Pre-copula acoustic behaviour of males in the malarial mosquitoes *Anopheles coluzzii* and *A. gambiae* s.s. does not contribute to reproductive isolation

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Summary Statement
*Anopheles gambiae* s.s. and *A. coluzzii* male mosquitoes display closely similar stereotypical acoustic behaviour in response to artificial tones at frequencies within the female wing-beat frequency range. Our findings strongly indicate that assortative mating between *A. coluzzii* and *A. gambiae* is unlikely to be based on this stereotypical pre-copula acoustic behaviour.

ABSTRACT
We reveal that males of two members of the *Anopheles gambiae* s.l. species complex, *A. coluzzii* and *A. gambiae* s.s. (hereafter *A. gambiae*), which are both malaria vectors, perform a stereotypical acoustic behaviour in response to pure tones at frequencies that encompass the frequency range of the female’s flight-tones. This behaviour resembles that described for *Culex quinquefasciatus* and consists of phonotactic flight initiated by a steep increase in wing-beat frequency (WBF) followed
by Rapid Frequency Modulation (RFM) of WBF when in close proximity to the sound source. RFM was elicited without acoustic feedback or the presence of a live female, but it appears to be a stereotypic behaviour in the immediate lead up to copula formation. RFM is an independent and different behavioural process from harmonic convergence interactions used by male-female pairs for mate recognition at earlier stages of mating. Acoustic threshold for RFM was used to plot behavioural audiograms from free-flying *A. coluzzii* and *A. gambiae* males. These audiograms were almost identical (minima ~400 Hz) and encompassed the WBF ranges of *A. coluzzii* (378-601 Hz) and *A. gambiae* females (373-590 Hz), indicating that males of both species share similar frequency tuning and range. Furthermore, no differences were found between the two species in their WBFs, RFM behaviour or Harmonic Convergence Ratios. These results indicate that assortative mating between *A. coluzzii* and *A. gambiae* is unlikely to be based on male-specific acoustic behaviours during RFM. The significance of these findings in relation to possible mechanisms for assortative mating is discussed.

INTRODUCTION

The complexity of malaria epidemiology and control is due in part to the remarkable degree of genetic variation among the species of the genus *Anopheles* (della Torre et al., 2005; Coetzee et al., 2013). This is particularly evident in the species complex *Anopheles gambiae s.l.*, found across much of sub-Saharan Africa and comprising at least nine morphologically similar species that vary in vector status, geographic distribution and ecology (Coetzee et al., 2013; Crawford et al., 2015). *Anopheles gambiae s.l.* species frequently occur in partially reproductively isolated and differentiated subpopulations, which in some cases led to rapid ecological speciation (Costantini et al., 2009; Coetzee et al., 2013; Crawford et al., 2015). In the context of public health, these speciation processes are of epidemiological importance because they influence vectorial capacity, vector distribution range and, consequently, species-specific means of control (Lehmann and Diabaté, 2008).

*Anopheles coluzzii* and *A. gambiae s.s.* (hereafter *A. gambiae*) are morphologically indistinguishable species, until recently considered to be two different molecular forms of the same species (M and S molecular forms, respectively) (Coetzee et al., 2013). They share an extensive geographical range in Central and West Africa (with over 90% of the range of *A. coluzzii*
overlapping with that of *A. gambiae* (Lehmann and Diabaté, 2008). However, they can exhibit marked local habitat segregation, with *A. coluzzii* having an extended distribution into more arid environments and *A. gambiae* mainly found in more humid habitats (Diabaté et al., 2006, 2009; Lehmann and Diabaté, 2008; Dabiré et al., 2013; Sawadogo et al., 2013). The causes for this habitat segregation are complex and involve phenotypic differences across all life stages (reviewed in Lehmann and Diabaté, 2008), but appears to be primarily associated with differential larval adaptations to exploit temporary or permanent freshwater habitats (Diabaté et al., 2008; Lehmann and Diabaté, 2008). Reproductive isolation between populations of *A. coluzzii* and *A. gambiae* is facilitated by assortative mating caused by temporal and spatial segregation of male swarms (Diabaté et al., 2009; Sawadogo et al., 2013).

Intriguingly, some natural sympatric populations of *A. coluzzii* and *A. gambiae* form mixed swarms with very low hybridization rates, suggesting the existence of other assortative mating processes (Tripet et al., 2001; Diabaté et al., 2006; Dabiré et al., 2013; Sawadogo et al., 2013) which appear to be mediated by as yet unidentified pre-mating, within-swarm mate recognition mechanism. Given the well-known observation that male mosquitoes locate females by flying towards the source of the female flight tone (Child, 1894; Roth, 1948; Wishart and Riordan, 1959; Charlwood and Jones, 1979; Belton, 1994), previous studies have investigated the possible role of flight-tone (Brogdon, 1998; Tripet et al., 2004) or harmonic convergence (Pennetier et al., 2010) in mate- and species-recognition between these two *Anopheles* species, but without unequivocal conclusions.

Rapid Frequency Modulation (RFM) behaviour, recently described in male *Culex quinquefasciatus*, is an acoustic response to the fundamental frequency of female flight-tones immediately prior to mating sequences (Simões et al., 2016). Significantly, this is a stereotypical behaviour that can be exploited to derive behavioural audiograms from free-flying male mosquitoes (Simões et al., 2016). The investigation of this behaviour in *A. coluzzii* and *A. gambiae* reported here has provided an opportunity to extend knowledge of the pre-mating behaviour in anopheline mosquitoes and to discover if the RFM behaviour could form a basis for assortative mating in these two species.

Here, we characterize and quantify the RFM acoustic behaviour of *A. coluzzii* and *A. gambiae* free-flying male mosquitoes. RFM in both species is elicited by tones at frequencies that encompass the frequency range of the two species’ female flight-tones. We used this stereotypical behaviour in *Culex quinquefasciatus* as a basis for exploring its potential role in species recognition and assortative mating in *A. coluzzii* and *A. gambiae*.
behaviour to derive behavioural audiograms for each species. Comparisons of the acoustic parameters of RFM, audiograms and WBFs show that no inter-specific differences were found between *A. coluzzii* and *A. gambiae*, indicating that assortative mating in these species is unlikely to be based on male-specific auditory behaviours during the RFM phase of mating. We discuss the consequences of these findings in relation to other possible mechanisms of assortative mating.

**MATERIALS AND METHODS**

**Mosquitoes**

*Anopheles coluzzii* Coetzee & Wilkerson (formerly M molecular form) and *Anopheles gambiae* Giles (formerly S molecular form) mosquitoes were obtained from Dr. K.R. Dabiré (Institute de Recherche en Sciences de la Santé, Bobo Dioulasso, Burkina Faso). These colonies were derived from populations in which mix-swarm assortative mating was reported (Diabaté et al., 2006); *Anopheles coluzzii* from larvae collected in village VK7 and *A. gambiae* from larvae collected in Soumousso, both in Burkina Faso. The colonies were lab-reared, maintained and bred in controlled-environment chambers (70-75% rH, 26±2°C and 12 h light: 12 h dark cycles). Adult mosquitoes 4-14 days post-emergence were tested during the first 3 h of the scotophase.

**Behavioural set-up**

The acoustic behaviour of free-flying mosquitoes was recorded inside a wire-framed arena of 30 cm sides which was covered by white cotton tubular-gauze and placed on a vibration damped table (Newport®, Irvine, Ca, USA) inside an sound attenuated booth (IAC Ltd, Winchester, UK). For the video/audio recordings, the metal frame was covered with matt-black cotton fabric, which is non-reflective to infra-red light, while the front side was covered by transparent acrylic enabling the camera to view the chamber’s interior. The ceiling was covered with white cotton gauze to allow the chamber to be illuminated by two infra-red multi-LED lights positioned 1 m above the cage.

Tone stimuli generated using the sine wave function of Test Tone Generator 4.4 (EsserAudio®, 2011) software were delivered to the cage from a sound source consisting of a 0.5 cm diameter plastic probe tip, damped with acoustic foam, connected via a 1 cm diameter polythene tube to an adapted Audio Technica® ATH A700AX speaker (5-35,000 Hz range with
flat frequency response 100-25,000 Hz). Sound from the speaker and flight-tones from the mosquitoes were monitored using a particle velocity microphone (Knowles NR-3158, Ithaca NY, USA) that was calibrated (Goßpfert and Robert, 2001) and mounted ~4 cm from the speaker probe tip. A pressure microphone (Knowles 23132, Ithaca NY, USA) mounted at the focal point of an 18” parabolic reflector (Edmunds), was placed on one side of the flight arena to monitor the sound inside. Signals from each of the microphones were amplified 100-fold with a purpose built two-channel preamplifier and the output of each channel was digitized at 192 kHz using a Fireface® UC sound card. The digital outputs were then recorded using Spectrogram 16 (Visualization Software, LLC) at a sampling rate of 48 kHz and frequency resolution of 5.9 Hz. Spectrogram 16 was also used to analyse and extract data on the time, frequency and amplitude of all acoustic signals.

For video recordings, an infra-red video camera (Swann® Pro-880) was placed 30 cm in front of the clear wall of the chamber and connected to the computer. Digital video recordings at 30 FPS of the flying mosquitoes were obtained using Debut Video Capture Software v1.88 (NCH® Software). The flight paths were then digitised using Kinovea (Version 0.8.23) software.

**Behavioural audiograms**

Male mosquitoes were placed inside the flight arena at the time of spontaneous circadian activity and left to fly freely during the recordings. After ~10 min period of adaptation to conditions inside the booth, the mosquitoes started to fly spontaneously, whereupon sound recording and stimuli presentation were initiated. All behavioural experiments were conducted at a room temperature of 30±2°C, which is within the range of temperatures of the natural habitat of *A. gambiae s.l.* mosquitoes (Huestis et al., 2012).

The behavioural audiograms of male mosquitoes were derived by recording the threshold of the RFM response relative to the particle velocity of the sound stimulus for tone frequencies between 200-1000 Hz (20 Hz increments until 700 Hz, 100 Hz increments thereafter). In each replicate (N=6), a group of 7-10 males was placed in the flight arena under illumination simulating dusk, when they are normally active. Upon initiation of spontaneous flight, a continuous tone of fixed frequency was presented to the swarming mosquitoes. The tone level was increased at a rate of 0.4 dB s⁻¹ from ~1x10⁻⁸ ms⁻¹ output until an RFM response was elicited from at least one male or until the maximum operating level (4x10⁻⁷ ms⁻¹) was reached. The sound stimulus was then
terminated and the particle velocity that elicited the response and the WBF of the responding male immediately before the onset of RFM were stored. After a 5-10 s rest period without stimulation, the procedure was repeated for another stimulus frequency. Particle velocity values were expressed as $\log_{10}$ for graphical display and statistical testing. Even when several males were swarming at the same time, the spectrogram analysis permitted the detection and isolation of the RFM response of individual males because the responses of an individual close to the microphone, which measured particle velocity rather than pressure, was much louder than the humming of the swarm in the background. The presence of higher harmonics of flight-tones provided a further basis for distinguishing between the WBFs of individual males.

The Harmonic Convergence Ratio (HCR) for each male was calculated by dividing the stimulus frequency (which simulates the WBF of a female) by the WBF just prior to the onset of RFM elicited by the stimulus. The inverse of the HCR corresponds to the harmonic relation of the two sound frequencies; e.g. HCR=0.5=1/2 indicates a 2:1 harmonic relation, i.e. the frequency of the 2$^{nd}$ harmonic of the female-like sound is equal to the fundamental WBF, whereas, HCR=0.667=2:3 indicates a 3:2 harmonic relation, which would correspond to a frequency convergence between the 3$^{rd}$ harmonic of the stimulus and the 2$^{nd}$ harmonic of the WBF. Although the stimulus frequencies were sinusoidal pure tones, harmonics of these pure tones are produced in the vibrations of the male’s antenna and JO upon sound detection, so males can potentially use these tones to reach harmonic convergence (Cator et al., 2009; Warren et al., 2009; Pennetier et al., 2010).

RESULTS

Males of both *A. coluzzii* and *A. gambiae* exhibited Rapid Frequency Modulation (RFM) behaviour, an acoustically driven flight response, when stimulated with pure tones at frequencies similar to the fundamental frequency of the female flight-tones. RFM in *Anopheles* males comprises three phases with distinct spectrographic and flight characteristics. This behaviour pattern is very similar to that reported for *C. quinquefasciatus* (Simões et al., 2016) and consists of the Onset, the Modulation or main phase, and the Offset (Fig. 1). The Onset phase is characterised by a steep increase in WBF of ~100 Hz in ~80ms (Table 1), which corresponds to a remarkable rate of 1250 Hz/s, and is associated to the phonotatic flight approach of the male to the sound
source (Fig. 2A and B).

The Modulation or main phase follows the fast WBF elevation of the Onset.
Spectrographically, the frequency modulation comprises fast and variable upward and downward
shifts in WBF that ranged from ~20 - 200Hz in amplitude at the fundamental frequency (Fig. 1 and
Fig. 2B). The peak-to-peak interval of an individual frequency shift was ~80 ms (Table 1), which
corresponds to approximately 12.5 modulations per second. The total duration of the Modulation
phase was variable and ranged from ~150 ms up to more than 2 seconds (Table 1). During this
phase, the male was flying in close proximity (4 cm or less) of the sound source while displaying
tight loops around it (Fig. 2). In some interactions the male touched the sound source without
ceasing RFM. The Modulation phase was followed by the Offset phase (Fig. 1), during which the
WBFs gradually decreased over a period of ~250 ms (Table 1) until it reach a frequency similar to
that before the RFM. This phase was concomitant with the male flying away from the sound
source (Fig. 2).

The total duration of RFM behaviour, from the Onset (steep frequency spike) until the
Offset (end of the final frequency drop) was approximately 1 second for both mosquito species.
The WBFs of the free-flying A. coluzzii and A. gambiae males were not significantly different and,
crucially, all the measured characteristics of the RFM behaviour and its different phases also
showed no significant differences between the two Anopheles species (Table 1).

The behavioural audiograms for A. coluzzii and A. gambiae males are shown in Figure 3A.
Both species had similar thresholds of response (Table 2) and RFM responses were elicited within
the same frequency range (280-620Hz; Fig. 3A). The particle velocity threshold of the RFM
response was dependent on the stimulus frequency and was lowest in both species for frequencies
between 360-500 Hz (Fig. 3A; Table 2), which encompasses the WBF ranges of their conspecific
females (Fig. 3A; Table 3).

The average WBF of females and the sound intensity of their wing beats were also
statistically similar between A. coluzzii and A. gambiae species (Table 3). Tethered-flying females
generated particle velocities of ~4.5x10⁻⁵ ms⁻¹ 2 cm in front of their heads (dashed lines in Fig.
3A), which considerably exceeds the behavioural threshold of the males. Anopheles males
responded within the range of the most sensitive frequencies to particle velocities between
8.7x10⁻⁷ ms⁻¹ and 7.3x10⁻⁶ ms⁻¹ at a reference point 2 cm from the speaker, which is ~25 dB below
the average sound intensity of the female flight-tones.
The positive correlation between WBF measured just prior to the onset of RFM and the frequency of the stimulus shows that Anopheles males flying at lower WBFs tend to respond to the lower frequencies of the stimulus range, while males flying at higher WBFs respond more often to higher stimulus frequencies (Fig. 3B). The slope and range of this correlation are similar in the two species, and, as reported for C. quinquefasciatus (Simões et al., 2016), suggest that the detection of female-like tones (and consequently the expression of RFM) by male Anopheles is dependent on their own WBFs.

The Harmonic Convergence Ratio (HCR) was calculated in order to discover if frequency tuning and RFM behaviour might be related to the frequency matching of flight-tone harmonics as described for both these Anopheles species (Pennetier et al., 2010). The HCRs of A. coluzzii and A. gambiae, plotted as a function of the stimulus frequency, are similar and not centred on any particular value (Fig. 3C). Rather, in both species the HCRs increase proportionally with stimulus frequency, which indicates that the initiation of the RFM response by the males is independent of any harmonic convergence between their flight-tones and the stimulus. Interestingly, the most sensitive RFM responses (elicited by low particle velocity levels, as indicated by the bubble areas in Fig. 3C) lie roughly between HCRs of 0.45-0.7, a range which encompasses the harmonic convergences $2\varphi:1\delta$ (HCR=0.5) and $3\varphi:2\delta$ (HCR=0.666).

DISCUSSION

Here we describe and quantify the Rapid Frequency Modulation (RFM) acoustic behaviour of free-flying males of Anopheles coluzzii and A. gambiae. The RFM response performed by Anopheles males is a stereotypical, open loop behaviour in response to tone stimulation at frequencies within the range of the fundamental component of female flight-tones and the pattern of behaviour is identical to that observed for Culex quinquefasciatus mosquitoes (Simões et al., 2016). Similarly, this behaviour also involves, particularly at the Onset and Modulatory phases, very fast changes in WBF of the flying males (>1250 Hz s\(^{-1}\)). The fact that RFM was observed both in the Culex and Anopheles genera is significant because it indicates that this pre-copulatory behaviour is shared by the Culicinae and Anophelinae subfamilies which diverged ~200 Ma (Reidenbach et al., 2009). It also suggests that the RFM might be found throughout all the Culicidae family, particularly in mosquito species with sexual dimorphism in their flight-tones; in
this context, it will be particularly interesting to determine if mosquito species without this sexual dimorphism, such as *Toxorhynchites brevipalpis* (Steffan and Evenhuis, 1981; Gibson and Russell, 2006) have lost this pre-copulatory behaviour.

Overall, no inter-specific differences were found between *A. coluzzii* and *A. gambiae* males in their free-flight WBFs, pre-copulatory behaviour, and behavioural audiograms. Likewise, no differences were found in the WBF and sound intensity of the females of both species. The average free-flight WBF of males and females do not differ between species and corroborates the data published by Tripet et al. (2004). Curiously, and albeit non-significant in both studies, the average WBF of *A. coluzzii* males (M form in Tripet et al., 2004) is slightly higher (~15 Hz) than that of *A. gambiae* (S form in Tripet et al., 2004) males, while the average WBFs of the females is almost identical. However, and taking in account their frequency range, it is unlikely that this slight frequency difference would reflect any basis for specific differences between the two *Anopheles* species.

No inter-specific differences were found in the acoustic parameters of RFM response of males to pure tones, either in changes of frequency, duration or frequency modulation. The RFM response probably serves as a controlled flight to reach and maintain a close-range position while attempting to seize and engage terminalia with the female (Roth, 1948; Wishart and Riordan, 1959; Charlwood and Jones, 1979; Simões et al., 2016). These similarities suggest that this pre-copulatory behaviour and the associated flight parameters are, in structure and function, indistinguishable between the two *Anopheles* species and should not provide a basis for the isolation of these two species. Furthermore, the Harmonic Convergence Ratio (HCR) for both species is very similar, and not centred on any particular value, increasing proportionally with stimulus frequency. This indicates that, as in *C. quinquefasciatus* (Simões et al., 2016), initiation of the RFM response in *Anopheles* males is independent of any harmonic convergence between the male flight-tones and the stimulus. Significantly, these results show that it is unlikely that harmonic convergence, at least by the males, during the initiation of RFM behaviour can be used as mechanism for species recognition in *Anopheles* (Pennetier et al., 2010). However, little is known about the role of harmonic convergence in the earlier phases of mating behaviour.

The behavioural audiograms for the *A. coluzzii* and *A. gambiae* males are very similar and have identical frequency ranges. Furthermore, males of both species are more sensitive to the same range of frequencies (360-500Hz), which encompasses the WBF range of free-flying females.
Similar hearing range and sensitivity indicates that the pre-mating isolation between these two Anopheles species is not related to morphological or physiological differences between their hearing organs. Moreover, the finding that A. coluzzii and A. gambiae males share the same hearing range and sensitivity further indicates that they should not be able to identify and discriminate conspecific females based solely on their WBF.

Culex males use acoustic distortion to hear female-like tones (Simões et al., 2016). Acoustic distortion can be seen as the generation of new vibrations – intermodulation distortion products – as a consequence of the interaction between two simultaneous tones of different frequencies in the mosquito’s antenna (Warren et al., 2009; Pennetier et al., 2010; Lapshin, 2012; Simões et al., 2016). In flight, this corresponds to the interaction between the fundamental frequency of the male’s own flight tone and the flight tone of a nearby flying female generating a third frequency equal to the arithmetic difference between the first two. The male’s hearing organ - the Johnston’s organ (JO) - is rather insensitive to the two flight tones but very sensitive to their frequency difference, which is amplified up to 100 times before the signal is transmitted to the insect’s brain (Simões et al., 2016). Thus, is it probable that Anopheles males hear female flight-tones by detecting distortion products produced by the frequency differences in their WBFs, as reported for Culex? We found a strong positive correlation between the male WBFs and the stimulus frequency that elicited RFM, which suggests that the detection of female-like tones (and consequently the expression of RFM) by male Anopheles is dependent of their own WBFs. Furthermore, previous measures of the electrophysiological tuning of the JO of A. gambiae males (Pennetier et al., 2010) reported a minima frequency around 300 Hz, which is almost ~100Hz below the minimum frequency range for the female WBF. Also, in the same study Pennetier et al. (2010) found that distortion is indeed generated in the vibrations of the antenna of the A. gambiae males and detected in the electrical responses of the JO. Taken together, these observations suggest that male Anopheles might use distortion products to detect flying females.

Therefore, our results here and in C. quinquefasciatus (Simões et al., 2016) indicate that the pre-copulatory behaviour of male mosquitoes appears to be a stereotyped fixed action pattern elicited solely by the detection of non-specific tones within the range of the fundamental frequency female flight-tones. Conversely, this suggests that is improbable that these acoustic signals transmit any information to the male mosquitoes aside from the presence (and location) of a flying female mosquito. It also implies that female flight-tones do not convey information about
conspecificity and mate assessment to male mosquitoes.

Natural sympatric populations of *A. coluzzii* and *A. gambiae* can form mixed swarms (Diabaté et al., 2006; Dabiré et al., 2013; Sawadogo et al., 2013). Analysis of these swarms revealed a very low percentage of hybrids and few inter-specific copulae within them, which indicates the existence of assortative mating, most probably caused by pre-mating isolation mechanisms (Dabiré et al., 2013). However, Dao et al. (2008) showed that when both species congregate inside huts, cross-species is as frequent as within-species mating, indicating that assortative mating breaks down when mating occurs indoors. This is consistent with reports observing the absence of assortative mating in lab-reared *Anopheles* colonies (Benedict et al., 2009; Paton et al., 2013), which, overall, suggests that chemical cues such as pheromones and cuticular hydrocarbons (Dao et al., 2008) and flight tones (Dao et al., 2008; Tripet et al., 2004) do not play a major role in species recognition.

The precise mechanisms for observed assortative mating remain, however, unidentified, but several hypotheses can now be eliminated. First, our results suggest there are no inter-specific differences in male hearing capabilities or in male pre-copulatory behaviour. These results agree with those of Tripet et al. (2004), which excluded putative species-specific differences on WBF and/or WBF detection (“The Wingbeat Hypothesis”) as the causal agent for reproductive isolation between *A. coluzzii* and *A. gambiae*. In addition, Pennetier et al. (2010) proposed that harmonic convergence may play a role in reproductive isolation between these two species; this hypothesis was supported by the observation that tethered mixed-species pairs showed a lower incidence of harmonic convergence than same-species pairs. Our results would exclude a male-initiated harmonic convergence mechanism, either for sex- or species- recognition, at least during the final phase of pre-copulatory mating behaviour.

Interestingly, the conjunction of all these results indirectly suggests that harmonic convergence might be a behaviour mediated fundamentally by female mosquitoes. On the one hand, that could provide females a mechanism for selecting high-quality males (Cator et al., 2010; Pennetier et al., 2010), but, on other hand, it could also play a role in the assortative mating of *A. coluzzii* and *A. gambiae*. Crucially, the hypothesis that assortative mating could be mediated by females is supported by the results of a recent study by Aboagye-Antwi et al. (2015); behavioural assays in recombinants strains for the M and S markers in the X chromosome of both *Anopheles* species revealed that females, but not males, mated assortatively, indicating that a species
recognition mechanism appears to be female-dependent. This, however, does not mean that males
do not contribute to assortative mating in nature; in the field, males are known to contribute to
assortative mating via swarm spatial segregation (Diabaté et al., 2006; Dabiré et al., 2013;
Sawadogo et al., 2013; Aboagye-Antwi et al., 2015).

Two main conclusions can be drawn from the overall similarity of the pre-copulatory male
acoustic behaviour in *A. coluzzii* and *A. gambiae*, and indeed between those and *C.
quinquefasciatus*: Firstly, the results indicate that the RFM response and the associated flight
characteristics represent a stable mating strategy, probably shared by all sexually dimorphic
mosquito species. In this context, it predicts that male *Aedes*, a genus of equivalent medical
importance, would also exhibit the same behavioural processes. Secondly, the non-specificity of
the frequency range eliciting the male behaviour has implications for novel mosquito control tools,
particularly those designed to make use of sound signals as the basis for acoustic traps.

### List of abbreviations

- **JO** – Johnston’s organ
- **HCR** – Harmonic Convergence Ratio
- **RFM** – Rapid Frequency Modulation
- **WBF** – Wing Beat Frequency

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Burkina Faso) for providing mosquito eggs and James Hartley for designing and constructing
electronic components for sound generation and signal acquisition.

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### Competing Interests

No competing interests declared.

### Author contributions
PMVS, GG and IJR designed experiments, PMVS made the measurements, PMVS and IJR analysed the data, PMVS, GG, and IJR wrote the paper.

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**Table 1.** Wing Beat Frequency (WBF) and temporal characteristics of the RFM behaviour in free-flying *A. coluzzii* and *A. gambiae* males (range and x± s.e.m.).

<table>
<thead>
<tr>
<th>Species</th>
<th>A. coluzzii (N=91)</th>
<th>A. gambiae (N=88)</th>
<th>T value</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBF (Hz)</td>
<td>626-912</td>
<td>675-903</td>
<td>1.586</td>
<td>0.065</td>
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<tr>
<td></td>
<td>793±5.8</td>
<td>779±5.2</td>
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<td></td>
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<tr>
<td>Δ Onset (Hz)</td>
<td>43-228</td>
<td>54-193</td>
<td>1.668</td>
<td>0.097</td>
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<tr>
<td></td>
<td>109±3.9</td>
<td>101±2.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Onset duration (ms)</td>
<td>30-500</td>
<td>17-220</td>
<td>0.604</td>
<td>0.547</td>
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<tr>
<td></td>
<td>83±5.8</td>
<td>79±4.2</td>
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<tr>
<td>Modulation duration (ms)</td>
<td>167-2407</td>
<td>127-2186</td>
<td>1.831</td>
<td>0.069</td>
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<tr>
<td></td>
<td>642±46.1</td>
<td>766±49.2</td>
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<td></td>
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<tr>
<td>Single FM duration (ms)</td>
<td>87±2.4</td>
<td>83±2.1</td>
<td>1.253</td>
<td>0.212</td>
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<tr>
<td>Δ Offset (Hz)</td>
<td>18-140</td>
<td>26-188</td>
<td>1.603</td>
<td>0.111</td>
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<tr>
<td></td>
<td>66±2.7</td>
<td>73±3.1</td>
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<tr>
<td>Offset duration (ms)</td>
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<td>45-623</td>
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<td></td>
<td>250±13.9</td>
<td>242±15.5</td>
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<tr>
<td>Duration of RFM (ms)</td>
<td>422-3146</td>
<td>341-2668</td>
<td>1.437</td>
<td>0.153</td>
</tr>
<tr>
<td></td>
<td>976±54.4</td>
<td>1086±54.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2. ANOVA results for the behaviour audiograms measured as the threshold particle velocity against *Anopheles* species and Stimulus frequency.

<table>
<thead>
<tr>
<th>Source</th>
<th>d.f.</th>
<th>SS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species</td>
<td>1</td>
<td>0.21</td>
<td>1.52</td>
<td>0.220</td>
</tr>
<tr>
<td>Stimulus frequency</td>
<td>17</td>
<td>45.73</td>
<td>19.70</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Species x Stimulus freq.</td>
<td>17</td>
<td>2.40</td>
<td>1.04</td>
<td>0.425</td>
</tr>
<tr>
<td>Error</td>
<td>143</td>
<td>19.54</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Particle velocity values were expressed as log$_{10}$. Species: *A. coluzzii* and *A. gambiae*; Stimulus frequency range: 280-620 Hz. Asterisk denotes statistical significance.

Table 3. Wing Beat Frequency (WBF) and sound intensity of wing beats (measured in particle velocity) of *A. coluzzii* and *A. gambiae* females.

<table>
<thead>
<tr>
<th>Species</th>
<th>A. coluzzii</th>
<th>A. gambiae</th>
<th>T value</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range</td>
<td>378-601</td>
<td>373-590</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WBF (Hz)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>x ± s.e.m (N=30)</td>
<td>488±11.5</td>
<td>490±10.5</td>
<td>0.155</td>
<td>0.878</td>
</tr>
<tr>
<td>Sound intensity (ms$^{-1}$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>x ± s.e.m (N=8)</td>
<td>4.5x10$^5$±1.94x10$^{-6}$</td>
<td>4.6x10$^5$±2.05x10$^{-6}$</td>
<td>0.895</td>
<td>0.831</td>
</tr>
</tbody>
</table>

Sound intensity: particle velocity generated by tethered-flying females 2 cm in front of their heads.
FIGURE LEGENDS

Figure 1. Rapid Frequency Modulation (RFM) of Anopheles males. Spectrogram of the wing beat frequency (WBF) of two free-flying Anopheles gambiae males when stimulated with a 440Hz (lower red trace; 5x10^{-5} ms^{-1}). Tone stimulation evoked a RFM response in one of the flying male while the other male maintained his WBF. White bars indicate duration of Onset (On.), Modulation and Offset phases. Blue and white arrows on spectrogram correspond to the fundamental WBF and lower harmonics of the responding and non-responding male, respectively.

Figure 2. Flight path and spectrogram of RFM behaviour of Anopheles males. A) Flight path and B) Spectrogram of the WBF of two free-flying Anopheles coluzzii males when stimulated with a female-like tone (lowest trace; 10 s, 440Hz, 5x10^{-5} ms^{-1}). Blue and white paths (A) represent the spatial position of a responding male and a non-responding male, respectively. Arrows on flight path indicate direction of flight. Lighter interval in spectrogram (B) corresponds to the duration of the illustrated flight paths. Blue and white arrows on spectrogram correspond to the fundamental WBF and lower harmonics of the responding and non-responding male, respectively. The flight path of the responding male (blue) during phonotaxis to the speaker, the tight looped flight near it and the final departure correspond, respectively, with the Onset of the RFM, the modulation phase, and the Offset phase, as observed in the spectrogram. In contrast, the non-responding male (white) did not show any flight towards or near the speaker nor did it exhibit any conspicuous changes in WBF. Note a third mosquito male resting just under the speaker which remained flightless during the entire sequence.

Figure 3. Behavioural audiograms of Anopheles coluzzii and Anopheles gambiae s.s. males. A) Threshold of Rapid Frequency Modulation (RFM) behaviour (mean ± s.e.m. expressed as the particle velocity of the sound stimulus measured 2 cm from the front of the speaker) as a function of stimulus frequency (N=6 replicates for each species). Shading: frequency range of free-flying
female wing-beat frequencies (WBFs) (Red: A. coluzzii, Blue: A. gambiae, Purple: Common range). ♀WBpv: mean particle velocity generated by the wing beats of tethered-flying females when measured 2 cm in front of the head (A. coluzzii: $4.5 \times 10^{-5} \pm 2.1 \times 10^{-6}$ ms$^{-1}$, A. gambiae: $4.6 \times 10^{-5} \pm 1.9 \times 10^{-6}$ ms$^{-1}$, N=6 each). B) Correlation between WBF of responding males and stimulus frequency (A. coluzzii: Stimulus=1.1 x ♀WBpv - 389, Pearson's r=0.41; A. gambiae: Stimulus=1.0 x ♀WBpv - 365, Pearson's r=0.32). C) Relation between stimulus frequency that elicited RFM response and the Harmonic Convergence Ratio (HRC). Bubble areas are proportional to stimulus intensity.