FRONT MATTER

Title

Full title: "Mosquito sound communication: assessment of ecologically relevant ranges"

Short title: "Limits to ranges of mosquito sound communication"

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One-sentence summary

Given the exceptional hearing sensitivity of mosquitoes at close-range that enables auditory interactions between pairs (~ cm) and at long-range with artificially loud sounds (~ meters), we question how far away female mosquitoes can hear the sound of a large group of males (mating swarms) and how likely they can detect species-specific acoustic cues before entering a mating swarm.

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.

MAIN TEXT

Introduction

In mosquitoes, one-on-one male-female auditory interactions have been shown to be related to pre-mating behavior 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 centimeters 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 meters 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 meters 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)*. Behavior 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 behavior 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. Next, we conducted behavioral experiments in an environmentally controlled laboratory fitted with a soundproof chamber (Figure 1), by presenting sound recordings of swarming males to free-flying females. 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 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 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 that were played-back through the speaker according to acoustic propagation theory. 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 summarizes the experimental protocol and the raw results.

Results

Mosquito sounds stand out against ambient noise at least 3 m from the swarm

Relative flight-sound intensities of wild male *An. coluzzii* swarms were measured to characterize the sound profile of typical male swarms in relation to the background sounds of other twilight-active organisms, including humans, near rice fields in the Bama 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 (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 center 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 center 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 center, 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 1, by using the *An. coluzzii* first-harmonic amplitude as a normalization factor (see 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 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; 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 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 4B). 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 S1 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 behavioral 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 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 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 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 (see 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 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 2 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 behavioral 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 behavioral 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 behaviors (i.e. free-flight and mating behavior). 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 *(33-35)*, between the number of males and the marker size *(34)* or the marker type *(36)*. 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 center, 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 center, ~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 center 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 *(36)*. 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 center (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 very weak (Figure S2). Second, our behavioral 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, especially when the density of swarms is high *(37)*. In that case, 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 *(34)* or olfaction *(38, 39)* 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, 40)* or for predator avoidance *(22)*, providing the host/predator sound is loud enough and tuned to mosquito hearing.

Limitations of the experimental design

The main limitation of our experimental paradigm is that we used swarming females to test their response to male-swarm sound (see Methods section 'Experiment paradigm'). This means that when females were exposed to the swarm sound in the laboratory, they were already flying above a swarm marker, while in the field they would have been

responding to environmental stimuli leading them to a swarm marker. This may have induced females to continue swarming over the marker without altering their behavior when male sound was played-back, effectively waiting for males to approach the marker. However, we monitored all the likely flight variables (flight velocities, positions and wingbeat frequency changes), so it is unlikely that we overlooked any female reactions to sound and unlikely that females would not respond if they could hear a male sound.

Materials and Methods

Experimental paradigm

***Principle*.** It is known that male mosquitoes are attracted to a source of female flight tones, either the sound of a live female or a speaker emitting female flight tones. Males and females are both tuned to the 'difference-tone' between their respective wingbeat frequencies, which provides a relatively robust means of locating each other in mating swarms *(1-6, 8, 9, 41)*. These observations raise the hypothesis that females may be attracted to the sound of male swarms, and if so, might they hear larger swarms from a long distance (> 1m)? *(18)*. To test this possibility, 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. Methods section 'Formulae between sound level and distance' explains how to predict apparent distances between the receiver and the

sound source ‘image’ based on sound pressure levels. By ‘image’, we mean that, while the distance from the female to the speaker was always the same (±0.2 m), the sound levels 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 behavior; 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 that reproduced the males’ swarming flight tones was placed 0.9 m from the center 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 as test subjects and for production of sound stimuli. We had no difficulty in triggering robust swarming behavior 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, which confirmed that the experimental protocol was valid (male responsiveness to female-swarm sound was robust). Although it was more difficult to induce *An. gambiae s.s.* females to swarm, we recorded the sound of a swarm composed of 4 females at a time, *versus* 30 females at a time for *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. 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 *(42)*. The NRI colonies were kept in environmentally controlled laboratory rooms with a 12h:12h light:dark cycle, >60% relative humidity and ~24-26°C. Larvae were reared in plastic breeding trays filled with ~1 L of isotonic saline. Four days after egg hatching, four groups of 90 larvae were transferred to four fresh breeding trays filled with isotonic saline. Larvae were fed Tetramin® fish-flakes and rice powder. Adult males and females were separated < 12h post-emergence to ensure females were not inseminated. 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*.

Recording environment

Soundproof chamber. 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 covering all surfaces, producing a reverberation time ≤ 0.07 s for frequencies above 200 Hz

(measurements conducted in empty room by IAC Acoustics, manufacturers). Figure S4C displays the sound level per octave band when the soundproof chamber was silent (dashed lines). At low frequencies (<176 Hz), the sound pressure level (SPL) was ≥ 25 dB (ref 20 µPa). Between 176 Hz (lower limit of the 250-Hz octave band) and 1.4 kHz (upper limit of the 1-kHz octave band), i.e. the frequency range within the *An. coluzzii* mosquito's response is the highest *(3)*, the SPL was < 14 dB, which is 8 dB less than the quietest sound stimulus used in the study.

Sound monitoring. The wingbeats (aka, 'flight tones') of mosquitoes in the laboratory were recorded with a weatherproof microphone (Sennheiser MKH60; RF-condenser; super-cardioid polar pattern at 0.5-1 kHz, with amplitude decrease of > 15 dB beyond 90° from the microphone head) directed toward the swarm location. The microphone was located at a distance of 0.9 m from the center of the swarm area (Figure 1) and plugged into a Scarlett 18i8 audio interface on a Windows7 computer running Pro Tools First 12.8 (Avid Technology, Inc).

Flight track recording. The 3D flight trajectories of mosquitoes were captured at a sampling rate of 50 Hz with Trackit software (SciTrackS GmbH, Switzerland, *(43)*). Two video cameras (Basler, ace A640-120gm) were fitted with wide-angle lenses (Computar, T3Z3510CS, 1/3" 3.5-10.5mm f1.0 Varifocal, Manual Iris) to maximize 3D volume of video-tracking and infra-red bandpass filters (>840nm, Instrument Plastics Ltd UK). Ten IR lights (Raytec RM25-F-120 RAYMAX 25 FUSION) enabled the tracking system to detect flying mosquitoes as silhouettes against an IR-illuminated white back-wall made of thin cotton cloth (Figure 1). The dual IR/white lighting system enabled constant bright IR light (invisible to mosquitoes) for video-tracking flying mosquitoes. The 3D-flight

trajectories were smoothed using a cubic spline interpolation at a sampling frequency of 200 Hz on Matlab (version R2017a).

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), centered ~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 ~0.2 to 3 m away. The recordings were produced with an RF-condenser microphone (MKH 60 P48) plugged into a Scarlett Solo sound card, captured via Audacity (v2.2.1) software on a MacOS operating system (v10.12.6).

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 (NATURO, 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 (LEDs 5630, HMCO FLEXIBLE dimmer, and PLeD

software, custom-built). Lamps were arranged to mimic sunset lighting; a sharp horizon ~40 cm above the floor on one side of the room provided a ’sunset’ feature and a gradually decreasing light intensity with increasing height above the floor (Figure 1). Ceiling lights were dimmed over 30 min, while the horizon lights started to dim 10 min before the ceiling light turned off, whereupon the light intensity decreased gradually over 10 min and finally remained constant for 1 h to provide a constant very dim light intensity that favored prolonged swarming flight during the experiments. The visually conspicuous matt-black swarm marker triggered swarming behavior. The marker consisted of a circle of matt-black paper (Ø=30 cm), placed > 30 cm away from the closest netting. The location and height of the swarm marker was arranged according to the swarming behavior of each species in order to induce swarming flight at the same location in the room for the two species: therefore, the swarm marker for *An. gambiae s.s.* was raised by 6 cm and moved 0.8 m horizontally in the opposite direction of the dusk light, compared to the position of the *An. coluzzii* swarm marker (Figure 1), as previously reported *(34).*

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

Generation of sound stimuli

Recording of the reference sound-stimuli. Swarms of *An. coluzzii* females or males, and *An. gambiae s.s.* females or males were recorded in the soundproof chamber (Figure 1 and Figure S2). About 300 x 4-7 days-old males or 1-4 x 2-6 days-old females were released in the swarming arena two days before the experiment to acclimatize before their flight sounds were recorded. The standard environmental conditions in the room were: 12h:12h light:dark cycle with a 1h artificial dawn/dusk transition in light intensity, 21-28°C and ~60-75% RH. One recorded 7s sequence was selected for each sex/species, which began ~10 min after the first male/female started to swarm (Sound S1: *An. coluzzii* male swarm; Sound S2: *An. gambiae s.s.* male swarm; Sound S3: *An. coluzzii* female swarm; Sound S3: *An. gambiae s.s.* female swarm). The swarms were composed of 30-70 individuals (except for the *An. gambiae s.s.* female swarm: 4 individuals) flying in loops 0.3 m above the floor level with a horizontal diameter of 0.2 m. The start and the end of the recorded-sound amplitude were multiplied by a fading window of 1 s to avoid creating sound artefacts due to the signal truncation, and to make the stimulus more natural, i.e. mimicking the male swarm sound amplitude which continuously increases when the female gets closer to the swarm.

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

To playback the stimuli at natural sound levels, we first played them back in the same room and at a distance to a speaker (Genelec 8010A) identical to the distance 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, and using the same software and hardware settings).

Figure S2 gives the sound spectrum of the swarm sounds used in the assays. 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 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 silence

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

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

Response Parameters

Wingbeat parameter extraction from flight sound. Wingbeat frequency was tracked every 40 ms using a Fast Fourier Transform algorithm (256-ms FFT, Hanning-windowed). Since females and males do not have the same wingbeat frequencies and we always played-back opposite-sex sound stimuli to individuals, we had to operate differently for each sex. For females, their fundamental wingbeat frequencies were tracked between 370 Hz and 660 Hz (given that the mean female wingbeat frequency was 487 Hz) to avoid overlap with played-back wingbeat harmonics of swarming males (female wingbeat frequencies were always lower). For males, only the two first harmonics of female sound stimuli were played-back as explained above and then the male's third harmonic (3 x fundamental frequency) was tracked between 2190 and 2920 Hz (given that the mean male's third harmonic without sound stimulus was 2409 Hz), since it is the lowest harmonic that does not overlap with the sound stimulus (example of spectrogram in Figure 2C). When several wingbeat frequencies were tracked due to the presence of several mosquitoes over the swarming marker, their wingbeat frequencies were averaged. Male

harmonics were divided by three to get the 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 for each individual recording to reduce noise and since we are interested in the response difference between sound stimulation and no stimulation.

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 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 $avel = \Delta\theta / \Delta t$, 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:

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

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 tracked with the software Trackit every 2 ms when the light was switched off (i.e. creating a dark silhouette) simultaneously with the sound and was automatically detected on Matlab. 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. 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 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 *(44)*.

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).

Sound pressure level (SPL)

Measurement. Stimulus SPLs were measured at the females’ swarming position with a sound meter (Casella, CEL633C1, Class 1) set as follows: reference pressure of 20 µPa; no octave weighting (i.e. dB Z); slow octave time-constant (IEC 61672-1: 2002); octave and third-octave bands; calibrated twice a day (CEL-120/1, Class 1, at 94 dB / 1 kHz) before and after each measurement. The 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 behavioral 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 (Table 1; and Figure S4 for all third-octave values). This method enabled us to compare our sound stimulus levels to pure sounds used in previous studies on hearing sensitivity 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 which 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 hypothesis to simplify the problem. In a room, however, sound level can decrease (destructive interference) or increase (constructive interference) independently of the distance to the speaker. This effect was reduced by the semi-absorbent walls of the room but was still present because the room was not an anechoic chamber. Boundary-induced 'comb filtering' was reduced by locating the speaker close to the wall, but acoustic room modes were still present. We played-back the *An. coluzzii* male and female swarm stimulus and measured the sound level in a 0.2 m diameter sphere around the expected swarm center. The maximum error was about ±1 dB for the female sound stimulus and ±2 dB for male swarm stimulus. We ignored any reverberation effect, as we estimated its effect to < 0.4 dB at 800 Hz, using an acoustic room model of the ratio of direct and reverberant sound, given the reverberation times of the room provided by the soundproof chamber designer (IAC Acoustics Ltd).

The last type of measurement uncertainty arises when the estimated sound level should be estimated from the mosquito position. SPLs were measured at the expected center of the station-keeping swarm-flight of the mosquito. However, the distance between the female and the speaker varies between 72 and 113 cm (95%-CI, Figure 2A) due to the females' swarming-flight pattern and sound level changes, accordingly. We computed this error by

considering the fluctuating distance between the female mosquito and the speaker using equation 4.

Finally, using standard uncertainty-propagation theory, we calculated the total error of sound pressure level L_i at the location of the female exposed to male sound, resulting in a total error of ±3 dB SPL for the SPL. This error is considered to be conservative (at least 95%-CI) and were used to interpret the results of the experiments. For errors related to the difference between what we measured (sound pressure) and what mosquitoes detect (particle velocity), see Methods section '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 center than at the periphery *(18)* (Figure S3). 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 center. Swarms can be ovoid *(34, 18)*, but this is not an issue for our point-source assumption, because the oval dimension is often 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 (SVL) *(45)*. 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.

The root-mean square value (RMS) particle velocity v_{RMS} and the RMS sound pressure p_{RMS} can be related as follows for a point source radiating spherically (air impedance Z_{air}(28°C) = 408 N.s.m^{-3}; sound speed c(28°C)=348 m.s^{-1}) *(46)*:

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

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

$$L_v(r) = L_p(r) + \frac{1}{2}log_{10}\left(1+\left(\frac{c}{2\pi f r}\right)^2\right) \qquad (3)$$

Therefore, SVL 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 = 745 Hz (the smallest frequency value of the group of first harmonics of the swarming males at -12 dB below the peak at 857 Hz, Figure S2), then we can calculate that for r > 15 cm, $L_v(r)$ = $L_p(r)$ 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 SVL is, compared to the SPL. At 4 cm from the sound source, the SVL 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 SVL is equal to the SPL. As a consequence, monitoring the SPL 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 SPL label) to the virtual sound source (i.e. the played-back male swarm, or sound-source image) knowing the SPL L_i at the female's position at a distance r_i from the virtual swarm, and the SPL L_{ref} at position r_{ref} = 0.9 m known to be the distance to the reference sound stimulus source. The physical sound source is the speaker, at fixed distance r_{ref} from the swarm center (then from the female ± its movement above the swarming marker). The SPL is set to reproduce a natural swarm sound where its 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. $p_{RMS,i} \propto \frac{1}{r_i}$) *(46)*. Then the SPL difference ΔL_i can also be expressed as follows:

$$\Delta L_i \stackrel{\text{def}}{=} L_i - L_{ref} = 20log_{10}\left(\frac{r_{ref}}{r_i}\right) \qquad (4)$$

Then from equation 4 we get the distance r_i to the sound-source image as a function of the difference level ΔL_i and of 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:

$$r_i = r_{ref} 10^{\frac{-\Delta L_i}{20}} \qquad (5)$$

SPL 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, ($I \in \{1,2,3\}$) can be calculated from equation 5: they correspond to the SPLs 20 dB, 26 dB, 36 dB and 48 dB of a point-source 70-male swarm at a distance of 0.9 m, 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 the latest is unrealistic for the smallest distance (4 cm), 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 behavior 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 their behavior (i.e. non-swarming flight) could not have been controlled. As a consequence, we decided to use the 70-male swarm in the behavioral 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 behavioral 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 $10log_{10}(N)$ *(47)*. Let's assume a Nx70 -male swarm can be modelled as a single point (see 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 $r_{i,N\text{x}70}$ of a Nx70-male swarm with same SPL L_i as a 70-male swarm at distance r_i, knowing that the Nx70-male swarm has a SPL L_i + $10log_{10}(N)$ dB at distance r_i, by the following formulae derived from equation 4 (values are presented in Table 2 for a 300, 1,500, 6,000 and 10,000-male swarm):

$$r_{i,N\times 70} = r_i 10^{\frac{-\left(L_i-\left(L_i+10log_{10}(N)\right)\right)}{20}} = \sqrt{N} r_i \qquad (6)$$

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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.

Figures and Tables

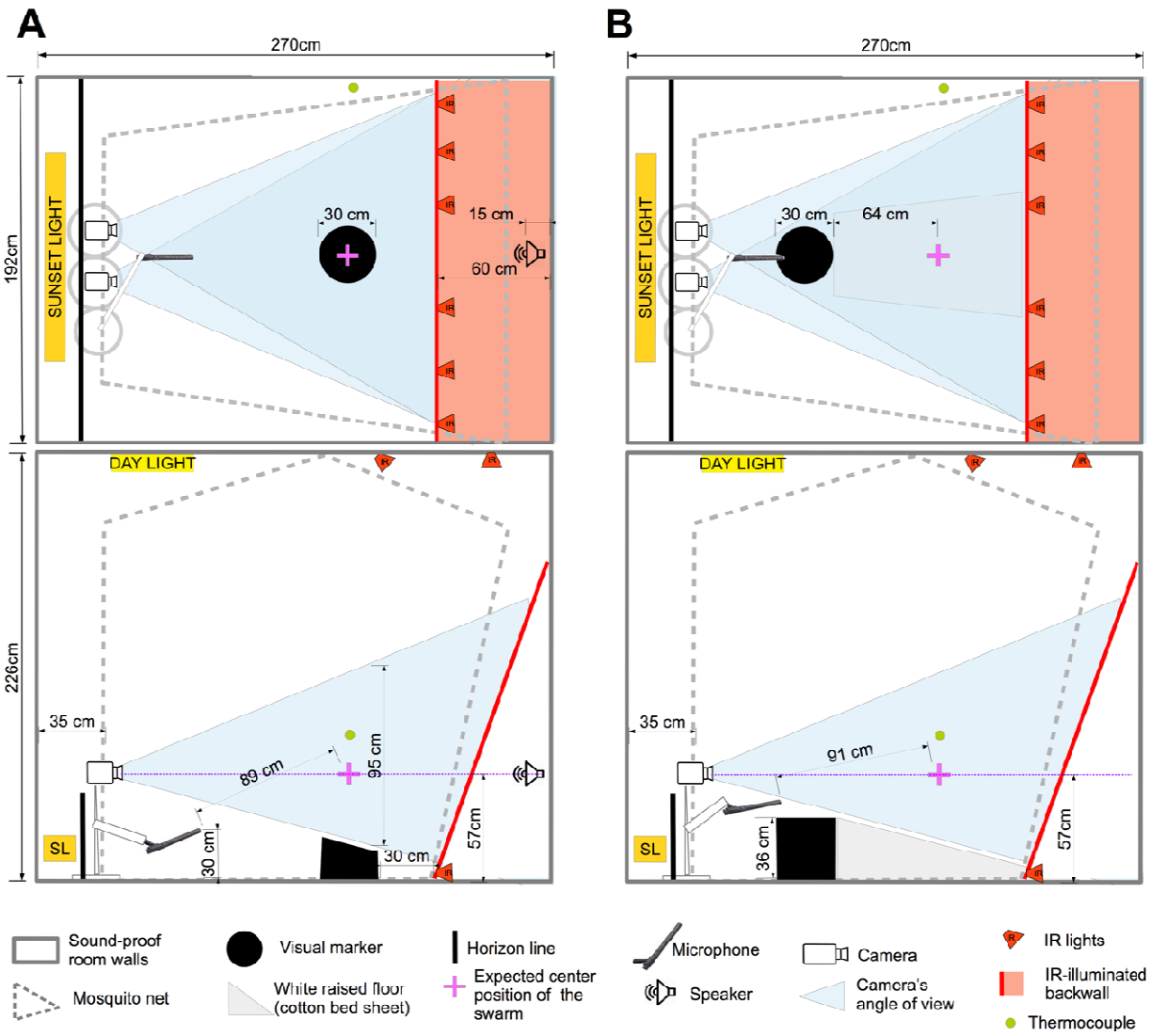


Figure 1. Soundproof chamber setup for recording sound and video of mosquito behavior. Bird's-eye and side views of soundproof chamber. Two IR-sensitive cameras fitted with IR pass filters tracked flying mosquitoes as black silhouettes against evenly lit IR- background. Separate lighting system provided 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 behavioral 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.

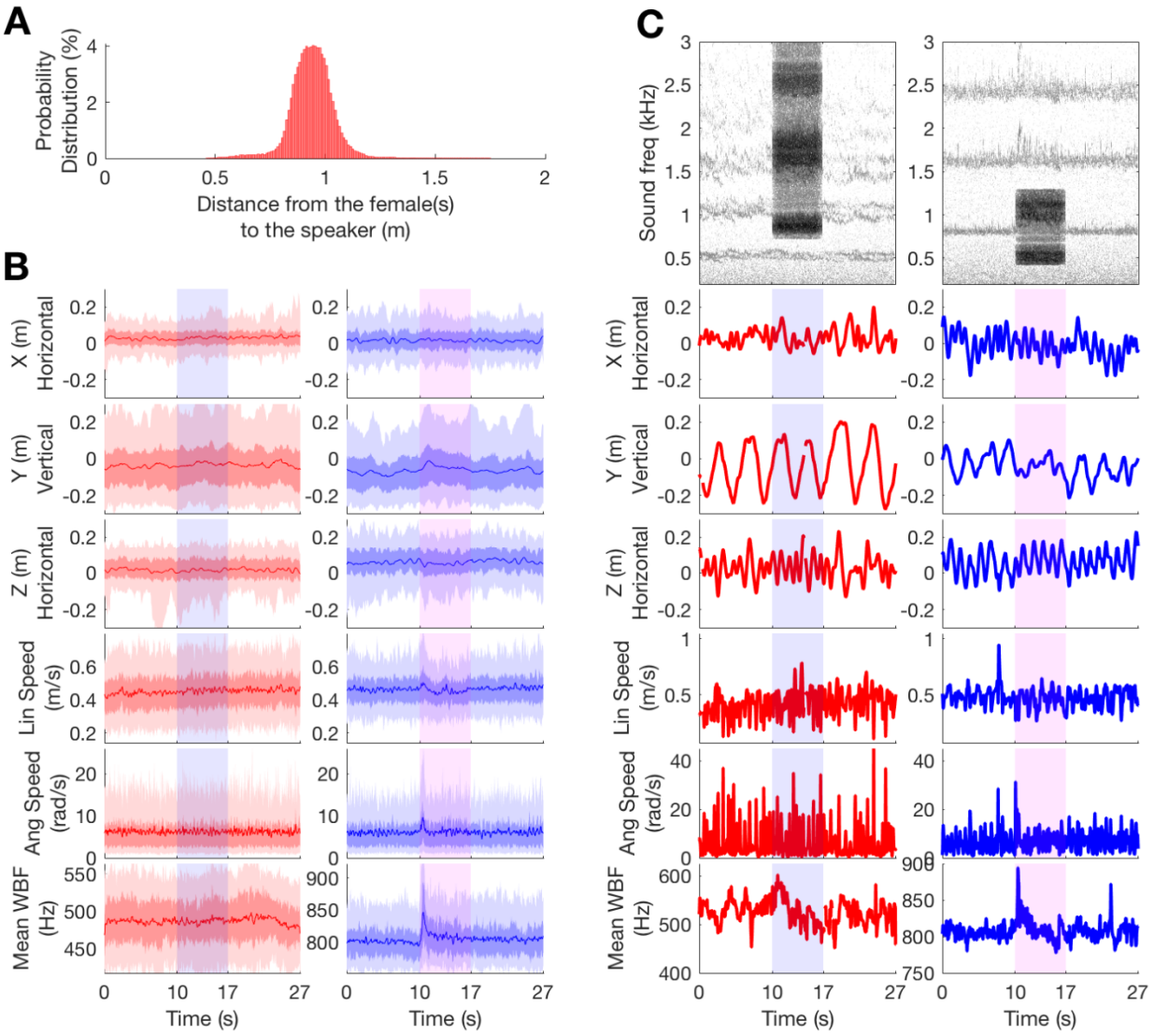


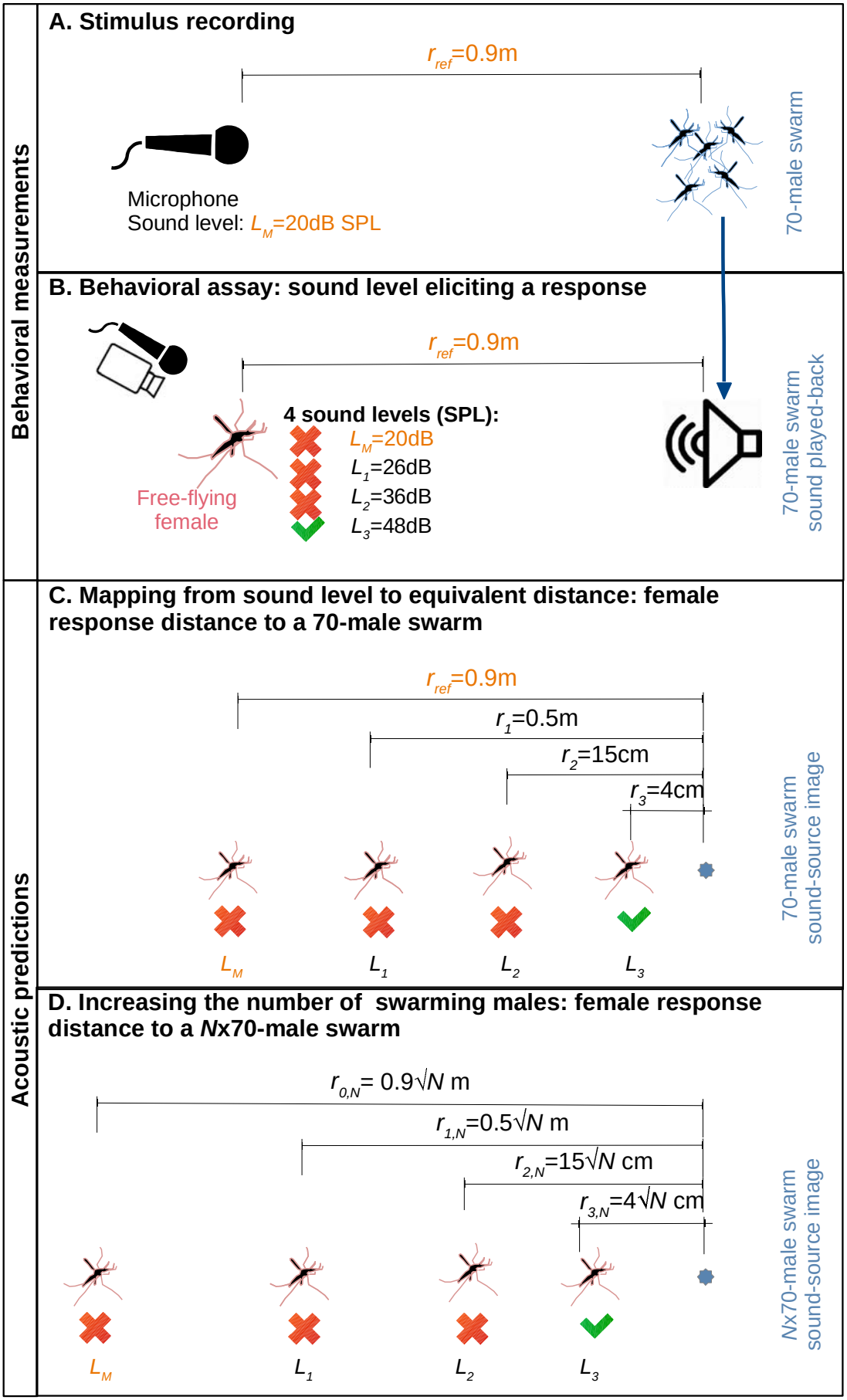
Figure 2. Flight and sound responses of females and males to sound stimuli. Female (red) and male (blue) flight-characteristics and wingbeat-frequencies before, during and after playback of male (blue rectangle) or female (red rectangle) sound stimuli.

(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) *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 behavior, is evident for males and females in 'XYZ' plots.


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

153

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 behavioral 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 2A). 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).

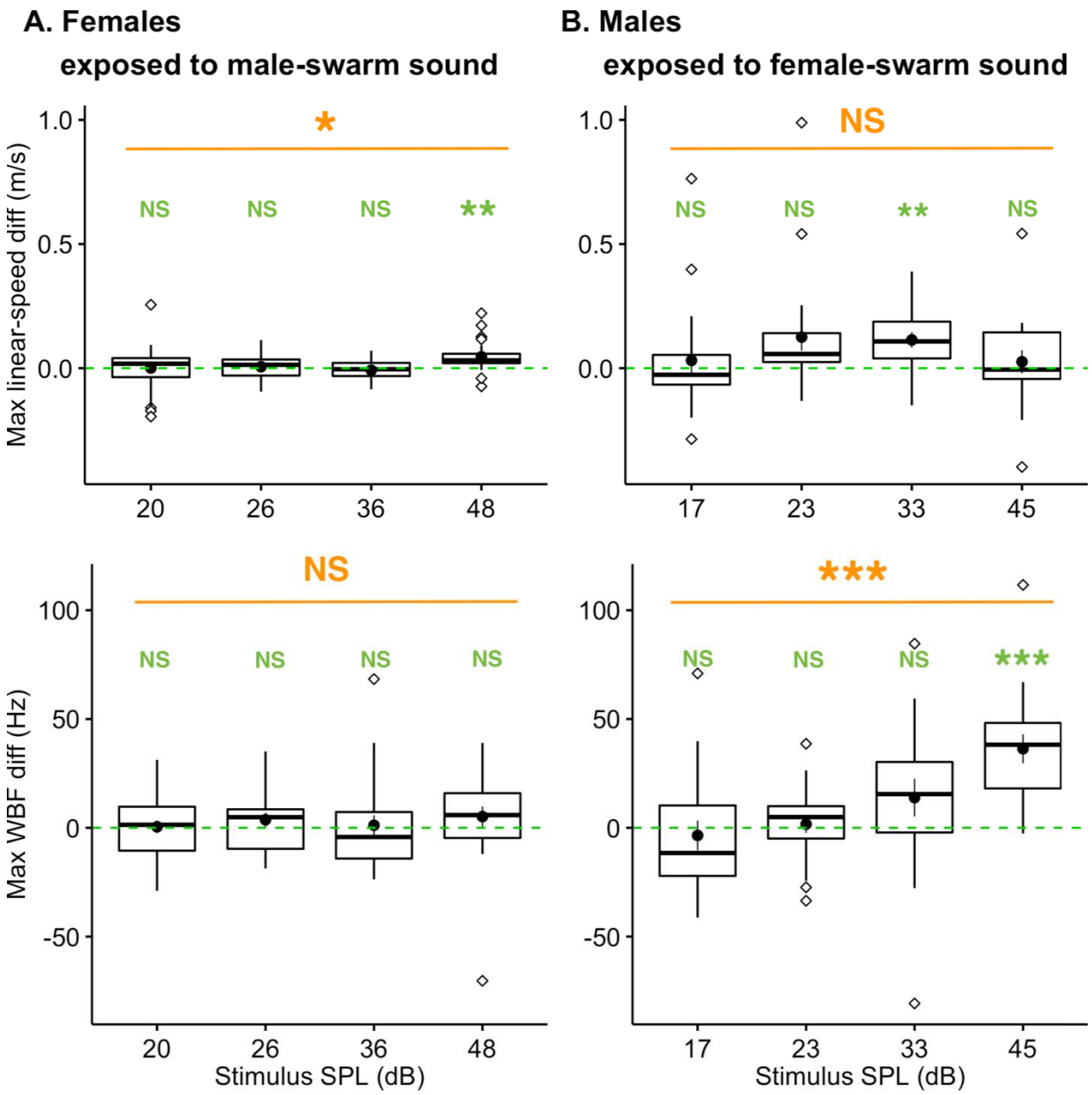


Figure 4. Results of behavioral experiment. One flight parameter (maximum linear speed difference, top row) and one acoustic parameter (maximum wingbeat frequency difference, bottom row) were extracted from flight tracks and wing-flapping sound for statistical analyses of female data (left column) and 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) *(44)*. 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.

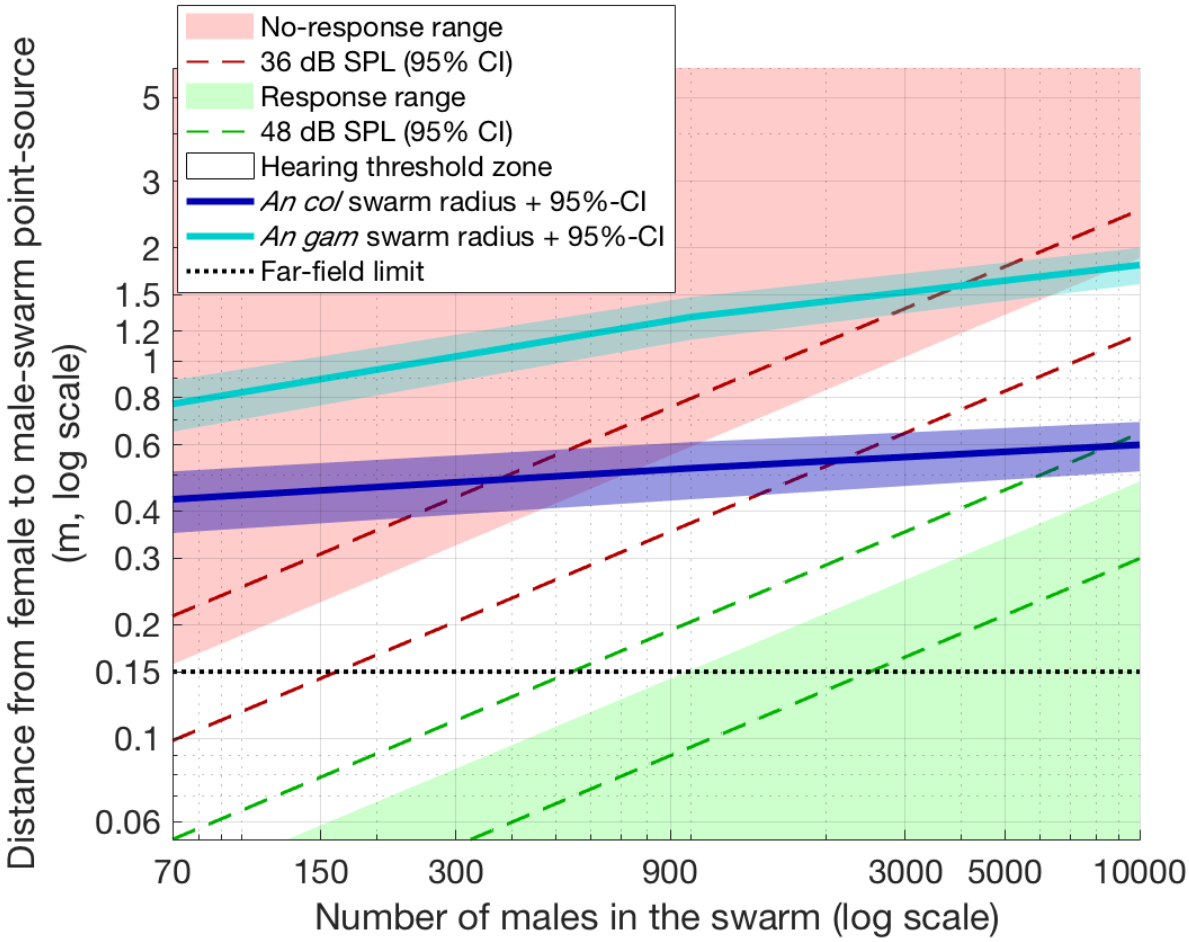


Figure 5. Estimated hearing-distance and swarm radius as a function of the number of males in the swarm. Green area covers the minimal response range, while red area indicates the minimal non-response range of a female to male swarm sound for both species, as a function of the number of males in a given swarm (X-axis) and the distance to the swarm center (Y-axis). These areas are based on our behavioral 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 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).

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 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 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 behavior (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 center 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 behavior above the visual marker (see Figure 2 and 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 Methods equation 4) 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 Methods equation 5). 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 Methods section 'Relationship between particle-velocity and pressure levels). The SPL measurements of *An. gambiae s.s.* sound stimuli are reported in Table 1; 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.

Supplementary Materials

Supplementary figures

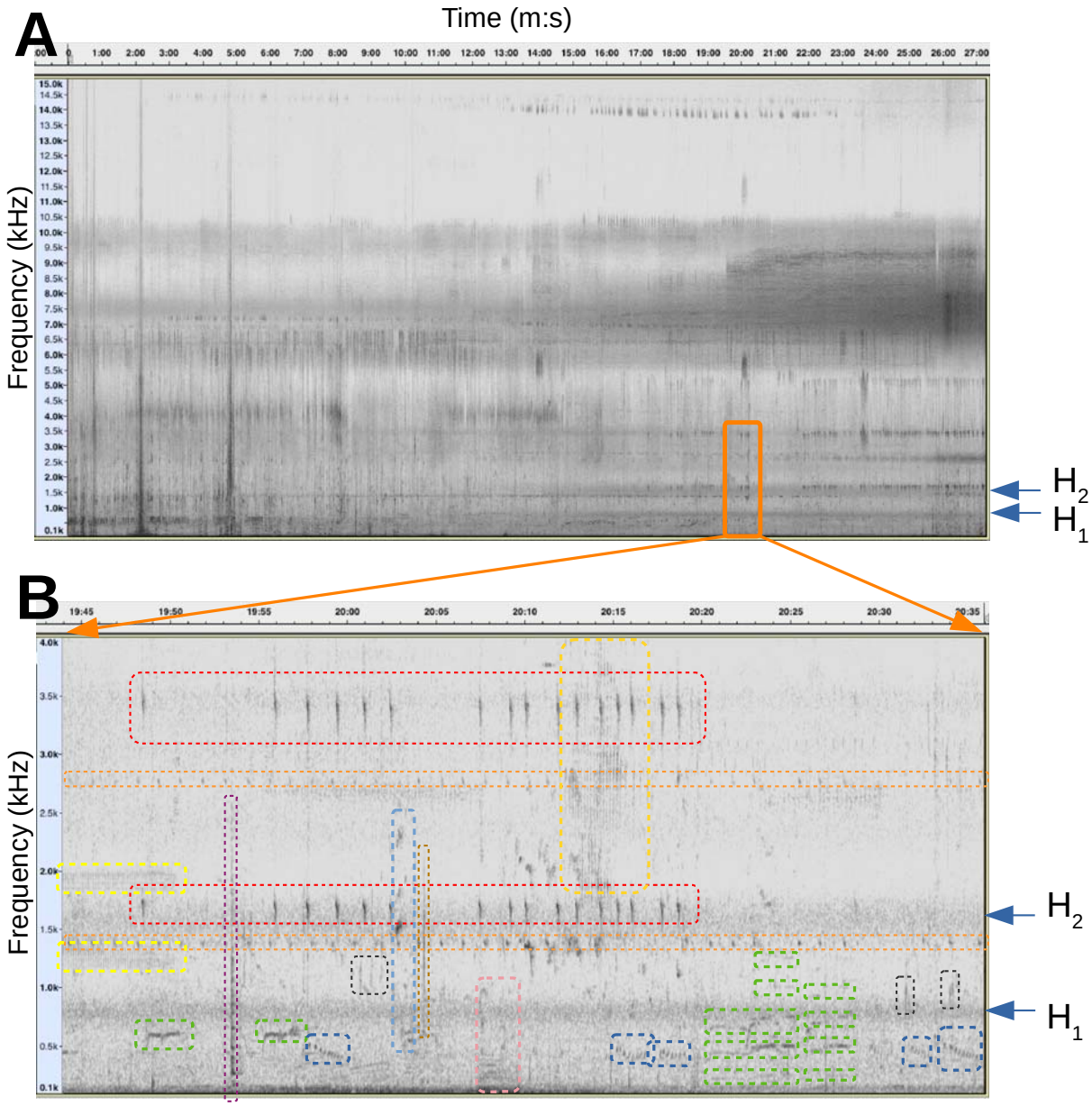


Figure S1. Spectrogram analysis of a typical field soundscape. Spectrogram analysis of background sounds in the field over 0-4 kHz in Bama VK5 village, 5 October 2017. X axis represents time, Y axis is the sound frequency and greyscale corresponds to the sound level at a given time and frequency.

(A) The spectrogram shows evening sound activity 18h05 - 18h33 (X axis), between 0.1 and 15 kHz (Y axis). Mosquitoes started to swarm ~ 1 min before sound recording began and they start being visible on the spectrogram (i.e. emerging from background sound noise) in the second half of the recording (first and second harmonic frequencies are

shown by arrows, H1 and H2, respectively). No sound activity was visible between 15 kHz and 20 kHz, so this frequency range was not displayed. The orange rectangle shows the selected time-frequency area for (B).

(B) 52-s spectrogram zoomed-in from orange selection shown in (A). The two arrows show the first and second harmonics of the swarming mosquitoes' wingbeat frequencies. Each colored rectangle shows a different non-mosquito sound source. At one meter from the swarm, the mosquitoes' first harmonics were the only sound-source in this frequency band, while the second harmonics were mixed with a range of bat and frog calls (red, orange, clear yellow, brown) or child voice (light blue). Gold yellow and dark blue correspond to bird sounds; green: mosque call to prayer; pink: motor; purple: cooking metal dish; black: frogs. Mosquito sounds appear to be the only steady sound in the 100-1000Hz frequency band. Third and fourth harmonic levels are almost invisible.

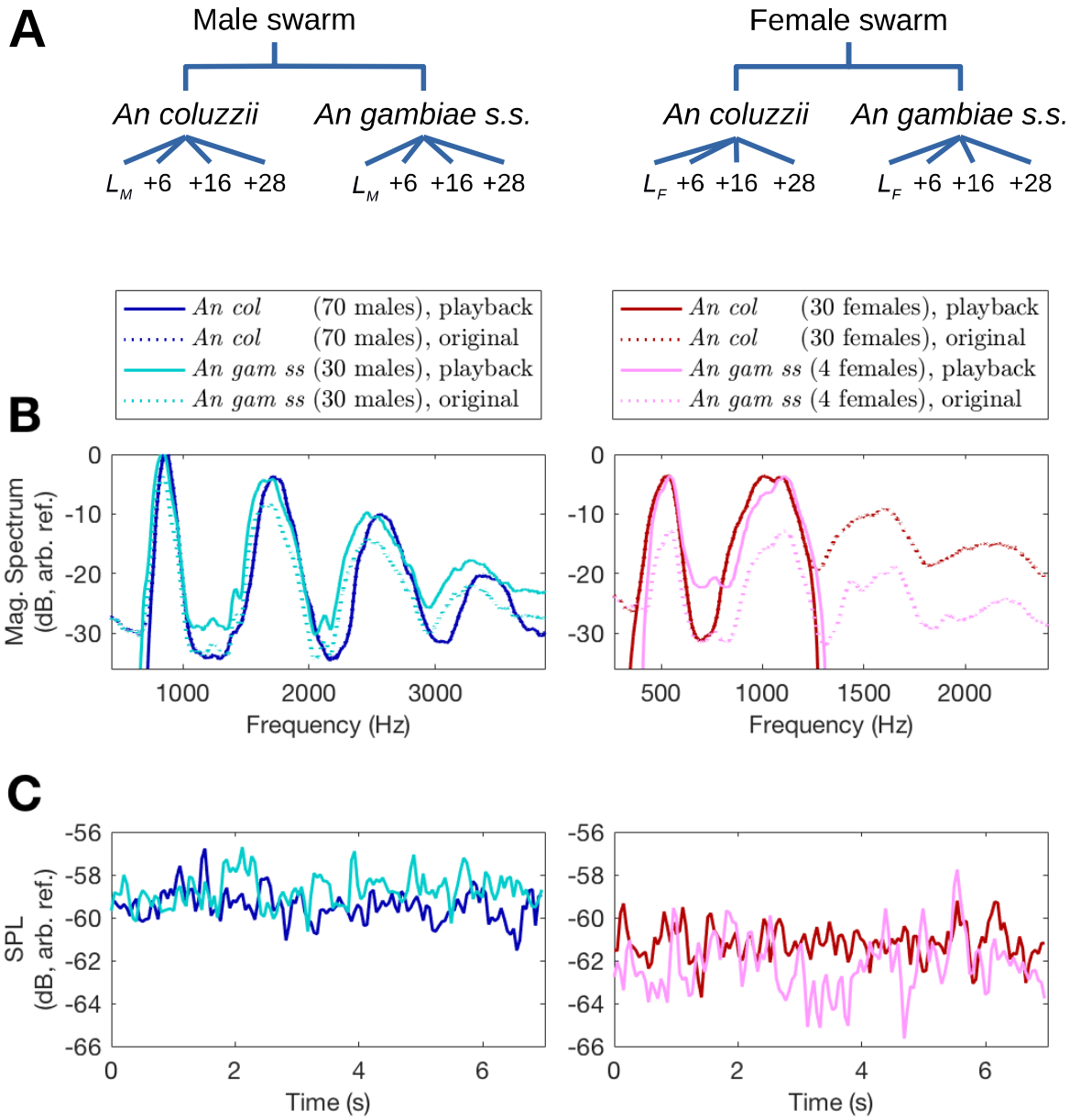


Figure S2. Spectral and temporal properties of sound stimuli for both sexes and both species. Spectral and temporal properties of sound stimuli of male *An. coluzzii* swarm (Sound S1), male *An. gambiae s.s.* swarm (Sound S2), female *An. coluzzii* swarm (sound S3) and female *An. gambiae s.s.* swarm (Sound S4).

(A) Sound stimulus variables: mosquito sex; species; and amount of sound level increase compared to a sex-specific reference level (L_M or L_F).

(B) Magnitude spectrum, computed over 7 s and averaged over 50-Hz windows, of stimuli of each species (dark colors for *An. coluzzii* and light colors for *An. gambiae s.s.*) and each sex (blue for males and red for females). Dotted lines represent recorded sounds, while solid lines indicate played-back sounds, the level for which was adjusted to one of *An. coluzzii*'s first-harmonics (see Methods section "Generation of sound stimuli"). High-frequencies of the female-swarm sound were filtered out to allow monitoring of the sound harmonics of the males, which otherwise would overlap with the females' higher harmonics (see Methods section "Wingbeat parameter extraction from flight sound"). The number of mosquitoes in the original sound stimuli is indicated in brackets in the legend. **(C)** Root-Mean-Square pressure level (0.1 s time windows with 0.05 s overlap), over the 7-s duration of the stimuli, for each species (dark colors: *An. coluzzii*, light colors: *An. gambiae s.s.*) and each sex (blue: males and red: females).

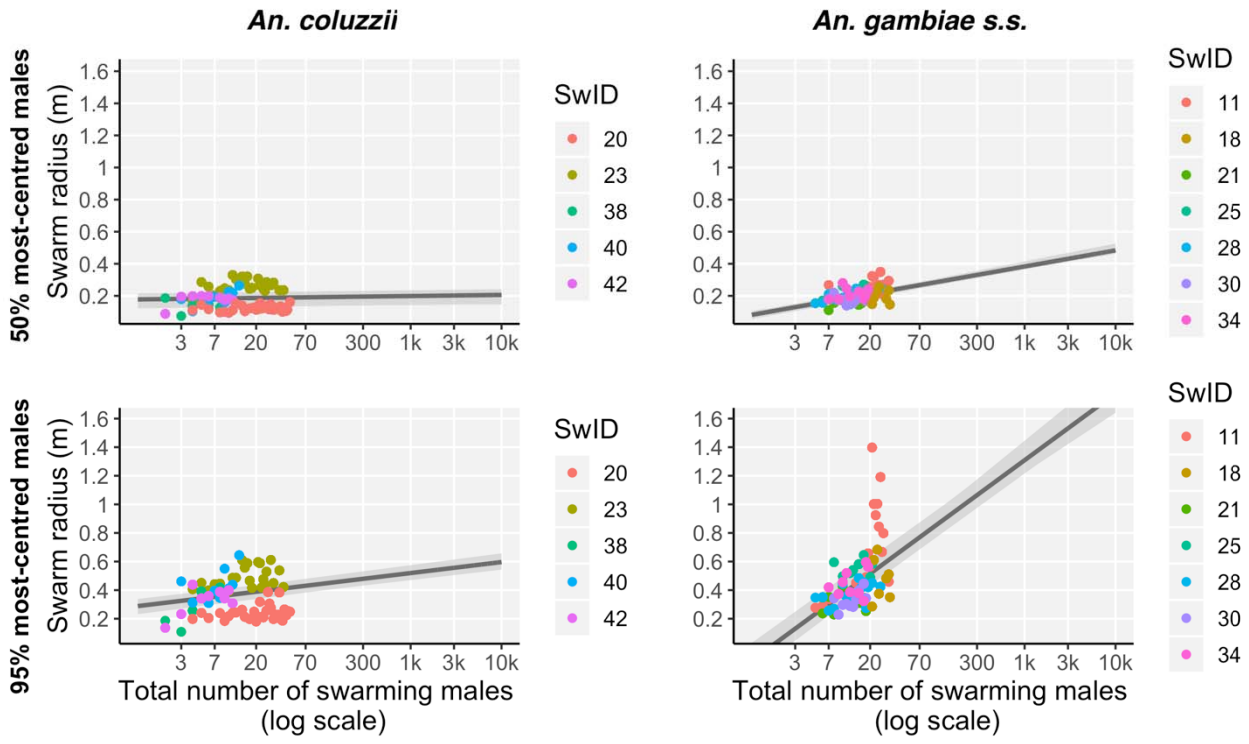


Figure S3. Radius of wild swarms of *An. coluzzii* and *An. gambiae s.s.* in Mali. Distance between swarm's centroid and the 50% (top row) or 95% (bottom row) closest males (data from *(18)*). For each column, color corresponds to a different swarm (left column: *An. coluzzii*.; right column: *An. gambiae s.s.*). Each point of each color corresponds to a measurement made at a different time. The thick black line and grey panel are the regression line of all swarms (random intercept model, *lmer* R function) and the associated 95%-CI. Related to Figure 5.

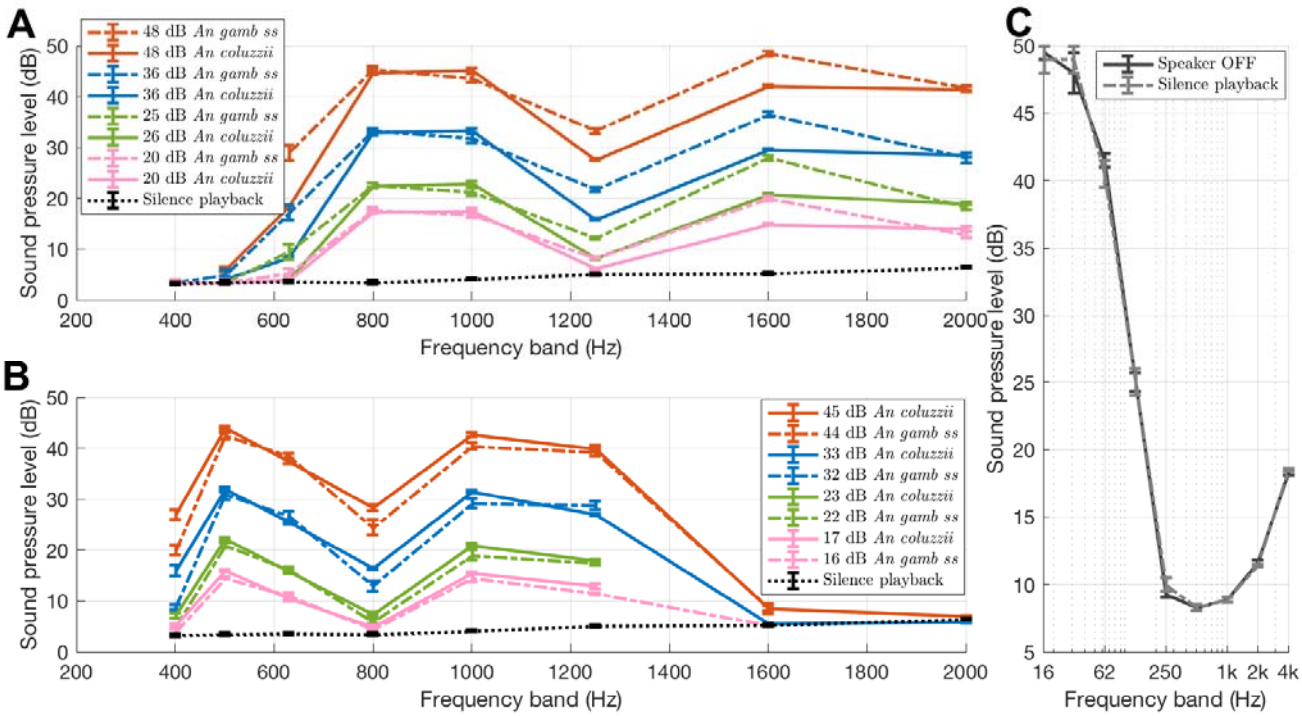


Figure S4. Calibrated sound-level measurements of played-back swarm sounds and background noise. SPLs (ref 20 µPa) were measured at the *An. coluzzii* swarming position 1) to estimate the sound level received by the tested mosquito and 2) to know the background noise level of the soundproof chamber. The X-axis values represent the central frequency f_c of the octave or 1/3-octave band filters. Each filter has a lower limit of $2^{-1/6}f_c$ and an upper limit of $2^{1/6}f_c$. For example, X=800 Hz represents the sound pressure level between 713 Hz and 898 Hz. The Y-axis error-bar represents maximum and minimum values measured during stimulus duration under a time constant of 1 s (slow mode, according to IEC 61672-1: 2002).

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

swarm stimuli (related to four distances from the male-swarm sound image, see Figure 3). Dashed lines represent the same, but for *An. gambiae* sound stimuli.

(B) As for A, except related to female sound stimuli. Not all points were measured above 1250 Hz which is of less interest because we filtered out sound frequencies above the second harmonics.

(C) SPL measurements (ref 20 µPa) in the soundproof chamber without playback and with 'silence' playback, along octave bands from 16 Hz to 4 kHz. The black dashed line shows the sound level when playing-back silence (i.e. speaker noise), while the plain black line corresponds to when the speaker is off (i.e. showing noise floor of sound-proof room). Both are the same, i.e. they show that the speaker has a very low noise level enabling us to playback low level sounds.

Supplementary tables

	Explanatory variables in the Anova model					
Response variable (diff)	SPL:species		species		SPL	
	χ_1^2	P	χ_1^2	P	χ_1^2	P
Maximum wingbeat frequency	1.01	0.31	1.19	0.27	0.46	0.50
Median wingbeat frequency	0.07	0.79	0.02	0.88	3.11	0.08
Maximum height	0.18	0.66	1.19	0.27	0.60	0.44
Median height	2.92	0.087	0.21	0.64	0.06	0.81
Minimum height	2.06	0.15	0.03	0.86	0.54	0.46
Maximum distance to speaker	0.014	0.90	1.62	0.20	0.26	0.61
Median distance to speaker	0.18	0.67	0.29	0.59	0.16	0.69
Minimum distance to speaker	0.04	0.84	1.04	0.31	3.69	0.055
Maximum angular speed	0.42	0.52	0.042	0.82	0.77	0.38
Maximum linear speed (1s)	0.052	0.82	1.56	0.21	1.09	0.30
Maximum angular speed (1s)	0.67	0.41	2.51	0.11	3.89	0.048*

Table S1. Statistical results from additional parameters computed from female wingbeat frequency or flight trajectory. Response parameters were calculated both during the sound stimulus playback and just before the playback for the same duration (7 s, except where indicated), and then were differentiated. LRT χ^2 was calculated between selected model pairs for which stepwise removal of terms was used. If the large number of tested parameters was not taken into account to adjust the *p*-values, only one of them was significant at the level of p=0.05 but no distribution was significantly different from the intercept, including the loudest sound stimulus, 48 dB: distribution (one-sample $t(22)$=0.063, BH-corrected p=0.063, mean=2.6 rad/s). Holm correction of *p*-values for multiple comparisons led to no statistically significant predictors of female response.

Other supplementary files

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-S1.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-S2.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.