Title

Mosquito sound communication: assessment of ecologically relevant ranges

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Abstract

Given the unsurpassed sound sensitivity of mosquitoes among arthropods and the sound-source power required for long-range hearing, we investigated the distance over which female mosquitoes might identify species-specific cues in the sound of mating swarms. We exposed free-flying *Anopheles coluzzii* females to pre-recorded sounds of male *An. coluzzii* and closely related *An. gambiae s.s.* swarms over a range of natural sound-levels, based on a reference recording. Sound-levels tested were related to equivalent distances between the female and the swarm for a given number of males, enabling us to infer

distances over which females can hear large male swarms. We show that even for the loudest swarms, a female hears an individual male at the edge of the swarm sooner than she would hear the swarm as a whole, due to the exponential increase in sound level at close-range. We conclude that inter-mosquito acoustic communication is restricted to close-range pair interactions.

Keywords

Anopheles gambiae, free-flying mosquitoes, long-range hearing, mating swarm, mosquito hearing, speciation

MAIN TEXT

Introduction

In mosquitoes, one-on-one male-female auditory interactions have been shown to be related to pre-mating behaviour in at least 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 dipteran flies *(11)*. It is assumed that the hearing distance between a male and a female is limited to a range of a few centimetres to ~ 10 cm *(12, 13)*. However, although their auditory organs are optimized for close-range hearing, they are not restricted to a given hearing distance *(14)*, because they are sensitive to an intrinsic component of sound *(15, 16)*. Consequently, males have been shown to respond to artificially-loud sound levels of played-back single

female flight-tones metres away from the sound source *(16)*. Thus, the debate about hearing distance should be strictly linked to sound-source power and the biological relevance of the sound-source in the field. In other words, is long-range inter-mosquito sound communication *(16)* a laboratory artefact or does it also occur under natural environmental conditions? Accordingly, to be heard at distances greater than ~10 cm the source of mosquito sound must be more powerful than that of an individual mosquito. Species of mosquito that form mating swarms can produce a relatively loud sound, easily discernible to the human ear a few metres away *(17)*, by forming relatively dense station-keeping aggregations *(18)*, consisting of up to thousands of males *(19-21)*. This raises the hypothesis that a female can be attracted from a distance to swarm sounds produced by males in established swarms.

Electrophysiology measurements show that mosquito auditory organs are the most sensitive among arthropods when exposed to the sound of an opposite-sex individual *(13)*, with females generally slightly less sensitive than males *(1, 16)*; but see the following reference *(22)*. Behaviour studies demonstrate that, although females have not been shown to move toward the sound source of an individual male (phonotaxis), they exhibit phonotaxis to locate a blood host *(23)* and they alter their wingbeat frequency when exposed to male sound *(1, 3, 24)* probably to hear the male better *(3, 6)*. An important lacuna in the literature remains; can a single female hear the sound of an entire swarm of conspecific males?

The two species of the *An. gambiae s.l.* complex are African malaria vectors we worked with from species complex (*An. coluzzii* and *An. gambiae s.s.)* are under-going speciation *(25)*. These species are found in sympatry and mainly mate assortatively. Subtle

differences in swarming behaviour between these closely related species can minimize hybridization. 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. Species-specific acoustic cues in *An. coluzzii* and *An. gambiae s.s*. have been reported based on studies of single male or male-female pair interactions. Laboratory-based research characterizing the flight tones of single males flying in cages found no significant differences between the fundamental frequencies of *An. coluzzii* and *An. gambiae s.s.*, although significant differences were found in the second harmonic amplitude *(26)*. In a distinct study, the rapid wingbeat frequency modulations associated with mating *(6, 8, 9)* were found to be similar when males of both species were exposed to pure tones mimicking the female's fundamental wingbeat frequency *(27)*. However, a separate 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 that frequency-matching occurred more consistently within pairs of the same species than in hetero-specific pairs *(4)*, and frequency matching was shown to be associated with mating success in *Aedes (8)*. These close-range studies are interesting, but they beg the question as to what occurs in the lead-up to close-range interactions. To our knowledge, the response of females to the species-specific sound of distant male swarms has not been tested quantitatively yet.

Accordingly, our hypothesis is that uninseminated *An. coluzzii* female mosquitoes detect distant sounds of swarming con-specific males at natural sound levels and respond to species-specific cues in the swarm sound. 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: mosquito sounds stand out against ambient noise at least 3 m from the swarm (see supplemental information). Thus, we decided to test our hypothesis under laboratory conditions.

Materials and Methods

Experimental principle based on behaviour assay and acoustic propagation theory

We conducted behavioural experiments in an environmentally controlled laboratory fitted with a soundproof chamber (Figure 1), by presenting sound recordings of swarming males to free-flying females (see Supplementary Methods section 'Generation of sound stimuli' and 'Sound pressure level'). 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 (see Supplementary Methods section 'Environmental conditions in soundproof chamber') to initiate swarming flight (figure-of-eight loops) over a visual marker, effectively confining them to a volume of 0.06 m^3 and within a fixed distance from the source of male swarm sound (Figure 2 A). Instead of changing the distance between the test female and the male swarm, we used a range of sound levels to mimic a range of distances between a female and swarming males; we altered the apparent distance between the female and the sound-source 'image' of the played-back swarm by changing the sound level produced by the speaker according to acoustic propagation theory (see Supplementary Methods section 'Formulae between sound level and distance'). Finally, the measured results were extrapolated to estimate how far away a female mosquito can hear a swarm of a given number of males (see Supplementary Methods section 'Formula relating hearing distance

and number of individuals in the swarm'). Figure 3 summarizes the experimental principle and the raw results.

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 *(33, 34)*. 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 *(33, 34)*, 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 (Genelec 8010A) 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 *(35)* (Figure 2 B, Figure 2 C) which enabled us to determine the distance between a mosquito and the speaker emitting mosquito sound (0.9±0.2 m ,95%-CI, Figure 2 A).

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. When at least one female started to swarm robustly over the marker, a first sequence of sound stimuli was played. 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 stimuli 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.

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 *(36)*. The NRI colonies were kept in environmentally controlled laboratory rooms with a 12h:12h light:dark cycle (lights went off at 15h00),

>60% relative humidity and ~24-26°C. Larvae were fed Tetramin® fish-flakes and rice powder. Adult males and females were separated < 12h post-emergence to ensure females were not inseminated, and fed a solution of 10% sucrose in an isotonic saline *ad libitum*.

Statistics

Flight trajectories were measured by the 3D-tracking software *(35)* and wingbeat frequencies were extracted from the sound recording on Matlab (see Supplemental Methods section 'Response parameters'). 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. 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 in the reciprocal test). Each replicate was performed on a different day. The sound and video response parameters were analyzed 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. Sexes were 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 *(37)*. 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 3.5.3).

Results

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; see Supplemental Methods section ‘Sound pressure level’) 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 (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; Figure S2). Figure S2C shows the strong 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 S1, by using the *An. coluzzii* first-harmonic amplitude as a normalization factor (see Supplementary Methods section 'Sound stimuli').

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

We played-back the sound of male swarms to a group of 1-5 swarming *An. coluzzii* females at four different sound levels (Table S1) 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 2 A). 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; Figure S2).

Figure 2 B 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 4 A) show that free-flying females responded 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.

Males are known to be generally more sensitive to mosquito flight sounds than females *(13, 22, 28, 29)*. Accordingly, males were exposed to swarming female sounds, as an experimental control, to demonstrate the relevance of our protocol for assessing female responses to sound. This reciprocal test of male response to female sound stimuli resulted in a highly significant response (Figure 4 B). Indeed, for males, the effect of SPL was to increase the maximum WBF difference (LRT, χ_1^2=18.8, p<0.001), and the 45 dB 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 flight 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 S3 gives an extra eight parameters tested (acoustic and 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.

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 S2; see Supplementary 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 in 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 Supplementary 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 the acoustic prediction formulae. Figure 5 shows the female hearing ranges as a function of distance to the swarm and number of males in the swarm. The findings at 36 dB SPL (no-response) and 48 dB SPL (response) allow 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 S2 incorporates all the acoustic values related to the hearing or non-hearing of five orders of magnitude in the number of males.

Discussion

Hearing sensitivity of *An. coluzzii* females and males

Previous studies estimated the hearing threshold of tethered *An. gambiae s.l.* females was in the range 44-52 dB (particle velocity of 14±6 µm.s^{-1}, *n*=5) and tethered *Aedes aegypti* females around 55 dB SPL (*n*=10) by monitoring the activity of the Johnston's organ nerve *(4, 16)*. In the present study, the sound level eliciting a behavioural response in free-flying *An. coluzzii* females was 48±3 dB, with no response at 36±3 dB SPL. For free-flying *An. coluzzii* males, we found a significant response to 45±3 dB SPL, and a non-significant tendency at 33±3 dB, indicating that their hearing thresholds are likely to be < 45±3 dB for males. This is similar to reported values for tethered male *An. gambiae s.l.* (18±6 µm.s^{-1}, i.e. 38-39 dB SPL for the SD range in the far-field, *n*=5) from recording the Johnston's organ nerve with the antenna fibrillae extended *(4)*, and for tethered male *Culex pipiens pipiens* (32.0±4.4 dB sound particle-velocity level, n=74, equivalent to 32.0±4.4 dB SPL in the far-field) *(30)*. Overall, the results are in general agreement with previous electrophysiological studies.

To our knowledge, our study is the first report of sound sensitivity based on behavioural responses in free-flying mosquitoes. We expected a level of sensitivity higher than those reported in electrophysiological studies referred to above, since mosquitoes exhibit active

hearing *(7, 31)*, which could be triggered only by using natural behaviours (i.e. free-flight and mating behaviour). In the case of males, a possible reason for the unexpected similarity in sensitivity is that the sound stimuli were not strictly natural; we played-back the sound of a large group of swarming females (i.e. wide band tone) to test male sensitivity, which does not occur in the field. Accordingly, we still expect a greater sensitivity for free-flying males exposed to single-female sound (i.e. sharp-band tone corresponding to the sound of a single female), as noted previously *(12)*.

Number of males in swarms

In order to extrapolate the sound level of swarms that have more males than those we established under laboratory conditions, we needed to know the range of number of males in natural 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 *(19)*, 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 *(32)*, indicating the likelihood that there may be far more males in a swarm than these estimates. As many as 10,000 males in a swarm have been observed in the area of our field study (pers. com. Diabaté). We observed that larger swarms (numerically and spatially) occur in areas and times of year when *An. gambiae* s.l. population densities are highest, especially in the peak of irrigated rice growing during the wet season, and in non-irrigated areas during drier periods, swarms are regularly composed of 20-30 individuals at their peak *(18)*.

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 largest swarms, the hearing distance threshold is greater than the radius of the swarm elicited under laboratory conditions. 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 (Figure 5) shows the hearing range as 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 the 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 *(33, 34, 38)*, between the number of males and the marker size *(34)* or the marker type *(39)*. In one of our previous studies the relationship between the number of males and the swarm dimension, given a visual marker, was quantitatively measured *(18)*. *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 is to increase male density in the swarm centre and not throughout the entire volume of the swarm.

Figure S3 uses the data of five swarms of *An. coluzzii* and seven swarms of *An. gambiae s.s.* from *(18)* to predict swarm radius as a function of the total number of males and of two 'layers' of a swarm (50% and 95% of the most central males), with a random intercept and slope model to predict the radius of swarms consisting of greater number of males, up to the order of thousands. We consider the swarm radius to be defined by the radius of the sphere which encompasses 95% of the males nearest the swarm centroid. The results for *An. coluzzii* are consistent with observations of swarms with thousands of males which are usually < 1 m in radius *(39)*. For *An. coluzzii*, the predicted mean swarm 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 S3).The swarm radius

of an *An. gambiae s.s.* swarms is slightly larger for small swarms, but the predicted radius for large swarms is much larger (Figure S3).

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's 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 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 the same responses to the same environmental cues as their male counterparts, thereby enhancing the likelihood of encountering con-specific males. It is possible that long-range cues are not necessary for the female to arrive at a swarm site. In that case, females may use the close-range sound of a chasing male to avoid being inseminated by the wrong species *(4)*, however, investigations on long-range cues such as vision *(34)* or olfaction *(40, 41*) should be pursued in future research.

Long-range hearing is unlikely in inter-mosquito communication

To our knowledge, male swarms are the only serious candidate source of sound which is loud enough and fits the tuning of the mosquito organs to enable inter-mosquito acoustic communication at long-range. This study presents data that rejects the hypothesis that *An. coluzzii* female can hear a male swarm before entering it. It is also unlikely that a male hears a male swarm at long-range because, although males are more sensitive to sound than females *(13)*, their hearing organ is not tuned to male wingbeat frequencies. Finally, as we chose a mosquito species which produces large and loud swarms, we can claim that long-range interspecific acoustic-communication in mosquitoes is unlikely to occur before the female mosquito enters a swarm.

This study does not eliminate the hypothesis that long-range hearing can be used for host location *(23, 42)* or for predator avoidance *(22)*, providing the host/predator sound is loud enough and tuned to mosquito hearing.

References and Notes

(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) 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.

(13) 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.

(14) 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.

(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*, 407–429.

(16) 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.

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

(18) 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.

(19) 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.

(20) 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.

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

(22) 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.

(23) 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.

(24) 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*.

(25) 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.

(26) 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 *42*, 375–384.

(27) 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.

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

(29) 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.

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

(31) 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.

(32) 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.

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

(34) 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.

(35) 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.

(36) 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.

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

(38) 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.

(39) 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.

(40) 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

(41) 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

(42) 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.

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Competing interests

The authors declare no conflict of interest.

Data and materials availability

Custom audio-video code for parameter-extraction, audio-video synchronization (Matlab files), custom statistics code for data analysis and figure plot (R files), and dataset (Text file) are available at http://dx.doi.org/10.17632/hn3nv7wxpk.1. Raw sound files and tracked flight dataset are available on request.

Figure captions

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 *(34)*.

(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 S2 and Figure 5.

(B) *Anopheles coluzzii* response to highest sound-level *An. coluzzii* and *An. gambiae* sound-stimulus over 27 s of recording. 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 colored 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 color gradient represents the sound level given a frequency and a time (the darker the color, 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.

Figure 3. Steps to evaluate the distance a female mosquito can detect the sound of an *An. coluzzii* male swarm of a given number of individuals. This schematic explanation shows how methodologies from behavioural assays ('measurements') and acoustic theory ('predictions') were employed in this study, based on details

for *An. coluzzii* sound stimuli). The same procedure was repeated with sound stimuli of *An. gambiae s.s.* and the reciprocal experiment was performed with males exposed to sound stimuli of a female-swarm for both species.

(A) First, the reference stimulus (sound of 70 males swarming) was recorded at 0.9 m from the male swarm, producing a sound pressure level of 20 dB SPL.

(B) Second, this stimulus was played-back to 1-5 swarming (station-keeping) females in free-flight at four different sound levels (20, 25, 36 and 48 dB SPL) as measured at the mean females' distance to the speaker (see Figure 2 A). Only the loudest stimulus produced a response in females.

(C) Third, assuming the swarm sound emitted from the speaker to be a point source, and given the natural sound level of a 70-male swarm (L_M) at a distance of 0.9 m (r_{ref}), we can compute the natural distance to a similar swarm corresponding to the other three sound levels (see Methods).

(D) Fourth, the effect of multiplying the number of swarming males per N over the female hearing distance is predicted (see Methods).

Figure 4. Results of behavioural experiment. One flight parameter (maximum linear speed difference, top row) and one acoustic parameter (maximum wingbeat frequency difference, bottom row) were extracted from flight tracks and wing-flapping sound for statistical analyses of female data (left column) and male data (right column). 'Zero' (green dashed line) indicates 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). Black disk in each distribution shows mean and standard error.

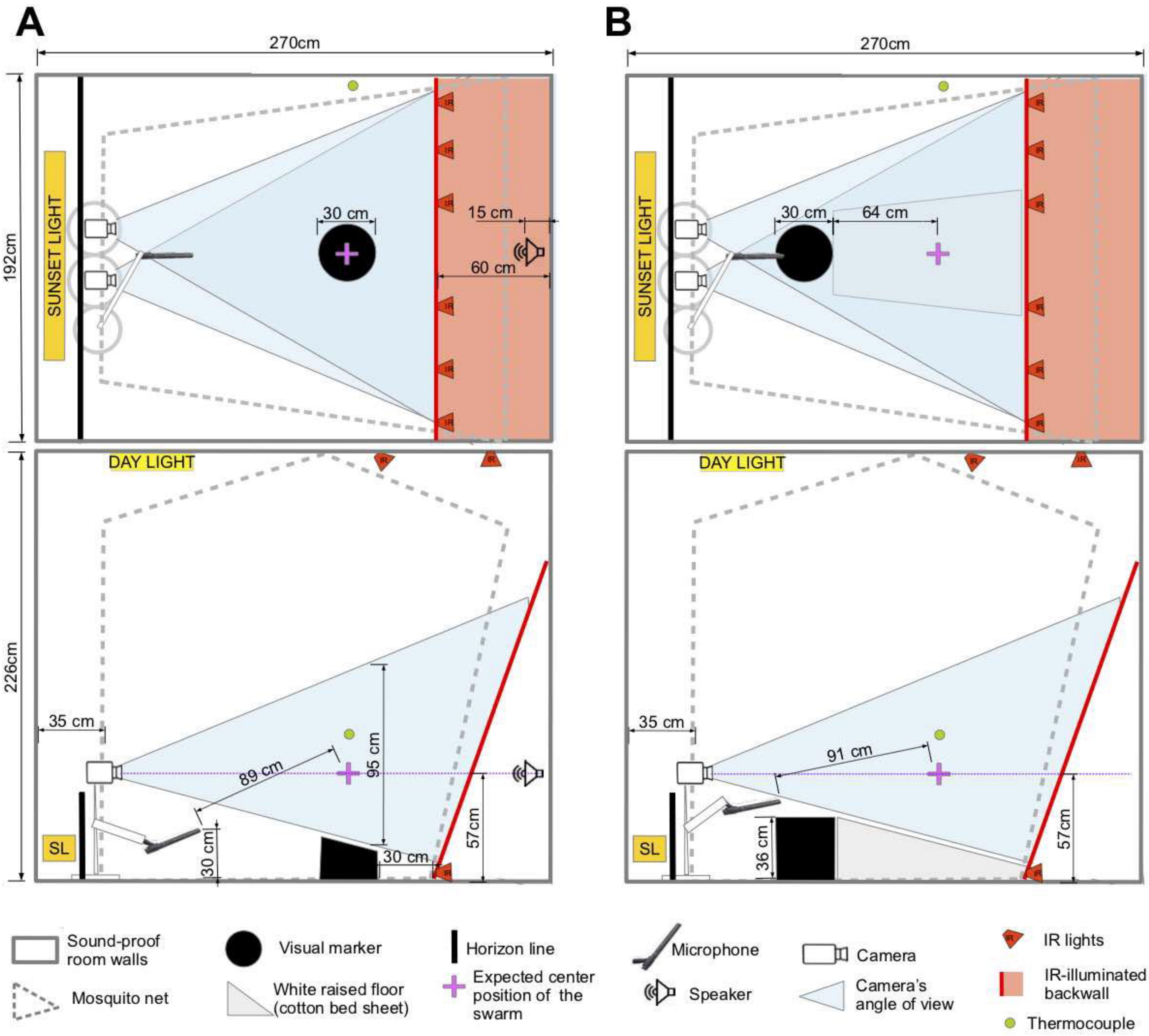
Two independent types of statistical tests were performed. Stepwise removal of terms was used for model selection, followed by LRT (likelihood ratio tests, see orange annotation for each of the four plots) *(42)*. An additional one-sample t-test with BF-correction for multiple comparisons (see green annotations above each boxplot) was performed independently for each distribution to measure significance of the mean to zero value (dashed green lines).

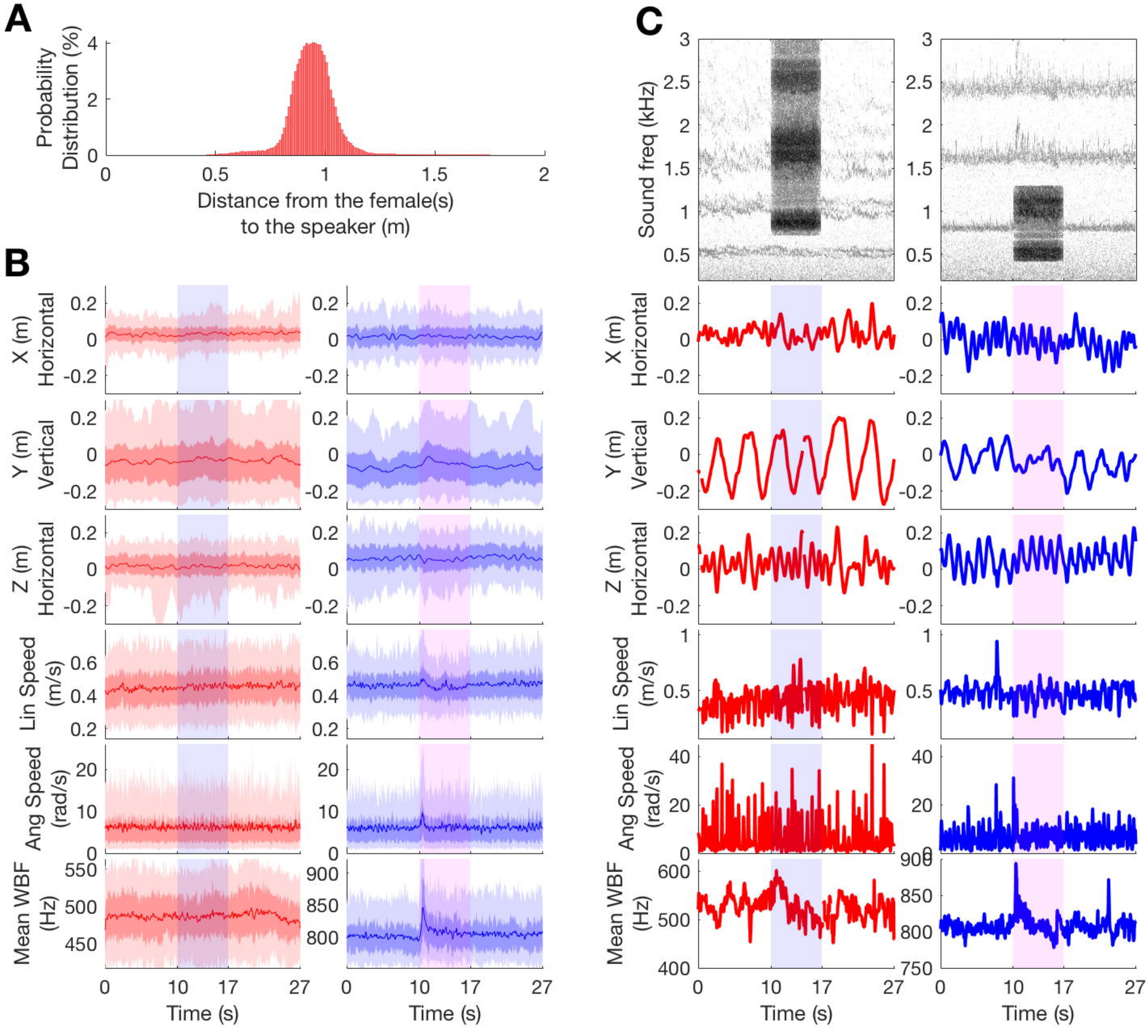
(A) Female *An. coluzzii* responses to *An. coluzzii* male-swarm sounds at four SPLs. For the parameter related to linear speed, there was an effect of SPL (LRT χ_1^2=4.34, p=0.037) with a significant BH-corrected one-sample t-test (t(22)=3.6, p=0.0067, *mean*=0.04 m/s).

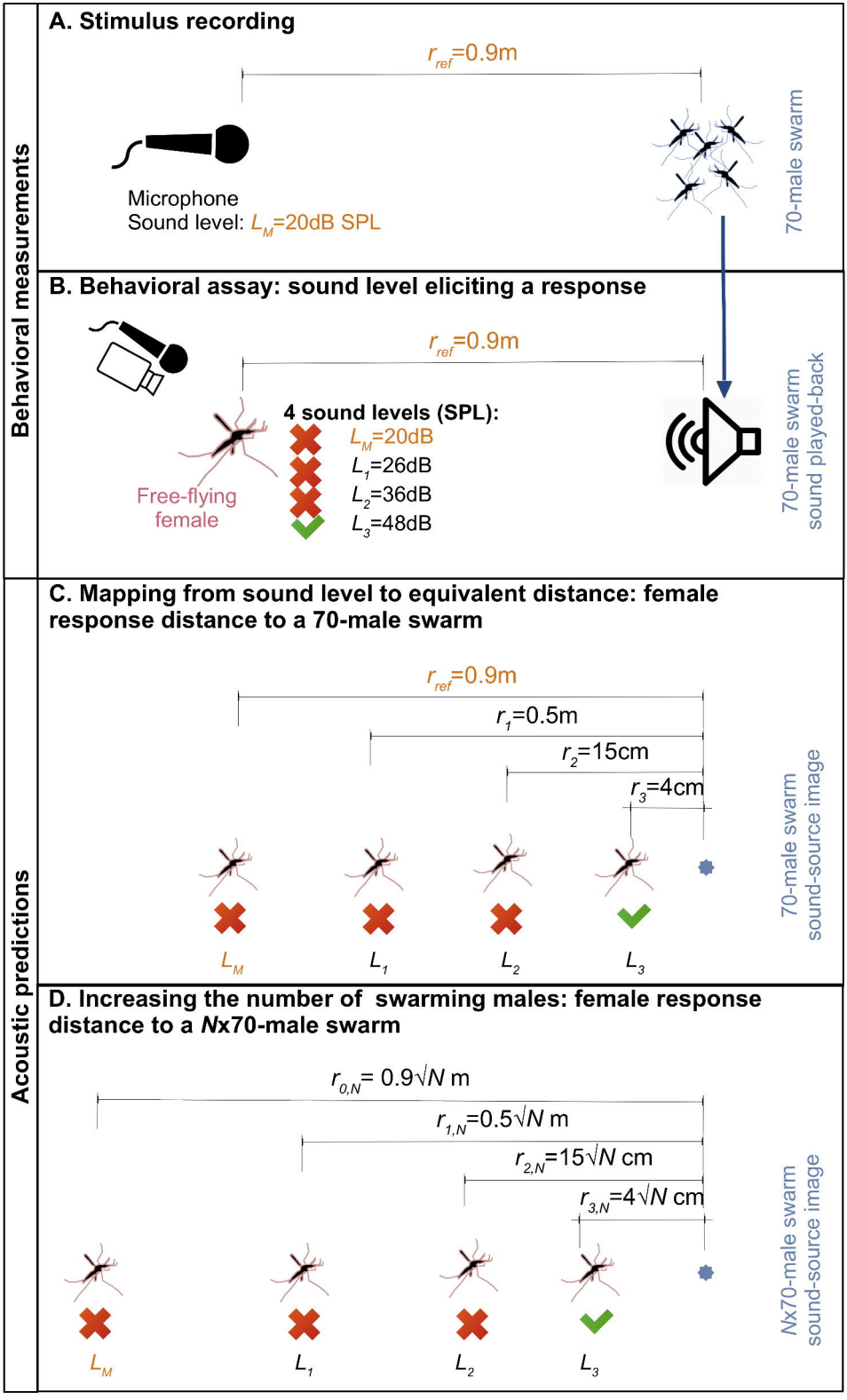
(**B)** Male *An. coluzzii* responses to *An. gambiae* female-swarm sounds at four SPLs. For the maximum wingbeat frequency, there was a strong effect of the SPL (LRT χ_1^2=18.87, $p < 0.001$), with a highly significant one-sample t-test for the 45 dB SPL distribution (one-sample t(17)=5.45, BH-corrected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

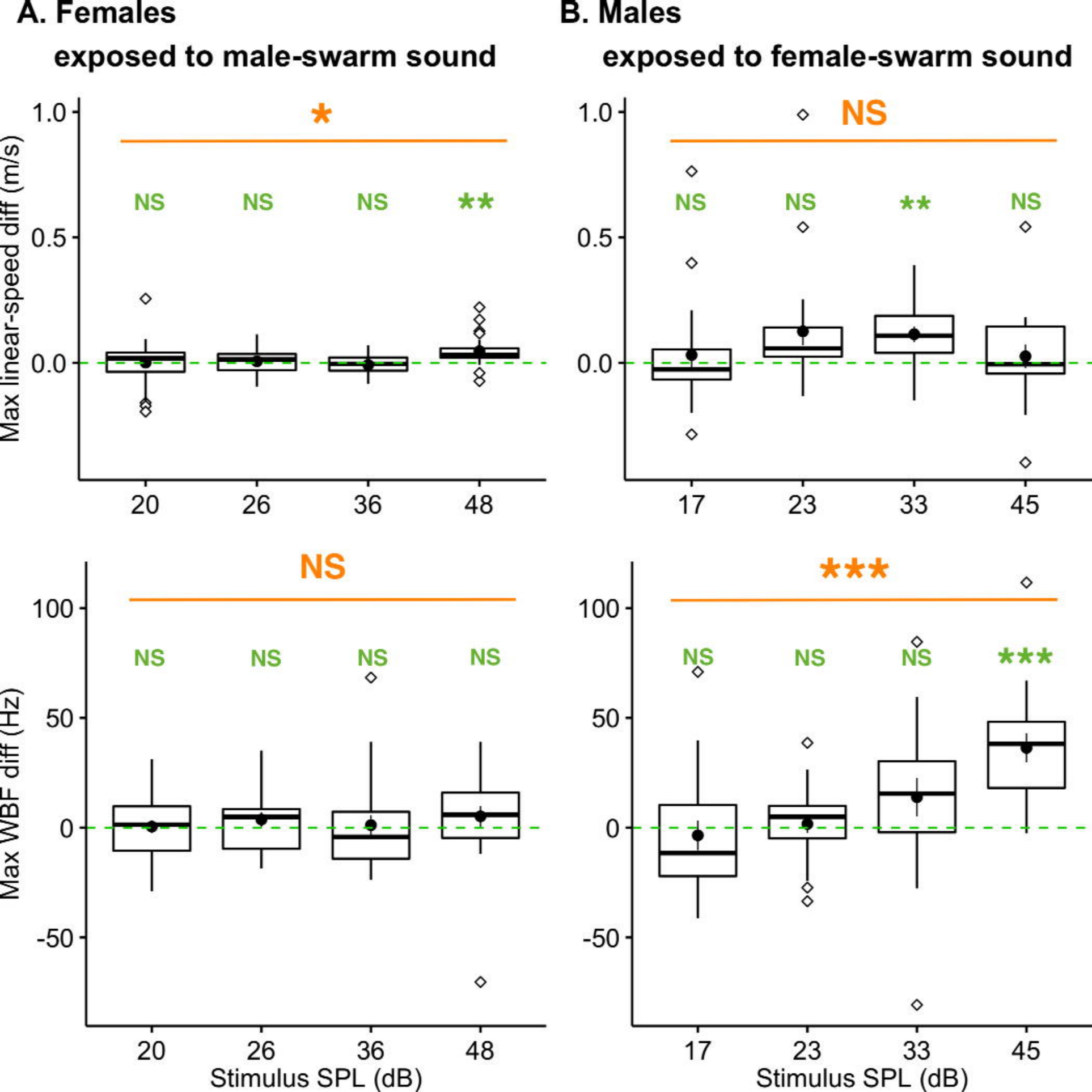
Figure 5. Estimated hearing-distance and swarm radius as a function of the number of males in the swarm. Green area covers the minimal response range, while red area indicates the minimal non-response range of a female to male swarm sound for both species, as a function of the number of males in a given swarm (X-axis) and the distance to the swarm centre (Y-axis). These areas are based on 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 95% confidence interval (dashed lines). The swarm is assumed to be a point source

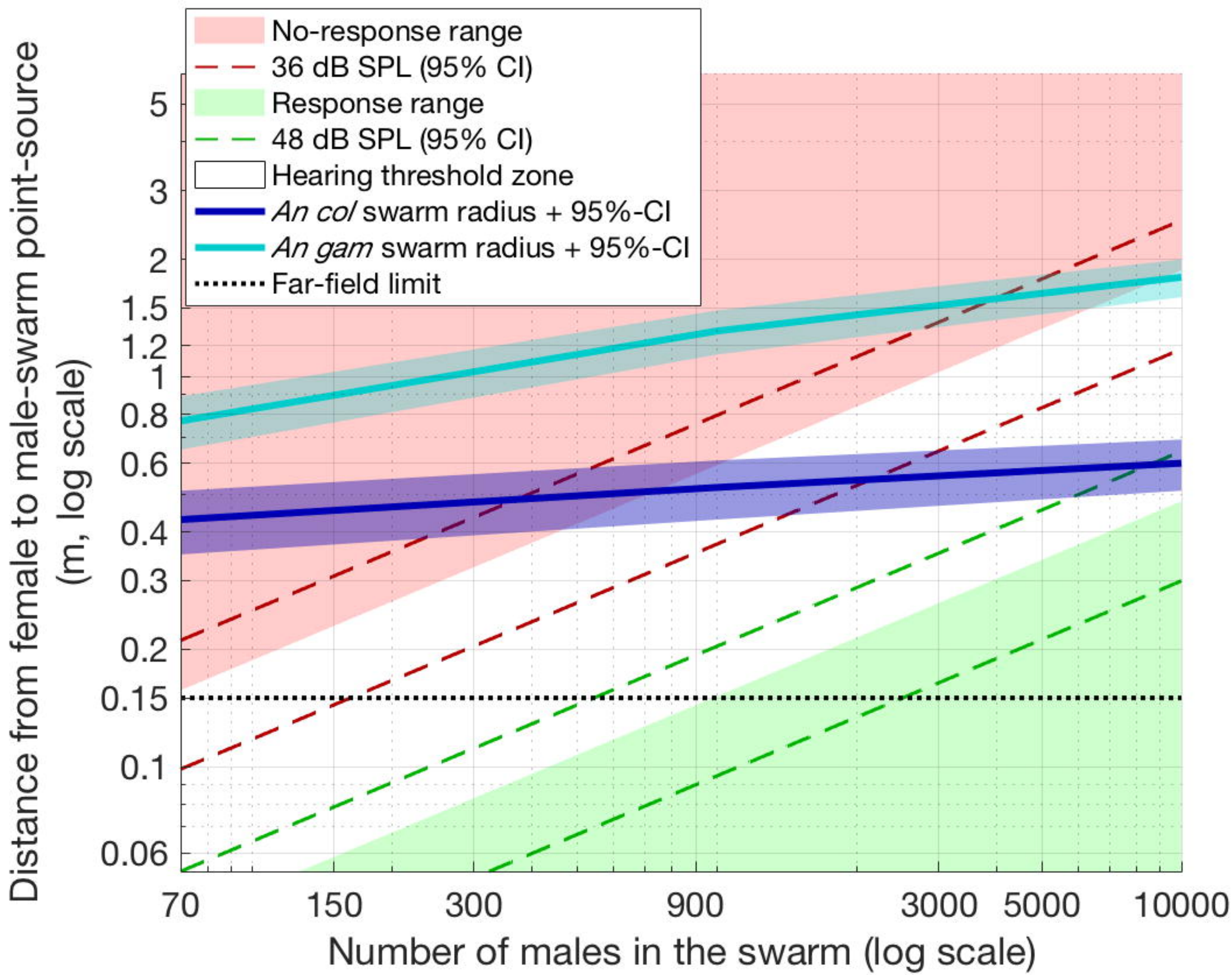
in the model and only the far-field component of the particle velocity is considered (see Supplementary Methods section ‘Acoustic assumptions and formulae’): above 0.15 cm (black dotted line), the near-field component of the particle velocity is negligible (< 1 dB); below 15 cm the smaller the distance, 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 S2).


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
Probability 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)


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√N cm
L_M
L_1
L_2
L_3
Nx70-male swarm sound-source image


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