1 **Pre-copula acoustic behaviour of males in the malarial mosquitoes *Anopheles coluzzii* and**

2 ***A. gambiae s.s.* does not contribute to reproductive isolation**

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16 **Running title**

17 *Anopheles* pre-copula acoustic behaviour

18

19 **Summary Statement**

20 *Anopheles gambiae s.s.* and *A. coluzzii* male mosquitoes display closely similar stereotypical

21 acoustic behaviour in response to artificial tones at frequencies within the female wing-beat

22 frequency range. Our findings strongly indicate that assortative mating between *A. coluzzii* and *A.*

23 *gambiae* is unlikely to be based on this stereotypical pre-copula acoustic behaviour.

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25

26 **ABSTRACT**

27 We reveal that males of two members of the *Anopheles gambiae s.l.* species complex, *A. coluzzii*

28 and *A. gambiae s.s.* (hereafter *A. gambiae),* which are both malaria vectors, perform a stereotypical

29 acoustic behaviour in response to pure tones at frequencies that encompass the frequency range of

30 the female's flight-tones. This behaviour resembles that described for *Culex quinquefasciatus* and

31 consists of phonotactic flight initiated by a steep increase in wing-beat frequency (WBF) followed

32   by Rapid Frequency Modulation (RFM) of WBF when in close proximity to the sound source.

33   RFM was elicited without acoustic feedback or the presence of a live female, but it appears to be a

34   stereotypic behaviour in the immediate lead up to copula formation. RFM is an independent and

35   different behavioural process from harmonic convergence interactions used by male-female pairs

36   for mate recognition at earlier stages of mating. Acoustic threshold for RFM was used to plot

37   behavioural audiograms from free-flying *A. coluzzii* and *A. gambiae* males. These audiograms

38   were almost identical (minima ~400 Hz) and encompassed the WBF ranges of *A. coluzzii* (378-601

39   Hz) and *A. gambiae* females (373-590 Hz), indicating that males of both species share similar

40   frequency tuning and range. Furthermore, no differences were found between the two species in

41   their WBFs, RFM behaviour or Harmonic Convergence Ratios. These results indicate that

42   assortative mating between *A. coluzzii* and *A. gambiae* is unlikely to be based on male-specific

43   acoustic behaviours during RFM. The significance of these findings in relation to possible

44   mechanisms for assortative mating is discussed.

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47   **INTRODUCTION**

48   The complexity of malaria epidemiology and control is due in part to the remarkable degree of

49   genetic variation among the species of the genus *Anopheles* (della Torre et al., 2005; Coetzee et al.,

50   2013). This is particularly evident in the species complex *Anopheles gambiae s.l.,* found across

51   much of sub-Saharan Africa and comprising at least nine morphologically similar species that vary

52   in vector status, geographic distribution and ecology (Coetzee et al., 2013; Crawford et al., 2015).

53   *Anopheles gambiae s.l.* species frequently occur in partially reproductively isolated and

54   differentiated subpopulations, which in some cases led to rapid ecological speciation (Costantini et

55   al., 2009; Coetzee et al., 2013; Crawford et al., 2015). In the context of public health, these

56   speciation processes are of epidemiological importance because they influence vectorial capacity,

57   vector distribution range and, consequently, species-specific means of control (Lehmann and

58   Diabaté, 2008).

59        *Anopheles coluzzii* and *A. gambiae s.s.* (hereafter *A. gambiae*) are morphologically

60   indistinguishable species, until recently considered to be two different molecular forms of the

61   same species (M and S molecular forms, respectively) (Coetzee et al., 2013). They share an

62   extensive geographical range in Central and West Africa (with over 90% of the range of *A. coluzzii*

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63  overlapping with that of *A. gambiae*) (Lehmann and Diabaté, 2008). However, they can exhibit

64  marked local habitat segregation, with *A. coluzzii* having an extended distribution into more arid

65  environments and *A. gambiae* mainly found in more humid habitats (Diabaté et al., 2006, 2009;

66  Lehmann and Diabaté, 2008; Dabiré et al., 2013; Sawadogo et al, 2013). The causes for this habitat

67  segregation are complex and involve phenotypic differences across all life stages (reviewed in

68  Lehmann and Diabaté, 2008), but appears to be primarily associated with differential larval

69  adaptations to exploit temporary or permanent freshwater habitats (Diabaté et al., 2008; Lehmann

70  and Diabaté, 2008). Reproductive isolation between populations of *A. coluzzii* and *A. gambiae* is

71  facilitated by assortative mating caused by temporal and spatial segregation of male swarms

72  (Diabaté et al., 2009; Sawadogo et al, 2013).

73       Intriguingly, some natural sympatric populations of *A. coluzzii* and *A. gambiae* form mixed

74  swarms with very low hybridization rates, suggesting the existence of other assortative mating

75  processes (Tripet et al., 2001; Diabaté et al., 2006; Dabiré et al., 2013; Sawadogo et al, 2013)

76  which appear to be mediated by as yet unidentified pre-mating, within-swarm mate recognition

77  mechanism. Given the well-known observation that male mosquitoes locate females by flying

78  towards the source of the female flight tone (Child, 1894; Roth, 1948; Wishart and Riordan, 1959;

79  Charlwood and Jones, 1979; Belton, 1994), previous studies have investigated the possible role of

80  flight-tone (Brogdon, 1998; Tripet et al., 2004) or harmonic convergence (Pennetier et al., 2010) in

81  mate- and species-recognition between these two *Anopheles* species, but without unequivocal

82  conclusions.

83       Rapid Frequency Modulation (RFM) behaviour, recently described in male *Culex*

84  *quinquefasciatus*, is an acoustic response to the fundamental frequency of female flight-tones

85  immediately prior to mating sequences (Simões et al., 2016). Significantly, this is a stereotypical

86  behaviour that can be exploited to derive behavioural audiograms from free-flying male

87  mosquitoes (Simões et al., 2016). The investigation of this behaviour in *A. coluzzii* and *A. gambiae*

88  reported here has provided an opportunity to extend knowledge of the pre-mating behaviour in

89  anopheline mosquitoes and to discover if the RFM behaviour could form a basis for assortative

90  mating in these two species.

91       Here, we characterize and quantify the RFM acoustic behaviour of *A. coluzzii* and *A.*

92  *gambiae* free-flying male mosquitoes. RFM in both species is elicited by tones at frequencies that

93  encompass the frequency range of the two species' female flight-tones. We used this stereotypical

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94 behaviour to derive behavioural audiograms for each species. Comparisons of the acoustic

95 parameters of RFM, audiograms and WBFs show that no inter-specific differences were found

96 between *A. coluzzii* and *A. gambiae*, indicating that assortative mating in these species is unlikely

97 to be based on male-specific auditory behaviours during the RFM phase of mating. We discuss the

98 consequences of these findings in relation to other possible mechanisms of assortative mating.

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101 **MATERIALS AND METHODS**

102 *Mosquitoes*

103 *Anopheles coluzzii* Coetzee & Wilkerson (formerly M molecular form) and *Anopheles gambiae*

104 Giles (formerly S molecular form) mosquitoes were obtained from Dr. K.R. Dabiré (Institute de

105 Recherche en Sciences de la Santé, Bobo Dioulasso, Burkina Faso). These colonies were derived

106 from populations in which mix-swarm assortative mating was reported (Diabaté et al., 2006);

107 *Anopheles coluzzii* from larvae collected in village VK7 and *A. gambiae* from larvae collected in

108 Soumousso, both in Burkina Faso. The colonies were lab-reared, maintained and bred in

109 controlled-environment chambers (70-75% rH, 26±2°C and 12 h light: 12 h dark cycles). Adult

110 mosquitoes 4-14 days post-emergence were tested during the first 3 h of the scotophase.

111

112 *Behavioural set-up*

113 The acoustic behaviour of free-flying mosquitoes was recorded inside a wire-framed arena of 30

114 cm sides which was covered by white cotton tubular-gauze and placed on a vibration damped table

115 (Newport®, Irvine, Ca, USA) inside an sound attenuated booth (IAC Ltd, Winchester, UK). For the

116 video/audio recordings, the metal frame was covered with matt-black cotton fabric, which is

117 non-reflective to infra-red light, while the front side was covered by transparent acrylic enabling

118 the camera to view the chamber's interior. The ceiling was covered with white cotton gauze to

119 allow the chamber to be illuminated by two infra-red multi-LED lights positioned 1 m above the

120 cage.

121 Tone stimuli generated using the sine wave function of Test Tone Generator 4.4

122 (EsserAudio®, 2011) software were delivered to the cage from a sound source consisting of a 0.5

123 cm diameter plastic probe tip, damped with acoustic foam, connected via a 1 cm diameter

124 polythene tube to an adapted Audio Technica® ATH A700AX speaker (5-35,000 Hz range with

125  flat frequency response 100-25,000 Hz). Sound from the speaker and flight-tones from the

126  mosquitoes were monitored using a particle velocity microphone (Knowles NR-3158, Ithaca NY,

127  USA) that was calibrated (Go□pfert and Robert, 2001) and mounted ~4 cm from the speaker

128  probe tip. A pressure microphone (Knowles 23132, Ithaca NY, USA) mounted at the focal point of

129  an 18" parabolic reflector (Edmunds), was placed on one side of the flight arena to monitor the

130  sound inside. Signals from each of the microphones were amplified 100-fold with a purpose built

131  two-channel preamplifier and the output of each channel was digitized at 192 kHz using a

132  Fireface® UC sound card. The digital outputs were then recorded using Spectrogram 16

133  (Visualization Software, LLC) at a sampling rate of 48 kHz and frequency resolution of 5.9 Hz.

134  Spectrogram 16 was also used to analyse and extract data on the time, frequency and amplitude of

135  all acoustic signals.

136  For video recordings, an infra-red video camera (Swann® Pro-880) was placed 30 cm in

137  front of the clear wall of the chamber and connected to the computer. Digital video recordings at

138  30 FPS of the flying mosquitoes were obtained using Debut Video Capture Software v1.88 (NCH®

139  Software). The flight paths were then digitised using Kinovea (Version 0.8.23) software.

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141  ***Behavioural audiograms***

142  Male mosquitoes were placed inside the flight arena at the time of spontaneous circadian activity

143  and left to fly freely during the recordings. After ~10 min period of adaptation to conditions inside

144  the booth, the mosquitoes started to fly spontaneously, whereupon sound recording and stimuli

145  presentation were initiated. All behavioural experiments were conducted at a room temperature of

146  $30\pm2°C$, which is within the range of temperatures of the natural habitat of *A. gambiae s.l.*

147  mosquitoes (Huestis et al., 2012).

148  The behavioural audiograms of male mosquitoes were derived by recording the threshold

149  of the RFM response relative to the particle velocity of the sound stimulus for tone frequencies

150  between 200-1000 Hz (20 Hz increments until 700 Hz, 100 Hz increments thereafter). In each

151  replicate (N=6), a group of 7-10 males was placed in the flight arena under illumination simulating

152  dusk, when they are normally active. Upon initiation of spontaneous flight, a continuous tone of

153  fixed frequency was presented to the swarming mosquitoes. The tone level was increased at a rate

154  of 0.4 dB $s^{-1}$ from $\sim1\times10^{-8}$ ms$^{-1}$ output until an RFM response was elicited from at least one male or

155  until the maximum operating level ($4\times10^{-4}$ ms$^{-1}$) was reached. The sound stimulus was then

156 terminated and the particle velocity that elicited the response and the WBF of the responding male

157 immediately before the onset of RFM were stored. After a 5-10 s rest period without stimulation,

158 the procedure was repeated for another stimulus frequency. Particle velocity values were

159 expressed as $\log_{10}$ for graphical display and statistical testing. Even when several males were

160 swarming at the same time, the spectrogram analysis permitted the detection and isolation of the

161 RFM response of individual males because the responses of an individual close to the microphone,

162 which measured particle velocity rather than pressure, was much louder than the humming of the

163 swarm in the background. The presence of higher harmonics of flight-tones provided a further

164 basis for distinguishing between the WBFs of individual males.

165 The Harmonic Convergence Ratio (HCR) for each male was calculated by dividing the

166 stimulus frequency (which simulates the WBF of a female) by the WBF just prior to the onset of

167 RFM elicited by the stimulus. The inverse of the HCR corresponds to the harmonic relation of the

168 two sound frequencies; e.g. HCR=0.5=1/2 indicates a 2:1 harmonic relation, i.e. the frequency of

169 the $2^{nd}$ harmonic of the female-like sound is equal to the fundamental WBF, whereas,

170 HCR=0.667=2:3 indicates a 3:2 harmonic relation, which would correspond to a frequency

171 convergence between the $3^{rd}$ harmonic of the stimulus and the $2^{nd}$ harmonic of the WBF. Although

172 the stimulus frequencies were sinusoidal pure tones, harmonics of these pure tones are produced in

173 the vibrations of the male's antenna and JO upon sound detection, so males can potentially use

174 these tones to reach harmonic convergence (Cator et al., 2009; Warren et al., 2009; Pennetier et al.,

175 2010).

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177

178 **RESULTS**

179 Males of both *A. coluzzii* and *A. gambiae* exhibited Rapid Frequency Modulation (RFM)

180 behaviour, an acoustically driven flight response, when stimulated with pure tones at frequencies

181 similar to the fundamental frequency of the female flight-tones. RFM in *Anopheles* males

182 comprises three phases with distinct spectrographic and flight characteristics. This behaviour

183 pattern is very similar to that reported for *C. quinquefasciatus* (Simões et al., 2016) and consists of

184 the Onset, the Modulation or main phase, and the Offset (Fig. 1). The Onset phase is characterised

185 by a steep increase in WBF of ~100 Hz in ~80ms (Table 1), which corresponds to a remarkable

186 rate of 1250 Hz/s, and is associated to the phonotatic flight approach of the male to the sound

187 source (Fig. 2A and B).

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

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

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

211 The average WBF of females and the sound intensity of their wing beats were also
212 statistically similar between *A. coluzzii* and *A. gambiae* species (Table 3). Tethered-flying females
213 generated particle velocities of $\sim 4.5 \times 10^{-5}\,\mathrm{ms}^{-1}$ 2 cm in front of their heads (dashed lines in Fig.
214 3A), which considerably exceeds the behavioural threshold of the males. *Anopheles* males
215 responded within the range of the most sensitive frequencies to particle velocities between
216 $8.7 \times 10^{-7}\,\mathrm{ms}^{-1}$ and $7.3 \times 10^{-6}\,\mathrm{ms}^{-1}$ at a reference point 2 cm from the speaker, which is ~25 dB below
217 the average sound intensity of the female flight-tones.

218    The positive correlation between WBF measured just prior to the onset of RFM and the

219    frequency of the stimulus shows that *Anopheles* males flying at lower WBFs tend to respond to the

220    lower frequencies of the stimulus range, while males flying at higher WBFs respond more often to

221    higher stimulus frequencies (Fig. 3B). The slope and range of this correlation are similar in the two

222    species, and, as reported for *C. quinquefasciatus* (Simões et al., 2016), suggest that the detection of

223    female-like tones (and consequently the expression of RFM) by male *Anopheles* is dependent on

224    their own WBFs.

225    The Harmonic Convergence Ratio (HCR) was calculated in order to discover if frequency

226    tuning and RFM behaviour might be related to the frequency matching of flight-tone harmonics as

227    described for both these *Anopheles* species (Pennetier et al., 2010). The HCRs of *A. coluzzii* and *A.*

228    *gambiae,* plotted as a function of the stimulus frequency, are similar and not centred on any

229    particular value (Fig. 3C). Rather, in both species the HCRs increase proportionally with stimulus

230    frequency, which indicates that the initiation of the RFM response by the males is independent of

231    any harmonic convergence between their flight-tones and the stimulus. Interestingly, the most

232    sensitive RFM responses (elicited by low particle velocity levels, as indicated by the bubble areas

233    in Fig. 3C) lie roughly between HCRs of 0.45-0.7, a range which encompasses the harmonic

234    convergences $2\female:1\male$ (HCR=0.5) and $3\female:2\male$ (HCR=0.666).

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236

237    **DISCUSSION**

238    Here we describe and quantify the Rapid Frequency Modulation (RFM) acoustic behaviour of

239    free-flying males of *Anopheles coluzzii* and *A. gambiae.* The RFM response performed by

240    *Anopheles* males is a stereotypical, open loop behaviour in response to tone stimulation at

241    frequencies within the range of the fundamental component of female flight-tones and the pattern

242    of behaviour is identical to that observed for *Culex quinquefasciatus* mosquitoes (Simões et al.,

243    2016). Similarly, this behaviour also involves, particularly at the Onset and Modulatory phases,

244    very fast changes in WBF of the flying males ($>1250\,\text{Hz s}^{-1}$). The fact that RFM was observed both

245    in the *Culex* and *Anopheles* genera is significant because it indicates that this pre-copulatory

246    behaviour is shared by the Culicinae and Anophelinae subfamilies which diverged ~200 Ma

247    (Reidenbach et al., 2009). It also suggests that the RFM might be found throughout all the

248    Culicidae family, particularly in mosquito species with sexual dimorphism in their flight-tones; in

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249 this context, it will be particularly interesting to determine if mosquito species without this sexual

250 dimorphism, such as *Toxorhynchites brevipalpis* (Steffan and Evenhuis, 1981; Gibson and

251 Russell, 2006) have lost this pre-copulatory behaviour.

252       Overall, no inter-specific differences were found between *A. coluzzii* and *A. gambiae* males

253 in their free-flight WBFs, pre-copulatory behaviour, and behavioural audiograms. Likewise, no

254 differences were found in the WBF and sound intensity of the females of both species. The average

255 free-flight WBF of males and females do not differ between species and corroborates the data

256 published by Tripet et al. (2004). Curiously, and albeit non-significant in both studies, the average

257 WBF of *A. coluzzii* males (M form in Tripet et al., 2004) is slightly higher (~15 Hz) than that of *A.*

258 *gambiae* (S form in Tripet et al., 2004) males, while the average WBFs of the females is almost

259 identical. However, and taking in account their frequency range, it is unlikely that this slight

260 frequency difference would reflect any basis for specific differences between the two *Anopheles*

261 species.

262       No inter-specific differences were found in the acoustic parameters of RFM response of

263 males to pure tones, either in changes of frequency, duration or frequency modulation. The RFM

264 response probably serves as a controlled flight to reach and maintain a close-range position while

265 attempting to seize and engage terminalia with the female (Roth, 1948; Wishart and Riordan,

266 1959; Charlwood and Jones, 1979; Simões et al., 2016). These similarities suggest that this

267 pre-copulatory behaviour and the associated flight parameters are, in structure and function,

268 indistinguishable between the two *Anopheles* species and should not provide a basis for the

269 isolation of these two species. Furthermore, the Harmonic Convergence Ratio (HCR) for both

270 species is very similar, and not centred on any particular value, increasing proportionally with

271 stimulus frequency. This indicates that, as in *C. quinquefasciatus* (Simões et al., 2016), initiation

272 of the RFM response in *Anopheles* males is independent of any harmonic convergence between the

273 male flight-tones and the stimulus. Significantly, these results show that it is unlikely that

274 harmonic convergence, at least by the males, during the initiation of RFM behaviour can be used

275 as mechanism for species recognition in *Anopheles* (Pennetier et al., 2010). However, little is

276 known about the role of harmonic convergence in the earlier phases of mating behaviour.

277       The behavioural audiograms for the *A. coluzzii* and *A. gambiae* males are very similar and

278 have identical frequency ranges. Furthermore, males of both species are more sensitive to the same

279 range of frequencies (360-500Hz), which encompasses the WBF range of free-flying females.

280  Similar hearing range and sensitivity indicates that the pre-mating isolation between these two
281  *Anopheles* species is not related to morphological or physiological differences between their
282  hearing organs. Moreover, the finding that *A. coluzzii* and *A. gambiae* males share the same
283  hearing range and sensitivity further indicates that they should not be able to identify and
284  discriminate conspecific females based solely on their WBF.

285       *Culex* males use acoustic distortion to hear female-like tones (Simões et al., 2016).
286  Acoustic distortion can be seen as the generation of new vibrations – intermodulation distortion
287  products – as a consequence of the interaction between two simultaneous tones of different
288  frequencies in the mosquito's antenna (Warren et al., 2009; Pennetier et al., 2010; Lapshin, 2012;
289  Simões et al., 2016). In flight, this corresponds to the interaction between the fundamental
290  frequency of the male's own flight tone and the flight tone of a nearby flying female generating a
291  third frequency equal to the arithmetic difference between the first two. The male's hearing organ
292  - the Johnston's organ (JO) - is rather insensitive to the two flight tones but very sensitive to their
293  frequency difference, which is amplified up to 100 times before the signal is transmitted to the
294  insect's brain (Simões et al., 2016). Thus, is it probable that *Anopheles* males hear female
295  flight-tones by detecting distortion products produced by the frequency differences in their WBFs,
296  as reported for *Culex*? We found a strong positive correlation between the male WBFs and the
297  stimulus frequency that elicited RFM, which suggests that the detection of female-like tones (and
298  consequently the expression of RFM) by male *Anopheles* is dependent of their own WBFs.
299  Furthermore, previous measures of the electrophysiological tuning of the JO of *A. gambiae* males
300  (Pennetier et al., 2010) reported a minima frequency around 300 Hz, which is almost ~100Hz
301  below the minimum frequency range for the female WBF. Also, in the same study Pennetier et al.
302  (2010) found that distortion is indeed generated in the vibrations of the antenna of the *A. gambiae*
303  males and detected in the electrical responses of the JO. Taken together, these observations
304  suggest that male *Anopheles* might use distortion products to detect flying females.

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

311    conspecificity and mate assessment to male mosquitoes.

312         Natural sympatric populations of *A. coluzzii* and *A. gambiae* can form mixed swarms
313    (Diabaté et al., 2006; Dabiré et al., 2013; Sawadogo et al., 2013). Analysis of these swarms
314    revealed a very low percentage of hybrids and few inter-specific copulae within them, which
315    indicates the existence of assortative mating, most probably caused by pre-mating isolation
316    mechanisms (Dabiré et al., 2013). However, Dao et al. (2008) showed that when both species
317    congregate inside huts, cross-species is as frequent as within-species mating, indicating that
318    assortative mating breaks down when mating occurs indoors. This is consistent with reports
319    observing the absence of assortative mating in lab-reared *Anopheles* colonies (Benedict et al.,
320    2009; Paton et al., 2013), which, overall, suggests that chemical cues such as pheromones and
321    cuticular hydrocarbons (Dao et al., 2008) and flight tones (Dao et al., 2008; Tripet et al., 2004) do
322    not play a major role in species recognition.

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

334         Interestingly, the conjunction of all these results indirectly suggests that harmonic
335    convergence might be a behaviour mediated fundamentally by female mosquitoes. On the one
336    hand, that could provide females a mechanism for selecting high-quality males (Cator et al., 2010;
337    Pennetier et al., 2010), but, on other hand, it could also play a role in the assortative mating of *A.*
338    *coluzzii* and *A. gambiae*. Crucially, the hypothesis that assortative mating could be mediated by
339    females is supported by the results of a recent study by Aboagye-Antwi et al. (2015); behavioural
340    assays in recombinants strains for the M and S markers in the X chromosome of both *Anopheles*
341    species revealed that females, but not males, mated assortatively, indicating that a species

342  recognition mechanism appears to be female-dependent. This, however, does not mean that males
343  do not contribute to assortative mating in nature; in the field, males are known to contribute to
344  assortative mating via swarm spatial segregation (Diabaté et al., 2006; Dabiré et al., 2013;
345  Sawadogo et al., 2013; Aboagye-Antwi et al., 2015).

346      Two main conclusions can be drawn from the overall similarity of the pre-copulatory male
347  acoustic behaviour in *A. coluzzii* and *A. gambiae*, and indeed between those and *C.*
348  *quinquefasciatus*: Firstly, the results indicate that the RFM response and the associated flight
349  characteristics represent a stable mating strategy, probably shared by all sexually dimorphic
350  mosquito species. In this context, it predicts that male *Aedes*, a genus of equivalent medical
351  importance, would also exhibit the same behavioural processes. Secondly, the non-specificity of
352  the frequency range eliciting the male behaviour has implications for novel mosquito control tools,
353  particularly those designed to make use of sound signals as the basis for acoustic traps.
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355  **List of abbreviations**
356  JO – Johnston's organ
357  HCR – Harmonic Convergence Ratio
358  RFM – Rapid Frequency Modulation
359  WBF – Wing Beat Frequency
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369  **Competing Interests**
370  No competing interests declared.
371

372  **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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528 **TABLES**

529

530 **Table 1.** Wing Beat Frequency (WBF) and temporal characteristics of the RFM behaviour in

531 free-flying *A. coluzzii* and *A. gambiae* males (range and x̄ ± s.e.m.).

| | Species | | T value | *P* |
|---|---|---|---|---|
| | *A. coluzzii* (N=91) | *A. gambiae* (N=88) | | |
| WBF (Hz) | 626-912 | 675-903 | | |
| | 793±5.8 | 779±5.2 | 1.586 | 0.065 |
| Δ Onset (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 |
| Δ Offset (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 |
| Duration of RFM (ms) | 422-3146 | 341-2668 | | |
| | 976±54.4 | 1086±54.8 | 1.437 | 0.153 |

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540 **Table 2.** ANOVA results for the behaviour audiograms measured as the threshold particle velocity

541 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 x Stimulus frequency | 17 | 2.40 | 1.04 | 0.425 |
| Error | 143 | 19.54 | | |

542 Particle velocity values were expressed as $\log_{10}$. Species: *A. coluzzii* and *A. gambiae*; Stimulus

543 frequency range: 280-620 Hz. Asterisk denotes statistical significance.

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550 **Table 3.** Wing Beat Frequency (WBF) and sound intensity of wing beats (measured in particle

551 velocity) of *A. coluzzii* and *A. gambiae* females.

| | | Species | | T value | P |
|---|---|---|---|---|---|
| | | *A. coluzzii* | *A. gambiae* | | |
| WBF (Hz) | Range | 378-601 | 373-590 | | |
| | $\bar{x} \pm$ s.e.m (N=30) | 488±11.5 | 490±10.5 | 0.155 | 0.878 |
| Sound intensity ($ms^{-1}$) | $\bar{x} \pm$ s.e.m (N=8) | $4.5 \times 10^{-5} \pm 1.94 \times 10^{-6}$ | $4.6 \times 10^{-5} \pm 2.05 \times 10^{-6}$ | 0.895 | 0.831 |

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

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**FIGURE LEGENDS**

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**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.

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**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.

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**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

587 female wing-beat frequencies (WBFs) (Red: *A. coluzzii*, Blue: *A. gambiae*, Purple: Common

588 range). ♀WBpv: mean particle velocity generated by the wing beats of tethered-flying females

589 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*:

590 $4.6 \times 10^{-5} \pm 1.9 \times 10^{-6}$ ms$^{-1}$, N=6 each). B) Correlation between WBF of responding males and

591 stimulus frequency (*A. coluzzii*: Stimulus=1.1 x ♂WBF-389, Pearson's r=0.41; *A. gambiae*:

592 Stimulus=1.0 x ♂WBF-365, Pearson's r=0.32). C) Relation between stimulus frequency that

593 elicited RFM response and the Harmonic Convergence Ratio (HRC). Bubble areas are

594 proportional to stimulus intensity.