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RESEARCH ARTICLE

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

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ABSTRACT

We reveal that males of two members of the *Anopheles gambiae s.l.* species complex, *Anopheles coluzzii* and *Anopheles 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* (373–590 Hz) females, indicating that males of the two 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.

KEY WORDS: Rapid frequency modulation, Free-flight, Insect hearing, Johnston's organ

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 of this habitat segregation are complex and involve phenotypic differences across all life stages (reviewed in Lehmann and Diabaté, 2008), but appear 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 an as yet unidentified pre-mating, within-swarm mate recognition mechanism. Given the well-known observation that male mosquitoes detect and 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 in the present study provided an opportunity to extend

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knowledge of the pre-mating behaviour in anopheline mosquitoes and to discover whether the RFM behaviour could form the basis for assortative mating in these two species.

Here, we describe 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 to derive behavioural audiograms for each species. Comparisons of the acoustic parameters of RFM, audiograms and wing-beat frequencies (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 and 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); *A. coluzzii* from larvae collected in village VK7 and *A. gambiae* from larvae collected in Soumousso, both in Burkina Faso. The colonies were lab reared and maintained inside controlled chambers (26±2°C, 70–75% relative humidity and 12 h light:12 h dark cycles). Adult mosquitoes (between 4 and 14 days after emergence) were tested during the first 3 h of scotophase.

Behavioural set-up

The behaviour of free-flying mosquitoes was recorded using the same experimental set-up as described in Simões et al. (2016) to study *C. quinquefasciatus* behaviour. In short, the set-up for audio recordings comprises a 30 cm-side metal-framed arena covered by white gauze and placed inside a sound attenuation chamber (IAC Ltd, Winchester, UK). For audio/video sequences, we used a similar frame but covered with black, non-reflective cotton fabric, except for the front side, which was covered by transparent acrylic; the removable top was made of white cotton gauze to allow infra-red illumination of the chamber (multi-LED lights 1 m above the arena) and access to the interior of the arena. The sound source consisted of a plastic probe tip damped with acoustic foam and connected by a polythene tube to an adapted Audio Technica® ATH A700AX speaker. Tones were generated using the sine wave function of Test Tone Generator 4.4 (EsserAudio® 2011). All sounds inside the arena were monitored and recorded using a calibrated (Göpfert and Robert, 2001) particle velocity microphone (Knowles NR-3158, Ithaca, NY, USA) placed ~4 cm from the sound source and by a pressure microphone (Knowles 23132) mounted on an 18 in parabolic reflector (Edmunds) placed outside the flight arena. A two-channel preamplifier amplified 100-fold the signals from the microphones and each channel's output was digitized using a Fireface® UC sound card. The digital outputs were recorded and analysed using Spectrogram 16 (Visualization Software, LLC; sampling rate: 48 kHz; frequency resolution: 5.9 Hz). Video sequences of the flying mosquitoes were recorded using a Swann® Pro-880 infra-red camera placed 30 cm in front of the acrylic front of the arena and digitally acquired using Debut Video Capture v1.88 (NCH® Software). The flight paths were then digitised using Kinovea (v0.8.23) software.

Behavioural audiograms

The protocol used to plot the RFM behavioural audiograms in free-flying mosquitoes was as previously described (Simões et al., 2016). Briefly, a continuous pure tone of fixed frequency and increasing level (0.4 dB s^{-1}) was presented to a swarm of free-flying males mosquitoes of each *Anopheles* species until a RFM response was observed from at least one male or until the maximum operating level of the speaker was reached. The minimum sound level (in particle velocity) eliciting a RFM, representing the threshold of response, was used to derive the behavioural audiograms for the entire tested frequency range (200–1000 Hz, with 20 Hz increments until 700 Hz, and 100 Hz increments thereafter). Additionally, the fundamental WBF of the responding male immediately before the onset of RFM was also recorded. Each replicate (*N*=6) consisted of a group of 7–10 free-flying male mosquitoes placed inside the flight arena at the time of their spontaneous circadian activity and tested for the same tone frequencies. Inter-trial interval between different stimulus frequencies varied between 5 and 10 s. Experiments were conducted under dusk-like illumination and an ambient 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). Particle velocity was expressed as $\log_{10}$ values for graphical display and statistical testing. The spectrographic analysis of the sound level and the harmonics of the recorded acoustic activity permitted an easier detection and isolation of individual RFM responses, even when several males were swarming at the same time.

The harmonic convergence ratio (HCR) for each male was calculated as defined by Simões et al. (2016), i.e. as the ratio between the stimulus frequency that elicited a RFM and the male's fundamental WBF just before the onset of response. The inverse of the HCR will thus correspond to the harmonic relationship of the two sound frequencies, e.g. HCR=0.667=2:3 indicates a 3:2 harmonic relationship, corresponding to a convergence between the 3rd harmonic of the stimulus frequency and the 2nd harmonic of the WBF. Although the acoustic stimuli were pure tones, the production of harmonics in the vibrations of the male's antenna and Johnston's organ (JO) upon sound detection (Cator et al., 2009; Warren et al., 2009; Pennetier et al., 2010) indicates that males can potentially use pure tones to reach harmonic convergence.

RESULTS

Males of both *A. coluzzii* and *A. gambiae* exhibited 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 sharp increase in WBF of ~100 Hz in ~80 ms (Table 1), which corresponds to a remarkable rate of 1250 Hz s^{-1}, and is associated with the phonotactic flight approach of the male to the sound source (Fig. 2).

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 to 200 Hz in amplitude at the fundamental frequency (Figs 1 and 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

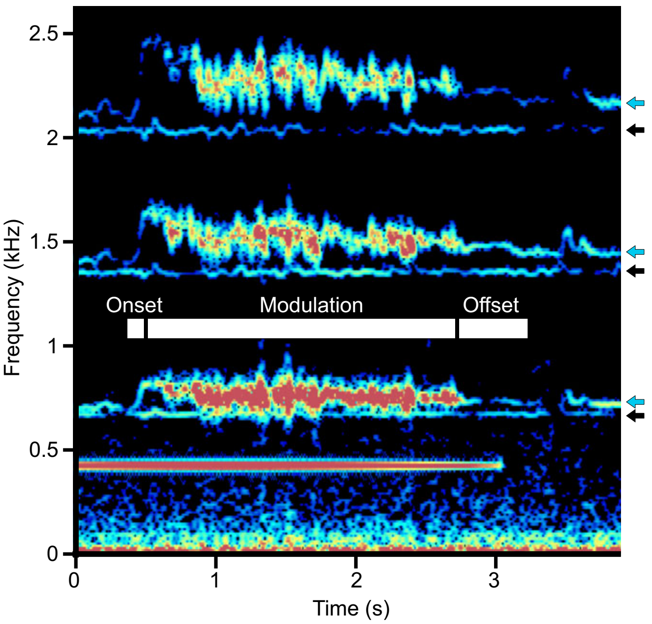

Fig. 1. Rapid frequency modulation (RFM) of *Anopheles* males. Spectrogram of the wing-beat frequency (WBF) of two free-flying *Anopheles gambiae* males when stimulated with a female-like tone (bottom trace; 440 Hz, 5×10^{-5} m s^{-1}). Tone stimulation evoked a RFM response in one of the flying males while the other male maintained his WBF. White bars indicate the duration of onset, modulation and offset phases. Blue and white arrows on the spectrogram correspond to the fundamental WBF and lower harmonics of the responding and non-responding male, respectively.

the modulation phase was variable and ranged from ∼150 ms up to more than 2 s (Table 1). During this phase, the male was flying near (≤4 cm) the sound source while displaying tight loops around it (Fig. 2A). In some interactions, the male touched the sound source without ceasing RFM. The acoustic behaviour terminated with the offset phase (Fig. 1), during which the WBF 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 departing from the vicinity of the sound source (Fig. 2).

Table 1. Wing-beat frequency (WBF) and temporal characteristics of the rapid frequency modulation (RFM) behaviour in free-flying *A. coluzzii* and *A. gambiae* males

	A. coluzzii (*N*=91)	*A. gambiae* (*N*=88)	*t*	*P*
WBF (Hz)	626–912	675–903		
	793±5.8	779±5.2	1.586	0.065
ΔOnset (Hz)	43–228	54–193		
	109±3.9	101±2.9	1.668	0.097
Onset duration (ms)	30–500	17–220		
	83±5.8	79±4.2	0.604	0.547
Modulation duration (ms)	167–2407	127–2186		
	642±46.1	766±49.2	1.831	0.069
Single FM duration (ms)	87±2.4	83±2.1	1.253	0.212
ΔOffset (Hz)	18–140	26–188		
	66±2.7	73±3.1	1.603	0.111
Offset duration (ms)	56–759	45–623		
	250±13.9	242±15.5	0.389	0.698
RFM duration (ms)	422–3146	341–2668		
	976±54.4	1086±54.8	1.437	0.153

Data are given as the range/mean±s.e.m.
WBF, wing-beat frequency; FM, frequency modulation.

The total duration of RFM behaviour, from the onset (steep frequency spike) until the end of the offset phase was approximately 1 s 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 Fig. 3A. The two species had similar thresholds of response (Table 2) and RFM responses were elicited within the same frequency range (280–620 Hz; 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 and 500 Hz (Fig. 3A, Table 2), which encompasses the WBF ranges of their conspecific females (Fig. 3A, Table 3).

The mean 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 ∼4.5×10^{-5} m s^{-1} 2 cm in front of their heads (dashed lines in Fig. 3A), which considerably exceeds the behavioural threshold of the males. Within the range of the most sensitive frequencies, *Anopheles* males responded to particle velocities between 8.7×10^{-7} and 7.3×10^{-6} m s^{-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 and the stimulus frequency indicates that *Anopheles* males flying at lower WBFs tend to respond to the lower frequencies of the stimulus range, while males flying at higher WBFs tend to respond 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 female-like tone detection by male *Anopheles* is dependent on their own WBFs.

The HCR was calculated in order to discover whether frequency tuning and RFM might be correlated to the frequency matching of flight-tone harmonics as described for both of these *Anopheles* species (Pennetier et al., 2010). The HCRs of *A. coluzzii* and *A. gambiae* are similar and are not centred on any particular value (Fig. 3C). Instead, and in both species, there is a proportional increase of HCR with stimulus frequency, which indicates that the RFM response 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 circle areas in Fig. 3C) lie roughly between HCRs of 0.45 and 0.7, a range which encompasses the harmonic convergences 2 female:1 male (HCR=0.5) and 3 female:2 male (HCR=0.666).

DISCUSSION

Here, we have described and quantified the RFM acoustic behaviour of free-flying *A. coluzzii* and *A. gambiae* males. The RFM response performed by *Anopheles* males is a stereotypical, open-loop behaviour elicited by 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 *C. 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 in both 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

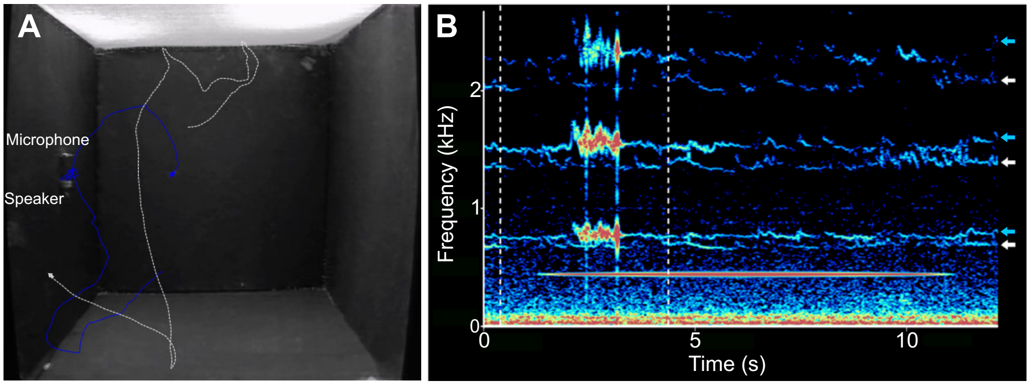

Fig. 2. Flight path and spectrogram of RFM behaviour of *Anopheles* males. Flight path (A) and spectrogram (B) of the WBF of two free-flying *Anopheles coluzzii* males when stimulated with a female-like tone (bottom trace; 10 s, 440 Hz, 5×10^{-5} m s^{-1}). Blue and white paths in A represent the spatial position of a responding male and a non-responding male, respectively. Arrows on the flight path indicate the direction of flight. The lighter interval in spectrogram in B corresponds to the duration of the illustrated flight paths. Blue and white arrows on the 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 remained flightless during the entire sequence.

throughout the Culicidae family, particularly in mosquito species with sexual dimorphism in their flight tones; in this context, it will be particularly interesting to determine whether 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 WBFs of males and females do not differ between species, which corroborates the data

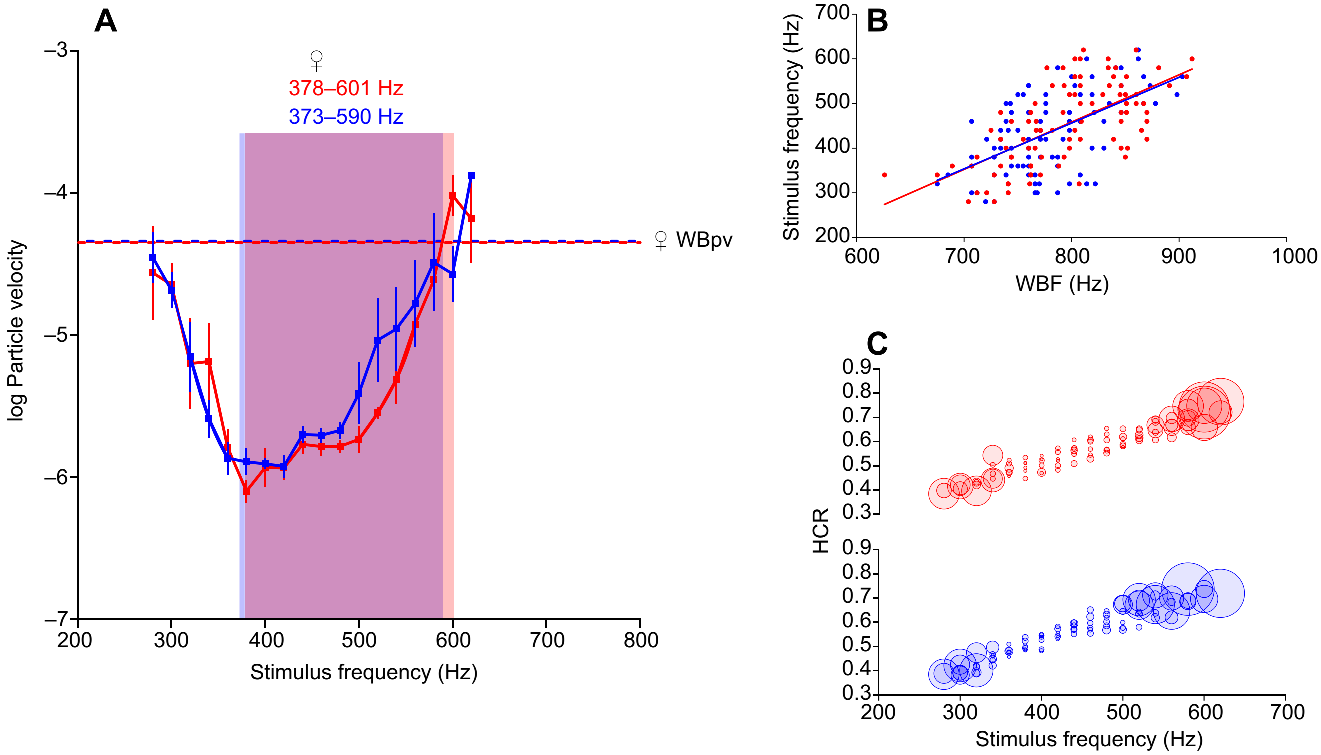

Fig. 3. Behavioural audiograms of *A. coluzzii* and *A. gambiae* males. (A) Threshold of RFM behaviour (means±s.e.m. expressed as the particle velocity of the sound stimulus in m s^{-1} measured 2 cm from the front of the speaker) as a function of stimulus frequency (*N*=6 replicates for each species). The shading indicates the WBF range of free-flying females (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*: 4.5×10^{-5}±2.1×10^{-6} m s^{-1}, *A. gambiae*: 4.6×10^{-5}±1.9×10^{-6} m s^{-1}, *N*=6 each). (B) Correlation between WBF of responding males and stimulus frequency (*A. coluzzii*: stimulus=1.1×♂WBF-389, Pearson's *r*=0.41; *A. gambiae*: stimulus=1.0×♂WBF-365, Pearson's *r*=0.32). (C) Relationship between stimulus frequency that elicited a RFM response and the harmonic convergence ratio (HRC). Circle areas are proportional to stimulus intensity.

Table 2. ANOVA results for the behaviour audiograms measured as the threshold particle velocity against *Anopheles* species and stimulus frequency

Source	d.f.	SS	*F*	*P*
Species	1	0.21	1.52	0.220
Stimulus frequency	17	45.73	19.70	<0.0001*
Species×stimulus frequency	17	2.40	1.04	0.425
Error	143	19.54		

Particle velocity values were expressed as $\log_{10}$. Species: *A. coluzzii* and *A. gambiae*; stimulus frequency range: 280–620 Hz. Asterisk denotes statistical significance.

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* males (S form in Tripet et al., 2004), while the average WBF of the females is almost identical for the two species. However, and taking into 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 the RFM response of males to pure tones, in terms of changes of frequency, duration or frequency modulation. The RFM response probably serves as a controlled flight to reach and maintain a close position near the female while attempting to seize her for copula (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 HCR for the two species is very similar and increases in proportion to 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 a 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 the two species show greater sensitivity to the same range of frequencies (360–500 Hz), 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 between 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 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 on their own WBFs. Furthermore, previous measures of the electrophysiological tuning of the JO of *A. gambiae* males (Pennetier et al., 2010) reported a minimum frequency around 300 Hz, which is almost ~100 Hz below the minimum frequency range for the female WBF. Also, 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 for *A. gambiae* and *A. coluzzii* (present study) and those for *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 of female flight tones. Conversely, this suggests that it 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 mating 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.

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

	N	*A. coluzzii*	*A. gambiae*	*t*	*P*
WBF (Hz)		378–601	373–590		
	30	488±11.5	490±10.5	0.155	0.878
Sound intensity (m s^{-1})	8	$4.5\times10^{-5}\pm1.94\times10^{-6}$	$4.6\times10^{-5}\pm2.05\times10^{-6}$	0.895	0.831

Data are given as the range and/or mean±s.e.m.
Sound intensity: particle velocity generated by tethered-flying females 2 cm in front of their heads.

The precise mechanisms for observed assortative mating remain 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 in 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. This could not only provide females with a mechanism for selecting high-quality males (Cator et al., 2010; Pennetier et al., 2010) but 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 recombinant 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 this and that of *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, this 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.

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Competing interests

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Author contributions

P.M.V.S., G.G. and I.J.R. designed experiments; P.M.V.S. took the measurements; P.M.V.S. and I.J.R. analysed the data; P.M.V.S., G.G. and I.J.R. wrote the paper.

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