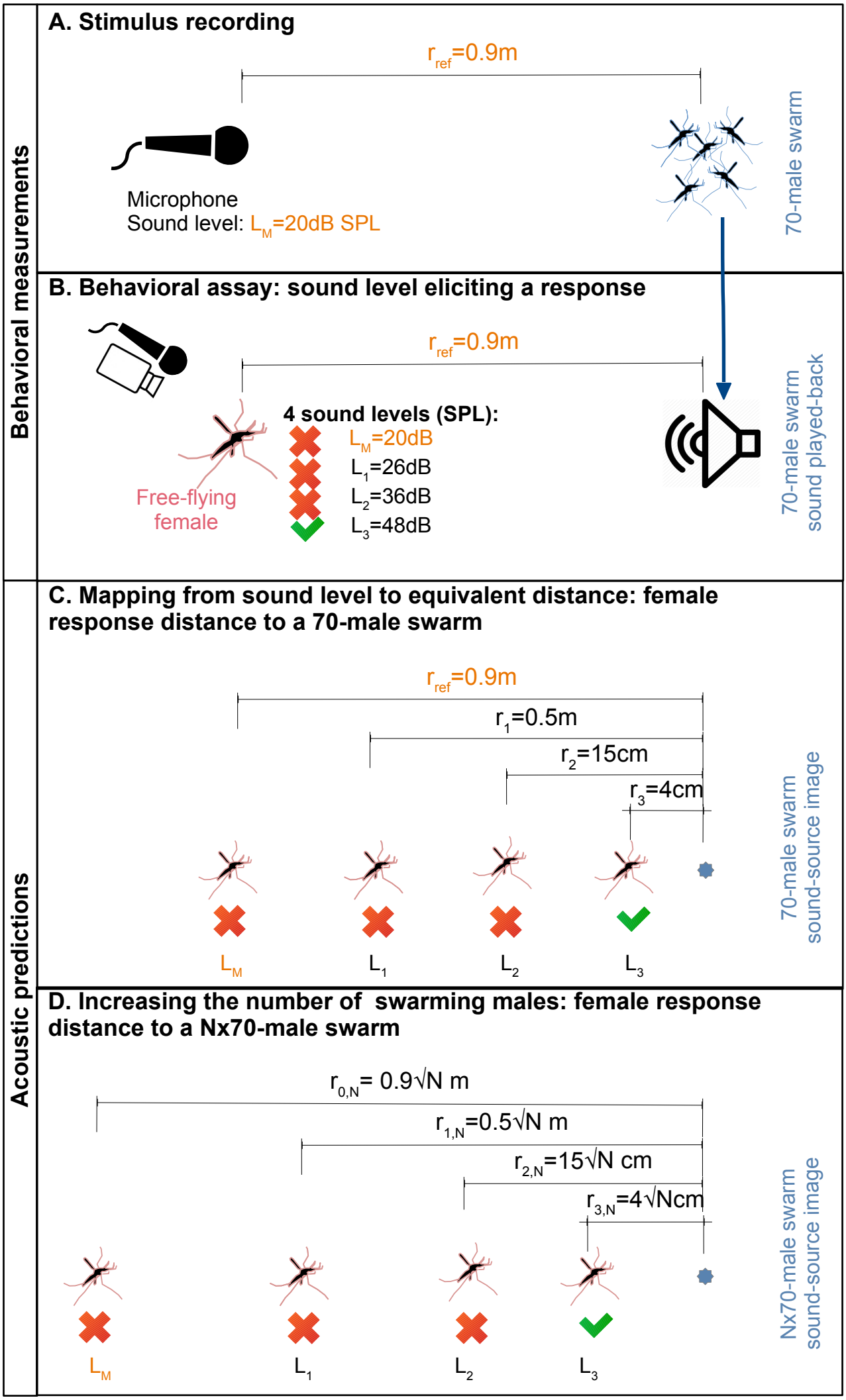
[55] Errede, P. S. (2020). Examples of Complex Sound Fields. In Lecture XII - Part 2, P406POM Lecture Notes (Urbana-Champaign, Illinois: Department of Physics, University of Illinois), https://courses.physics.illinois.edu/phys406/sp2017/Lecture_Notes/P406POM_Lecture_Notes/P406POM_Lect12_Part2.pdf (Last viewed May 15, 2020).

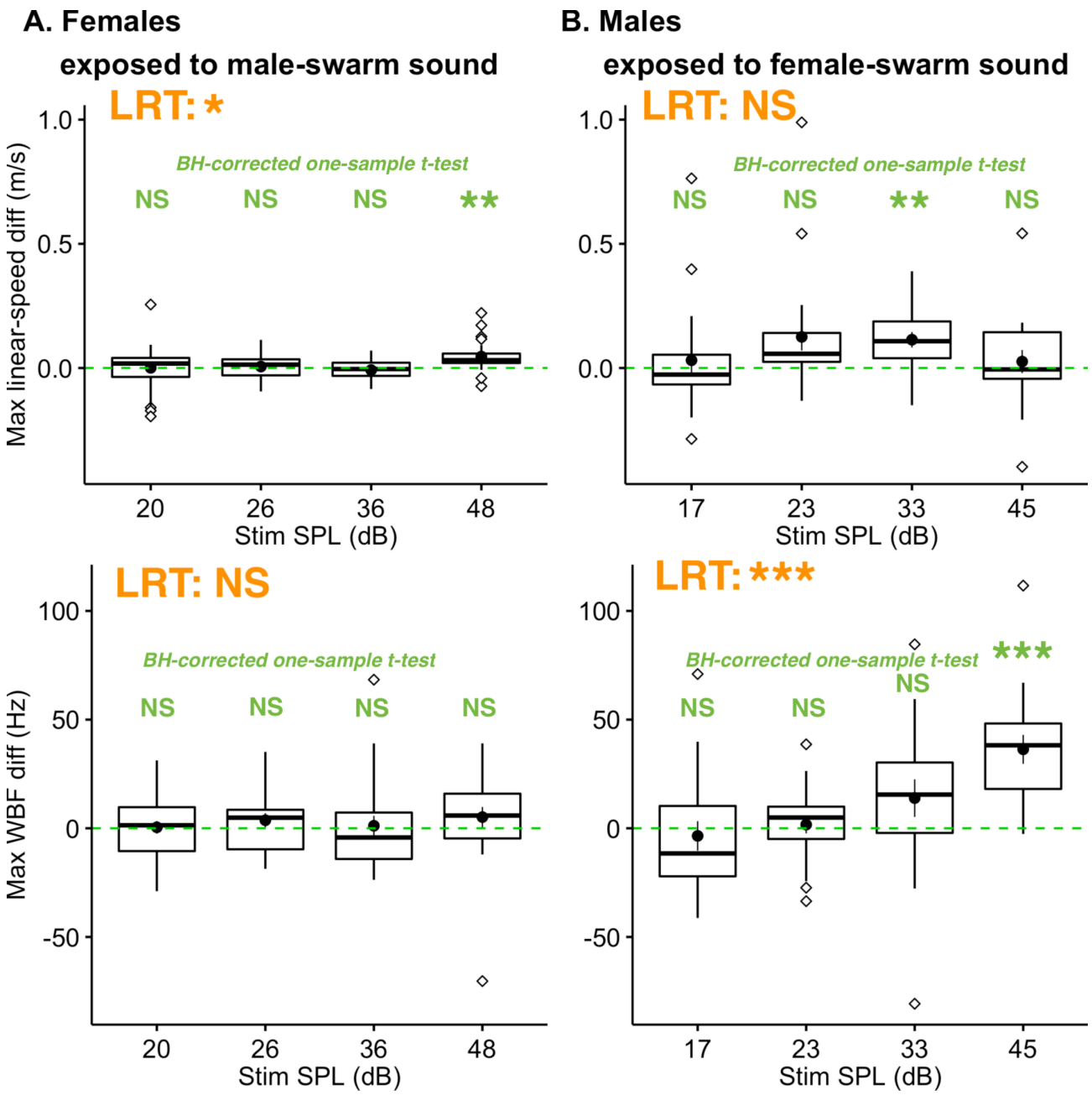
[56] Beranek, L. L. and Mellow, T. J. (2012). Acoustics: sound fields and transducer, Academic Press.

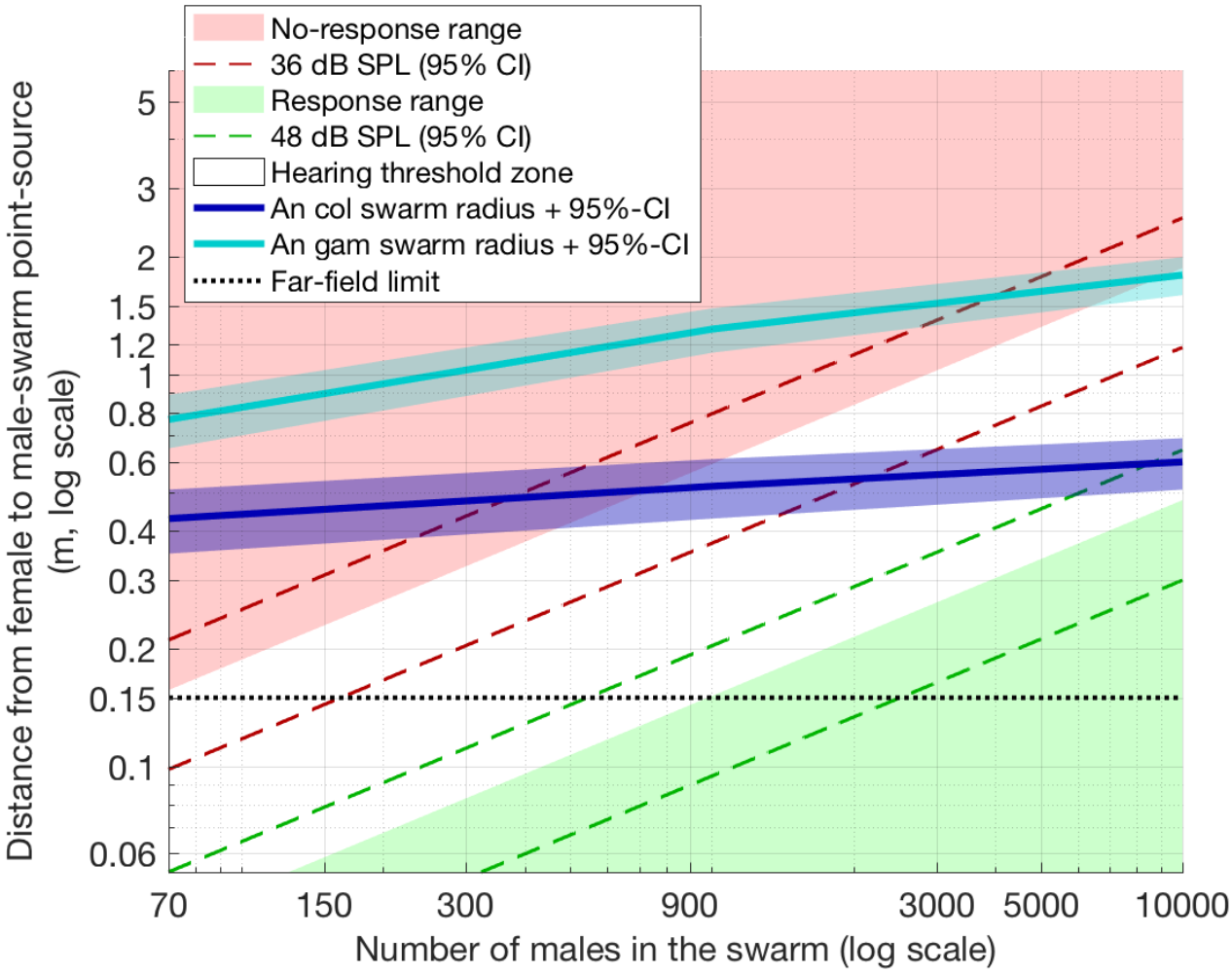
[57] Blauert, J. and Xiang, N. (2009). Acoustics for engineers: Troy lectures (Spinger)


A
270cm
192cm
SUNSET LIGHT
30 cm
15 cm
60 cm
DAY LIGHT
226cm
35 cm
89 cm
95 cm
30 cm
30 cm
57cm
SL
B
270cm
SUNSET LIGHT
30 cm
64 cm
DAY LIGHT
35 cm
91 cm
36 cm
57cm
SL
Sound-proof room walls
Mosquito net
Visual marker
White raised floor (cotton bed sheet)
Horizon line
Expected center position of the swarm
Microphone
Speaker
Camera
Camera's angle of view
IR lights
IR-illuminated backwall
Thermocouple


A
Probality Distribution (%)
Distance from the female(s) to the speaker (m)
B
X (m) Horizontal
Y (m) Vertical
Z (m) Horizontal
Lin Speed (m/s)
Ang Speed (rad/s)
Mean WBF (Hz)
Time (s)
C
Sound freq (kHz)
X (m) Horizontal
Y (m) Vertical
Z (m) Horizontal
Lin Speed (m/s)
Ang Speed (rad/s)
Mean WBF (Hz)
Time (s)


Behavioral measurements
A. Stimulus recording
r_ref=0.9m
Microphone
Sound level: L_M=20dB SPL
70-male swarm
B. Behavioral assay: sound level eliciting a response
r_ref=0.9m
4 sound levels (SPL):
L_M=20dB
L_1=26dB
L_2=36dB
L_3=48dB
Free-flying female
70-male swarm sound played-back
Acoustic predictions
C. Mapping from sound level to equivalent distance: female response distance to a 70-male swarm
r_ref=0.9m
r_1=0.5m
r_2=15cm
r_3=4cm
L_M
L_1
L_2
L_3
70-male swarm sound-source image
D. Increasing the number of swarming males: female response distance to a Nx70-male swarm
r_0,N= 0.9√N m
r_1,N=0.5√N m
r_2,N=15√N cm
r_3,N=4√Ncm
L_M
L_1
L_2
L_3
Nx70-male swarm sound-source image


A. Females
exposed to male-swarm sound
LRT: *
BH-corrected one-sample t-test
NS
NS
NS
**
Max linear-speed diff (m/s)
1.0
0.5
0.0
20
26
36
48
Stim SPL (dB)
B. Males
exposed to female-swarm sound
LRT: NS
BH-corrected one-sample t-test
NS
NS
**
NS
17
23
33
45
Stim SPL (dB)
LRT: NS
BH-corrected one-sample t-test
NS
NS
NS
NS
Max WBF diff (Hz)
100
50
0
-50
20
26
36
48
Stim SPL (dB)
LRT: ***
BH-corrected one-sample t-test
NS
NS
NS
***
17
23
33
45
Stim SPL (dB)


No-response range
36 dB SPL (95% CI)
Response range
48 dB SPL (95% CI)
Hearing threshold zone
An col swarm radius + 95%-CI
An gam swarm radius + 95%-CI
Far-field limit
Distance from female to male-swarm point-source
(m, log scale)
5
3
2
1.5
1.2
1
0.8
0.6
0.4
0.3
0.2
0.15
0.1
0.06
70
150
300
900
3000
5000
10000
Number of males in the swarm (log scale)