Masking of an auditory behaviour reveals how male mosquitoes use distortion to detect females

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Abstract

The mating behaviour of many mosquito species is mediated essentially by sound: males follow and mate with a female mid-flight by detecting and tracking the whine of her flight-tones. The stereotypical Rapid Frequency Modulation (RFM) male behaviour, initiated in response to the detection of the female's flight-tones, has provided a means of investigating these auditory mechanisms while males are free-flying. Mosquitoes hear with their antennae, which vibrate to near-field acoustic excitation. The antennae generate non-linear vibrations (distortion products, DPs) at frequencies that are equal to the difference between the two simultaneously presented tones, e.g. the male and female flight-tones, which are detected by mechanoreceptors in the auditory (Johnston's) organ (JO) at the base of the antenna. Recent studies indicated the male mosquito's JO is tuned not to the female flight-tone, but to the frequency difference

between the male and female flight-tones. To test the hypothesis that mosquitoes detect this frequency difference, *Culex quinquefasciatus* males were presented simultaneously with a female flight-tone and a masking tone, which should suppress the male's RFM response to sound. The free-flight behavioural and *in vivo* electrophysiological experiments revealed that acoustic masking suppresses the RFM response to the female's flight-tones by attenuating the DPs generated in the non-linear vibration of the antennae. These findings provide direct evidence in support of the hypothesis that male mosquitoes detect females when both are in flight through difference tones generated in the vibrations of their antennae due to interaction between their own flight-tones and those of a female.

Introduction

Acoustic masking has been used as a tool to measure frequency selectivity of the auditory system at all levels from the receptor to behaviour in mammals, including humans [1, 2]. More recently it has been used to study the sensory and neural mechanisms of acoustic detection in insects with tympanic hearing, such as in crickets or *Ormia* flies [3-8]. Here we use simultaneous acoustic masking to investigate the hearing mechanisms of the male mosquito *Culex quinquefasciatus*, an insect with antennal hearing.

Recent studies indicated that the male mosquito's auditory Johnston's organ (JO) is tuned not to the female flight-tone, but to the frequency difference between the male and female flight-tones [9-14]. To test the hypothesis that male mosquitoes listen and respond to this frequency difference, *Culex quinquefasciatus* males were presented simultaneously with a probe tone, which simulated the female flight-tone [9] and a masking tone, which was hypothesized to suppress the male's acoustic RFM response to sound, especially if the masking tone was close in frequency to that of the probe tone [2]. We used simultaneous acoustic

masking of the stereotypical, acoustically-driven behaviour – Rapid Frequency Modulation (RFM) – [9, 10] and of the electrical responses of the JO [9, 11-14] to explore what it is that male mosquitoes listen to when they detect the flight-tones of females and if their behaviour is determined by the non-linear properties and frequency responses of the JO.

Mosquitoes detect the near-field component of sound (air particle displacement) through vibrations of the antennae [15, 16]. Antennal vibrations are detected by and transduced into electrical signals by several thousand mechanosensory scolopidia, which compose the JO housed in the pedicel at the base of each of the antennae [17]. The mechanics of the antenna are non-linear; it behaves as a rod that becomes stiffer with increasing displacement [11]. When the antenna is vibrated by two tones, it generates a strong distortion product (vibration) at a frequency that is the arithmetic difference of these two tones, including those that mimic the wing beat frequency (WBF) of male and female mosquitoes [11-13]. Largely because of this non-linearity, the male JO is tuned overall to detect the difference in frequency between the male's own WBF and that of the female [9, 11-14]. The bandwidths of the sensory receptors of the JO set upper frequency limits on the phasic electrical responses that can be recorded from the JO [9, 11-16]. Male and female WBFs are above the frequency bandwidth of the JO. It is therefore hypothesized that male mosquitoes must fly to detect and locate females through listening to difference tones [9, 11-14].

It has long been established that male mosquitoes are attracted by female flight-tones [18-25]. In the case of *C. quinquefasciatus,* males aggregate over visual markers, forming swarms at dawn and dusk [26, 27]. Unmated females approach established swarms, whereupon flying males detect the female flight-tones and a mating chase ensues [17, 26, 27]. Male mosquitoes are also attracted to artificial sound sources emitting pure tones that simulate the flight-tones of a conspecific female mosquito [9, 10, 22-25]. Recently, we reported that free-flying male mosquitoes in two different taxonomic sub-families, *C. quinquefasciatus* [9] and

Anopheles gambiae s.l. [10], exhibit the stereotypical RFM behaviour in response to the fundamental frequency of female flight-tones. The RFM behaviour is defined by its spectrographic characteristics and by the male's flight path; it consists of a steep increase in WBF concomitant with the fast phonotactic flight towards the female (or artificial sound source), followed by rapid modulation of the WBF when in the immediate vicinity of a female or the female-like sound source [9]. RFM is performed only when the male mosquito has detected, located and reached (within ~5 cm) the sound source and represents an acoustic measure of a mating attempt by the male [9, 10]. Thus, this pre-copulatory behaviour provides an acoustic assay with which to monitor the conditions under which a male responds to a sound source emitting a female flight-tone.

The outcome of the free-flight behavioural and *in vivo* electrophysiological experiments reported here indicate that acoustic masking is caused through suppression of the DPs generated in the non-linear vibration of the antennae. Our findings provide direct behavioural evidence in support of the hypothesis that male mosquitoes detect females when both are in flight by detecting difference tones generated in the vibrations of their antennae through the interaction between their own flight-tones and those of a female.

Materials and Methods

Mosquitoes

Colonies of *Culex quinquefasciatus* Say (Muheza strain) were reared in controlled-environment chambers; 70-75% rH, 26±2°C and 12 h light: 12 h dark cycles. Larvae were reared on cat food pallets (Purina® PetCare, Gatwick, UK) and adults were provided with 10 % sugar solution *ad libitum*. Larval density was ~70 l^{-1} of water. Experiments were done with

adult male mosquitoes between 4-14 days post-emergence and during the first 3 h of the scotophase, when mating behaviour occurs under natural conditions.

Behavioural experiments

The set-up used to record the acoustic behaviour of free-flying mosquitoes consisted of a 30 cm sided metal-framed cube covered by white cotton gauze (the flight arena) placed on a vibration-damped table (Newport®, Irvine, CA, USA) inside a sound attenuated booth (IAC Ltd, Winchester, UK). Acoustic stimulation consisted of two different pure tones – a probe tone and a masking tone – delivered simultaneously to the flight arena from two different sound sources. The probe tone was delivered to the centre of the arena through a calibrated 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 Techniques® ATH A700AX speaker. The masking tone was delivered from a calibrated Beyerdynamics® DT 770 speaker placed on a side wall of the arena, thus at a distance of 15 cm from the probe speaker. In Experiment 1 the sounds produced by the two speakers and the male mosquitoes inside the arena were recorded using a calibrated [15] particle velocity microphone (Knowles® NR-3158, Itasca IL, USA) located ~2 cm from the probe speaker tip and a parabolic microphone consisting of a pressure microphone (Knowles® 23132, Itasca IL, USA) mounted at the focal point of an 18" parabolic reflector (Edmunds®) and located on one side of the cage. From the outcome of Experiment 1, we found that some males approached the probe speaker and others approached the masking speaker, so for Experiment 2 the set-up was altered to enable monitoring male approaches to the masking speaker by adding a second calibrated particle velocity microphone placed ~2 cm in front of the centre of the masking speaker. The acquired signals were amplified x100 with a purpose built 2-channel preamplifier and digitized with a RME® Fireface UC

sound card (sampling rate: 192 kHz). Digital sound outputs were recorded and analysed using Spectrogram 16 (Visualization Software, LLC).

Probe and masking tones were produced using the sine wave function of Test Tone Generator 4.4 (EsserAudio®, 2011) and cosine windowed to avoid acoustic transients. Three different probe tone frequencies were used, all within the stimulus range for eliciting an RFM response in males [9]: 340 Hz, which is within the 10 dB bandwidth of the JO; 400 Hz, within the best frequency of the male's behavioural audiogram, and; 450 Hz, within the higher frequency range of free-flying females. The particle velocity of the probe tones was set, using a calibrated particle velocity microphone, to be $5.7x10^{-5}$ ms^{-1} at a reference distance of 2 cm, which is similar to the sound intensity produced by tethered-flying females at the same distance [9]. The frequencies of the masking pure tones varied according to the experiments and probe tones used (Supplemental Tables 1 and 2), ranging between 100-1000 Hz. The masking sound source was set to deliver a particle velocity of ~$8x10^{-5}$ ms^{-1} at a reference distance of 2 cm.

One to four male mosquitoes were placed inside the flight arena and after ~10 min period of adaptation, the mosquitoes started to fly spontaneously, whereupon sound recording and stimuli presentation were initiated. The acoustic booth and flight arena remained in darkness throughout the experiments. The flight-tones of flying males appear in the spectrograms as constant lines corresponding to their instantaneous WBF (Figure S1). Spectrographic analysis of the sound levels and higher harmonics of the flight-tones enabled us to discern between the different individuals flying in the arena at the same time [9,10].

Acoustic stimulation consisted of the simultaneous presentation of a probe tone and a masking tone or the presentation of the probe tone alone (which provided the baseline to which acoustic masking was tested). All tone presentations lasted for 10 s and the interval between presentations was ~5 s. Flying males were stimulated successively with different probe/masking tone pairs, which were presented pseudo-randomly to avoid repetition.

Different tone pairs were presented until all males stopped flying or all probe/masking tone combinations pre-defined for the experiments were delivered (Supplemental Tables 1 and 2). Thus, an individual flying male was stimulated only once for each tone pair. Only flying males were considered for observation during tone presentation. Non-flying males were not considered for observation given they are not visible in the spectrograms.

The RFM acoustic response was used to indicate whether a male detected the presence of a female-like tone. Masking experiments were designed to quantify the occurrence of RFM response in the presence of a probe/masking tone pair. The presentation of a tone pair elicited one of three responses: *i*) the male flew toward the probe speaker and initiated RFM within ~ 5 cm of the probe speaker, *ii*) the flying male showed no conspicuous response to either speaker and maintained its WBF during the presentation, or *iii*) the male flew toward the masking speaker and initiated RFM towards the masking speaker (Figure S1). An RFM response was observed towards a speaker when the spectrogram showed the stereotypical frequency modulation of a male's WBF and the associated 20-30 dB increase in flight-tone level (Figure S1) [9]. Occasionally (<5% of the records), the presentation of a tone pair elicited RFM responses to both speakers; in this situation, we only registered the response towards the first speaker.

For each probe/masking tone pair, the proportion of RFM response was calculated by dividing the sum of the observed RFM responses by the total number of mosquitoes that were flying when that tone was presented. The number of total observations was predefined (n=26 for Experiment 1 and n=32 for Experiment 2) and was equal for all tones pairs presented. To achieve that number of observations we used a total of 36 males in Experiment 1 and 47 males in Experiment 2. Because consecutive presentations of female-like tones (460 Hz) do not cause a reduction in the probability of RFM occurring [9], we assumed the independence of the males' RFM response to a given tone presentation relative to their response to previous tone

presentations. Although males were tested in darkness and their auditory system and auditory behaviour indicate they are insensitive to tones in the range of the male's WBF [9-14], there may exist potential effects of group size on RFM expression during tone stimulation. To test this, G-tests of independence were carried for each probe/masking combination. William's correction for independence tests (which could be applied to tests with more than one degree of freedom) was used to avoid overestimation of significance when response counts are low [35]. Overall, no significant group size effect was observed in any of the experiments (Supplemental Tables 3 and 4). Therefore, the data from each group size was pooled within each probe/mask combination. The proportion of RFM responses to the probe tone alone provided the intrinsic null hypothesis used in G-tests for goodness-of-fit to test the effect of the experimental probe/masking tone pairs on the proportion of response [28]. G-tests for independence were used to compare different probe tones, while comparisons within the same probe tone were performed using G-tests for goodness-of-fit.

Electrophysiology

Mosquitoes were immobilized by cold narcosis and fixed with beeswax to a small brass block. The pedicel, head and legs were fixed using superglue. Female- and male-like pure tones were delivered through separate speakers to the preparation from a pair of modified Beyerdynamics® DT48 speakers, each coupled to a 7 mm plastic tube. The tip of each tube was positioned 1 cm from the mosquito on opposite sides of the head. Phasic, compound receptor potentials were measured from the JO with tungsten electrodes (5-7 MΩ, 1 μm tip, Microprobes.com. USA, part # WE30032.OH3) that were advanced with a Märzhäuser® PM10 manipulator so that the tip of the electrode just penetrated the wall of the pedicel. In this location, voltage responses from the JO are dominated by phasic compound receptor potentials from the local population of sensory cells [12], but still demonstrate a response twice the frequency of the acoustic

stimulus [17]. Signals from the electrodes were amplified (x10000) and low pass filtered (5 kHz) using custom built differential pre-amplifier. Probe tones of 82 ms duration with 8 ms rise/fall time were delivered via a 5 kHz low pass filter and calibrated against a known 94 dB SPL microphone (Bruel & Kjaer® 4230) [15, 16]. Voltage control signals for the sound system were generated and voltage signals from the electrodes were digitized at 250 kHz via a Data translation 3010 D/A A/D card using programs written in Matlab. The magnitude and phase of the phasic voltage signals were stored for further analysis. All measurements were made on a Newport® isolation table inside an IAC sound attenuated booth. Temperature control was provided by placing the preparation in a chamber machined in a Peltier controlled heat-sink [9, 29]. All recordings were made within 30 min of preparation.

Pairs of pure tones simulating the approximate fundamental flight-tones and particle velocities of male (f_1 = 700 Hz, $4x10^{-4}$ ms^{-1}) and female (f_2 = 400 Hz, $1x10^{-5}$ ms^{-1}) mosquitoes were delivered to the antennae of 4 sensitive males mounted in the recording setup. These parameters were chosen to simulate the likely stimuli received by the antennae of a free-flying male mosquito in the close presence of a female [9, 11]. The electrical responses of the JO were recorded from just below the cuticle of the pedicel, adjacent to the scolopidia, which generate graded, non-spiking potentials [12]. Pair tone acoustic stimulation resulted in electrical responses of the JO at the tone frequencies and at the resulting DP (300 Hz). Masking pure tones were generated by a Philips® PM5193 function generator and delivered simultaneously with f_1 and f_2 tones through a Beyerdynamics® DT 770 speaker positioned 7.5 cm in front of the preparation. Masking tones ranging between 125-450 Hz were delivered successively at increasingly particle velocity levels and their 10dB and 15dB suppression effects on the DP compound electrical potential generated in response the f1 and f2 tones were recorded.

Results

Acoustic masking of male mosquito RFM

In Experiment 1 we tested the effect of masking tones on the proportion of RFM responses that were directed towards the probe speaker by free-flying male mosquitoes. Probe-only tones elicited an RFM response towards the probe speaker in > 80% of the presentations (Figure 1A-C, dashed horizontal lines; probe 340 Hz: 81%; 400 Hz: 85%; 450 Hz: 88%). The proportion of mosquitoes that gave an RFM response was similar for all three probe tones (G-test of independence: G=0.596; d.f.=2; P=0.742).

Pure tone acoustic masking, regardless of the probe frequency, caused significant suppression of the RFM response (when compared to probe-only presentations) for masking frequencies between 300-550 Hz (G-test goodness-of-fit; probe: 340 Hz, $G \geq 5.16$, $p \leq 0.023$; probe: 400 Hz, $G \geq 3.87$, $p \leq 0.049$; probe: 450Hz, $G \geq 4.60$, $p \leq 0.032$) (Figure 1A-C, closed circles). Outside this range, the response proportion was similar to the probe-only stimulation (Figure 1A-C, open circles). The masking tones that caused maximum suppression of the RFM response fell within the same narrow frequency range (390-420 Hz), independently of the probe tone frequency (Figure 1, Supplemental Table 1).

Results shown in Figure 1 reveal that the proportion of RFM response in male mosquitoes can be reduced significantly or totally suppressed when a second pure tone is delivered simultaneously with the initial probe tone. Two possible processes can be considered for the observed behavioural masking: *i*) interference, in which the presence of a masking tone impairs the mosquito's ability to detect, locate and/or express RFM response to the probe tone; or *ii*) competition, in which the frequency of the masking tone is more attractive to the male than the frequency of the probe tone, resulting in an increased probability of RFM being expressed towards the masking speaker.

To address these possibilities, Experiment 2 was conducted with a second particle velocity microphone placed close to the masking speaker, in addition to the one located near the probe speaker. This arrangement enabled us to identify to which of the two speakers males directed their RFM responses (Figure S1). The same probe frequencies were used as in Experiment 1 and the masking frequencies ranged between 200-550 Hz. The masking tone frequency limits were based on the results from Experiment 1 (Supplemental Table 2).

The effect of simultaneous acoustic masking on the proportion of RFM response to each sound source is shown in Figure 2. Probe-only presentations elicited a high proportion of RFM responses towards the probe speaker (Figure 2A-C; probe 340 Hz: 75%; 400 Hz: 81%; 450 Hz: 84%), in agreement with the results from Experiment 1. Similarly, the presentation of probe/masking tone pairs caused significant suppression of the RFM response towards the probe speaker (Figure 2, range of blue bars) when compared to probe-only presentations (Figure 2A-C, probe 340 Hz: blue bar = 300-500 Hz, $G \geq 5.31$, $p \leq 0.021$; probe 400 Hz: blue bar = 320-550 Hz, $G \geq 4.37$, $p \leq 0.037$; probe 450 Hz: blue bar = 250-500 Hz, $G \geq 9.01$, $p \leq 0.003$).

Instead of being attracted towards the probe speaker, as indicated by exhibiting RFM behaviour, male mosquitoes can instead direct their response towards the masking speaker or they can display no conspicuous response, flying without frequency modulation (Figure S1). Suppression of attraction towards the probe appears to be dominated by competition from tones emitted by the masking speaker; indeed, attraction (i.e. the RFM response) towards the masking speaker occurred significantly more often than towards the probe speaker (Figure 2, red shading) for masking frequencies between 360 Hz and 470 Hz (Figure 2A-C, probe 340 Hz: red shading = 360-450 Hz, $G \geq 4.98$, $p \leq 0.026$; probe 400 Hz: red shading = 390-470 Hz, $G \geq 18.22$, $p \leq 0.001$; probe 450 Hz: red shading = 400-470 Hz, $G \geq 5.15$, $p \leq 0.023$).

However, the competition effect, i.e. the attractiveness of the masking frequency relative to the probe frequency, does not account for all the observed behavioural masking because masking tones caused significant RFM suppression to *either* speaker (Figure 2, grey shading). This interference effect by the masking tones on the overall RFM response was observed for all probe frequencies (Figure 2A-C, probe 340 Hz: grey shading = 320-400 Hz, $G \geq 11.53$, $p \leq 0.001$; probe 400 Hz: grey shading = 320-470 Hz, $G \geq 6.14$, $p \leq 0.013$; probe 450 Hz: grey shading = 280-470 Hz, $G \geq 4.85$, $p \leq 0028$).

Acoustic masking relative to JO tuning

Maximum masking of the behavioural responses to the probe tones (Figure 3A) coincides with the frequency range of the flight-tones of female *C. quinquefasciatus* mosquitoes but outside the 10dB bandwidth of the JO (244-364 Hz) [9]. A possible hypothesis for this mismatch is that male mosquitoes do not detect probe tones *per se* but detect their difference in frequency with respect to their own WBF. To test this, the difference between the WBF of the responding males, measured just prior to the onset of an RFM, and the masking tone frequency was calculated for each response. For non-responding males, the WBF was measured ~1 s after the start of stimulation. The calculated differences were binned in 25 Hz intervals (50 Hz intervals in the extreme differences) and the proportion of RFM response re-plotted for these groups (Figure 3B).

When the suppression of the RFM response is plotted as a function of the difference between WBF and the masking tone, maximum masking is within the 10dB bandwidth of the JO, regardless of the probe tone frequency (Figure 3B). Masking tone frequencies that cause maximum attraction towards the masking speaker also fall within the 10dB bandwidth of the JO, when plotted as the difference between WBF and masking tone. The maximum is centred on the JO 10dB bandwidth when using the 450 Hz probe tone, but moves to the low-frequency

boundary that bandwidth for the 350 and 400 Hz probe tones (Figure 3B). These relations indicate that the masking tones suppress the formation of DPs in the vibrations of the antenna [11] or the detection of DPs by the JO.

These results imply that RFM behaviour (and its suppression) in male mosquitoes may be dependent on adjustment of their WBF in relation to the frequencies of the stimulus tones. Analysis of variance (Supplemental Table 5) indicates that the WBF during simultaneous probe/masking tone stimulation differed significantly between probe tones, but not between masking tones. Crucially, the WBF of males when stimulated with a probe tone of 340 Hz was 722 ±1.7 Hz (average ± S.E.M.), which is a significantly lower WBF than those observed for 400 Hz (732±2.0 Hz) and 450 Hz (735±2.0 Hz) probe tone stimulation. Overall, these results suggest that male mosquitoes may adjust their WBF with respect to the stimulus tones to maintain the difference tone DP within the most sensitive bandwidth of the JO.

Attenuation of DPs generated by difference tone in the compound electrical responses of the JO

The particle velocity level required to suppress the magnitude of DP electric responses by 10 dB and 15 dB as a function of the masking tone frequency are shown in Figure 4. Analysis of variance revealed a significant effect of the masking tone frequency on the suppression of the DP electrical response (ANOVA; 10 dB: $F=7.34$, d.f=13, $p<0.001$; 15 dB; $F=2.77$, d.f.=10, $p=0.031$). Both suppression tuning curves have their minima outside the range female WBFs, but centred within the 10dB bandwidth of the JO threshold tuning curve (Figure 4). This finding supports the hypothesis from the behavioural experiments that acoustic masking of the RFM behaviour is due to the suppression of the DPs generated at frequencies in the most sensitive frequency range of the JO.

Discussion

We report here that the RFM behaviour of free-flying *C. quinquefasciatus* male mosquitoes can be significantly suppressed by simultaneous pure tone acoustic masking. Although background noise masking has been reported in *Drosophila* [30], from our knowledge this is the first study describing pure tone-on-tone acoustic masking in insects with antennal hearing. RFM behaviour represents an acoustic measure of a mating attempt by a male, which in mosquitoes is a function mediated essentially by sound [9-13, 22-25, 31, 32]. The most effective masking frequency range encompasses the fundamental frequency range of female flight-tones (430-527 Hz), which are similar to the most sensitive frequencies of the male behavioural audiograms (340-560 Hz) [9]. Acoustic masking is mediated by both competition and interference processes; a masking pure tone can significantly suppress the RFM response by being more attractive than a female-like probe tone and/or by interfering with the ability of the males to detect or locate the probe tone.

Significantly, suppression by acoustic masking of RFM behaviour towards pure tone sources provides direct evidence that male mosquitoes hear females through detection of difference tone DPs [9, 11-14]. Maximum RFM suppression occurred at similar masking frequencies for the three probe tones and within the range of the most sensitive frequencies of male behavioural audiogram [9]. Had the male mosquitoes been listening to the probe tones *per se* then the acoustic responses towards the probe tones would have been expected to be suppressed maximally at masking frequencies centred on the probe tones [1, 2]. This is because in non-linear systems, such as the electrical responses of hair cells in the mammalian cochlea, probe and masking tones suppress themselves mutually when these tones both fall within the sensitive bandwidth of the receptor [33, 34]. In the case of male mosquitoes, this should occur for tone frequencies falling within the sensitive 10 dB bandwidth of the JO (244-364 Hz) [9].

However, RFM responses to the 340 Hz probe tone were completely suppressed by masking tones between 400 and 450 Hz. Crucially, behavioural suppression (for all probe tones) and the 10dB bandwidth of the JO coincide only if it is expressed as a function of the frequency difference between the male fundamental flight-tone and the masking stimulus.

This behavioural finding was supported by the electrophysiology; DPs in the electrical responses of the JO generated by two tones, simulating the fundamental frequencies of the male and female flight-tones, were maximally suppressed by masking tones with frequencies within the 10dB bandwidth of the JO. Auditory masking is likely to occur at the level of the antennae where the male and female flight-tones interact non-linearly to generate difference tones in the antennal vibrations [11]. Given that maximum suppression by pure tone acoustic masking is centred on the most sensitive frequency of the auditory receptor [1, 2], masking of acoustic behaviour in male mosquitoes, as confirmed by the electrophysiology, is due to suppression of the DPs that are generated at frequencies in the most sensitive frequency range of the JO.

The WBF of males differed significantly between probe tones; their WBF, measured during the final approach phase just before RFM, is lowest for the 340 Hz tone and highest for the higher probe tones. This result, in conjunction with the finding that maximum RFM suppression occurred at similar masking frequencies indicates that male mosquitoes may adjust their WBF with respect to the stimulus tones to maintain the difference tone DP within the most sensitive bandwidth of the JO. In this context, it is also possible that harmonic frequency matching [11, 13, 32] could be a consequence of the attempts by the male (and perhaps female) mosquito to maintain the difference tones DP within the "sweet spot" of the JO.

Acoustic masking in free-flying male mosquitoes is due not only to suppression of the RFM behaviour but is also due to attraction to masking tone. We have separated these two processes to understand the basis for the suppression. Attraction of male mosquitoes to the masking sound source, i.e. competition, is likely to be related to the free-flight paradigm; probe

and masking sound sources are spatially separated, so if both tone frequencies are attractive, males can respond towards whichever tone appears loudest. Evidently, the perceived sound level will be dependent on the spatial location of the mosquito relative to the sound sources when stimulation occurs. It may also depend on the WBF of the male; slight changes in WBF will alter the frequency of the difference tone DP and could alter the apparent loudness of one tone relative to the other. It is also possible that a mechanism like the one found in the *Ormia ochracea* flies is present [8]; in these parasitoid flies the localization of two conflicting, spatially separated, sound sources is solved by a precedent effect, whereby the detection of small time differences (~10 ms) in sound reception are used to determine location of the first source detected.

Under natural conditions, *C. quinquefasciatus* males form relatively dense swarms while waiting for sexually receptive females [26, 27]. Given that masking frequencies above 600 Hz did not suppress RFM behaviour, male-male acoustic interactions within the swarm should not impair the ability of an individual male mosquito to detect and locate potential mates and perhaps other males [11, 12, 31]. In other words, from a male mosquito's perspective, swarms appear not to be a source of acoustic noise, although one-to-one encounters between pairs of males may cause them to shift apart their WBFs [11, 31].

Acoustic masking of RFM behaviour is most effective for masking frequencies similar to those of the female flight-tones. In this way, the extreme sensitivity of male mosquitoes to these frequencies brings with it the potential cost of high susceptibility to signal distortion and attenuation if two similar, female-like, tones were to be detected simultaneously. Under natural conditions, this would occur only if a male within a swarm was to detect the flight-tones of two nearby females at the same time and for a sustained period. This situation, however, would occur only with unrealistically high densities of unmated females nearby or within the swarm. Wishart and Riordan [23] studied the attractiveness to various sounds in *Aedes aegypti* males

and found the most attractive frequencies were, as in *C. quinquefasciatus* [9] and *A. gambiae* species [10], centred on the female fundamental frequency and ranged optimally between 400-600 Hz. Crucially, their work showed that two or more pure tones, which are each attractive on their own, are not attractive when presented together in the same speaker; in some frequency pairs (450Hz/500Hz and 500Hz/550Hz), this resulted in a > 95% reduction in the number of males trapped by their sound-lure vacuum trap. The cause for this marked decrease was not determined, but it appears that, as presented here, acoustic masking could be the underlying process.

The findings reported here support the hypothesis that mosquitoes must fly to hear and that hearing in male mosquitoes is an active process mediated by the detection of intermodulation distortion products. Nonetheless, a more complete model of acoustic masking in male mosquitoes could lead to the development of new strategies to control mosquitoes based on acoustic tools capable of disrupting swarming and mating in nature.

Author contributions

Experiments were conceived and designed by P.M.V.S., G.G. and I.J.R. Experiments were conducted and data was analysed by P.M.V.S. and R.I. Manuscript was written by P M.V.S. and I.J.R. with contributions from G.G. and R.I.

Ethics

No vertebrate animals were used in this research. Mosquitoes were cold anaesthetized prior to preparation for electrophysiological recordings. All applicable international, national, and institutional guidelines for the care and use of animals were followed.

Data accessibility

Data supporting this article can be accessed at Dryad Digital Repository: http://dx.doi.org/XXXXXX/dryad.XXXX (to be added after submission) and have also been uploaded as electronic supplementary material.

Competing interests

We have no competing interests.

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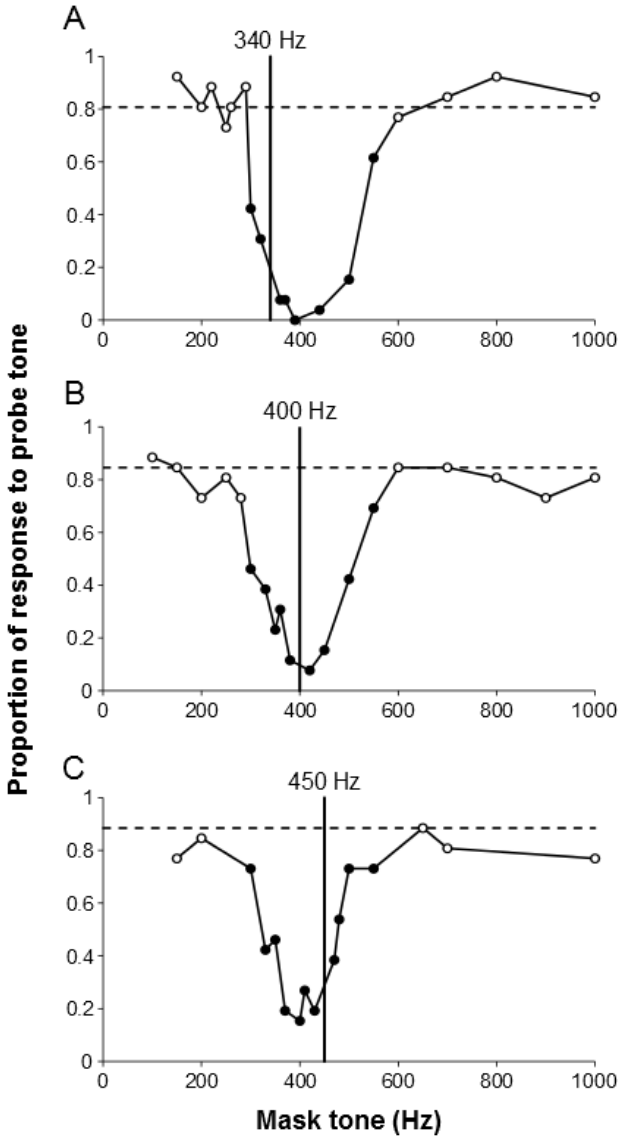

Figure 1. Acoustic masking of RFM behaviour of free-flying male mosquitoes to a speaker emitting a probe tone. The proportion of mosquitoes initiating an RFM response towards the probe speaker is plotted as a function of the masking frequency (n=26 for each data point). Probe tone: A) 340 Hz; B) 400 Hz; C) 450 Hz. Horizontal dashed line: proportion of male mosquitoes expressing the RFM response to the probe-only tone. Closed symbols: Proportion of responses significantly lower ($p<0.05$) than responses to probe-only tone. Open symbols: Proportions of responses not significantly different from responses to probe-only tone.

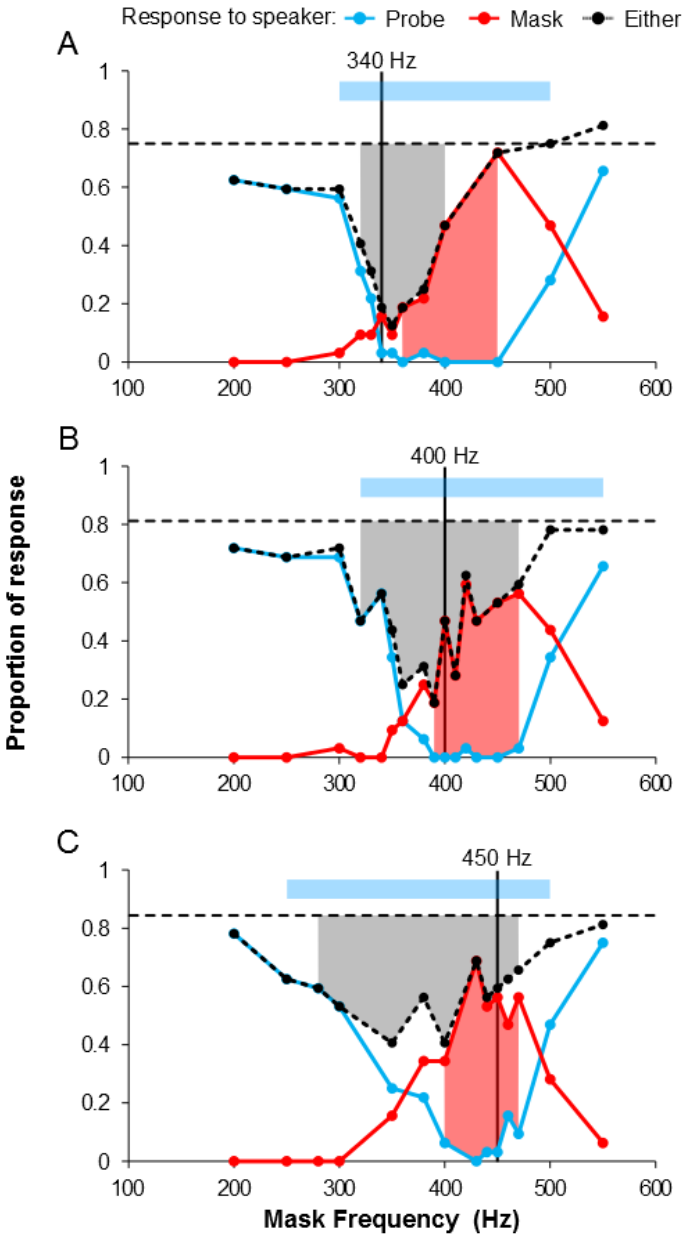

Figure 2. Interference and competition of masking tone. Probe tone: A) 340 Hz; B) 400 Hz; C) 450 Hz. The proportion of male mosquitoes exhibiting RFM behaviour towards the probe speaker (blue line) or masking speaker (red line) plotted as function of the masking frequency (n=32 for each data point). The black dashed line between data points represents the proportion of response to either speaker. Horizontal dashed line: proportion of response to the probe-only tone. Blue shading bar: masking frequencies causing significant ($p<0.05$) acoustic masking towards the probe speaker. Red shading: masking frequencies causing a significantly higher proportion of RFM response towards the masking speaker than to the probe speaker. Grey shading: masking frequencies causing a significantly lower proportion of RFM response to either speaker relatively to the probe-only presentations.

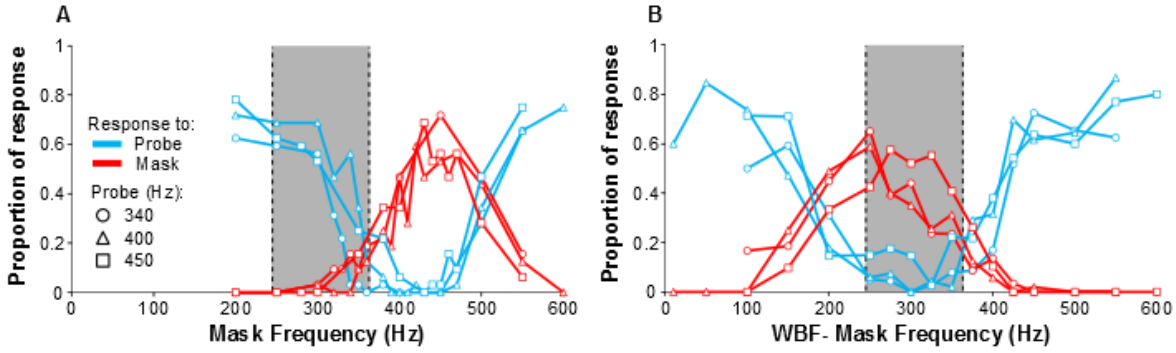

Figure 3. Acoustic masking of an RFM response correlates with JO tuning when the proportion of response is calculated as function of frequency difference between the male's WBF and the masking frequency. A) Proportion of RFM response to the probe tone (blue curve) as a function of the masking tone frequency (as in Figure2), is strongly reduced in the presence of the masking tone, especially for masking frequencies between 350-450 Hz, regardless of probe tone frequency. The proportion of RFM response to the masking tone speaker is greatly increased (red curve) during maximum masking. B) Proportion of RFM response calculated as a function of the frequency difference between the male's WBF measured immediately prior the RFM response and the masking frequency. Grey range: 10 dB bandwidth of the JO frequency threshold tuning curve (244-364 Hz) [9].

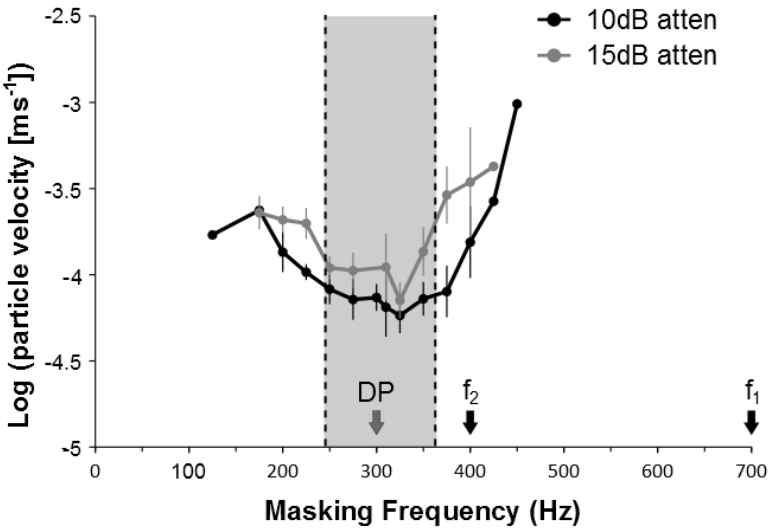

Figure 4. Attenuation of difference tone distortion products generated in the compound electrical responses of the JO. Difference tones (DP= f_1- f_2 = 300 Hz) were generated by the simultaneous presentation of two tones simulating male (f_1 = 700 Hz, $4x10^{-4}$ ms^{-1}) and female (f_2 = 400 Hz, $1x10^{-5}$ ms^{-1}) flight-tones. Curves represent the masking tone levels (ms^{-1}) required to suppress the magnitude of the DP response by 10dB and 15dB. Each point is mean ± SD from 4 preparations. Grey range: 10 dB bandwidth of the JO frequency threshold tuning curve (244-364 Hz) [9].

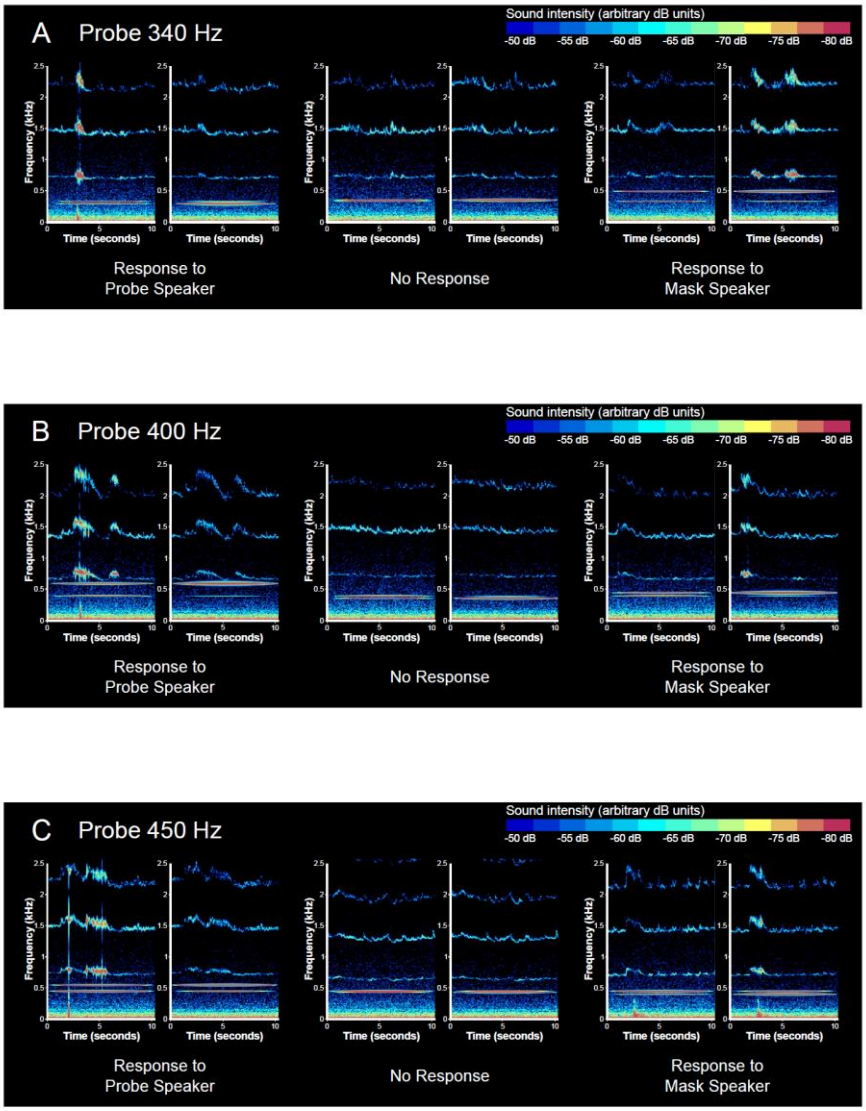

Figure S1. Spectrograms of the WBFs of free-flying male mosquitoes when stimulated simultaneously with a probe tone and a masking tone. Probe tone: A) 340 Hz; B) 400 Hz; C) 450 Hz. In response to pair tone stimulation, males showed one of three responses: directed RFM behaviour towards probe speaker, no measurable response, or directed RFM behaviour towards mask speaker (left, centre, and right columns, respectively). For each sequence shown here, the left spectrogram displays the sound recorded by the microphone near the probe speaker while the right spectrogram displays the sound recorded by the microphone near the mask speaker. The speaker towards which a male directed RFM was identified by observing the WBF sound level between the 2 sound channels: the sound level of an RFM near a microphone registered responses 20-30 dB higher than the furthest microphone. Occasionally (<5% of the records), a single probe/masking presentation would elicit an RFM response to both speakers; in this case, we registered the response towards the first speaker the male directed his response.

SUPPLEMENTAL TABLE 1

Behavioural Masking - Experiment 1 (26 observations per probe/mask tone pair)

Probe: 340 Hz

Mask tone	# RFM	Prop RFM
0	21	0.81
150	24	0.92
200	21	0.81
220	23	0.88
250	19	0.73
260	21	0.81
290	23	0.88
300	11	0.42
320	8	0.31
360	2	0.08
370	2	0.08
390	0	0
440	1	0.04
500	4	0.15
550	16	0.62
600	20	0.77
700	22	0.85
800	24	0.92
1000	22	0.85

Probe: 400 Hz

Mask tone	# RFM	Prop RFM
0	22	0.85
100	23	0.88
150	22	0.85
200	19	0.73
250	21	0.81
280	19	0.73
300	12	0.46
330	10	0.38
350	6	0.23
360	8	0.31
380	3	0.12
420	2	0.08
450	4	0.15
500	11	0.42
550	18	0.69
600	22	0.85
700	22	0.85
800	21	0.81
900	19	0.73
1000	21	0.81

Probe: 450 Hz

Mask tone	# RFM	Prop RFM
0	23	0.88
150	20	0.77
200	22	0.85
300	19	0.73
330	11	0.42
350	12	0.46
370	5	0.19
400	4	0.15
410	7	0.27
430	5	0.19
470	10	0.38
480	14	0.54
500	19	0.73
550	19	0.73
650	23	0.88
700	21	0.81
1000	20	0.77

SUPPLEMENTAL TABLE 2

Behavioural Masking - Experiment 2 (32 observations per probe/mask tone pair)

Probe:340 Hz

Mask tone	RFM (Probe)	RFM (Mask)	No RFM	Prop(Probe)	Prop(Mask)	Prop(NoRFM)
0	24	0	8	0.75	0	0.25
200	20	0	12	0.62	0	0.38
250	19	0	13	0.59	0	0.41
300	18	1	13	0.56	0.03	0.41
320	10	3	19	0.31	0.09	0.59
330	7	3	22	0.22	0.09	0.69
340	1	5	26	0.03	0.16	0.81
350	1	3	28	0.03	0.09	0.88
360	0	6	26	0	0.19	0.81
380	1	7	24	0.03	0.22	0.75
400	0	15	17	0	0.47	0.53
450	0	23	9	0	0.72	0.28
500	9	15	8	0.28	0.47	0.25
550	21	5	6	0.66	0.16	0.19

Probe:400 Hz

Mask tone	RFM (Probe)	RFM (Mask)	No RFM	Prop(Probe)	Prop(Mask)	Prop(NoRFM)
0	26	0	6	0.81	0	0.19
200	23	0	9	0.72	0	0.28
250	22	0	10	0.69	0	0.31
300	22	1	9	0.69	0.03	0.28
320	15	0	17	0.47	0.0	0.53
340	18	0	14	0.56	0.0	0.44
350	11	3	18	0.34	0.09	0.56
360	4	4	24	0.13	0.13	0.75
380	2	8	22	0.06	0.25	0.69
390	0	6	26	0	0.19	0.81
400	0	15	17	0	0.47	0.53
410	0	9	23	0	0.28	0.72
420	1	19	12	0.03	0.59	0.38
430	0	15	17	0	0.47	0.53
450	0	17	15	0	0.53	0.47
470	1	18	13	0.03	0.56	0.41
500	11	14	7	0.34	0.44	0.22
550	21	4	7	0.66	0.13	0.22

Probe:450 Hz

Mask tone	RFM (Probe)	RFM (Mask)	No RFM	Prop(Probe)	Prop(Mask)	Prop(NoRFM)
0	27	0	5	0.84	0	0.16
200	25	0	7	0.78	0	0.22
250	20	0	12	0.63	0	0.38
280	19	0	13	0.59	0	0.41
300	17	0	15	0.53	0	0.47
350	8	5	19	0.25	0.16	0.59
380	7	11	14	0.22	0.34	0.44
400	2	11	19	0.06	0.34	0.59
430	0	22	10	0	0.69	0.31
440	1	17	14	0.03	0.53	0.44
450	1	18	13	0.03	0.56	0.41
460	5	15	12	0.16	0.47	0.38
470	3	18	11	0.09	0.56	0.34
500	15	9	8	0.47	0.28	0.25
550	24	2	6	0.75	0.06	0.19

SUPPLEMENTAL TABLE 3: Behavioural Experiment 1 – Group Size

Probe: 340 Hz

Mask (Hz)	Group size	RFM		G-test (Williams correction)		
		no	yes	G value	d.f.	p
0	**1**	1	3	0.197	3	0.978
	2	2	10			
	3	1	5			
	4	1	3			
150	**1**	0	2	0.713	3	0.870
	2	1	7			
	3	1	11			
	4	0	4			
200	**1**	0	1	1.354	3	0.716
	2	0	2			
	3	4	11			
	4	1	7			
220	**1**	0	2	2.266	3	0.519
	2	0	8			
	3	2	10			
	4	1	3			
250	**1**	0	2	3.707	3	0.295
	2	2	2			
	3	4	8			
	4	1	7			
260	**1**	0	1	0.798	3	0.850
	2	2	12			
	3	1	2			
	4	2	6			
290	**2**	1	11	2.745	2	0.254
	3	0	6			
	4	2	6			
300	**1**	0	1	5.443	3	0.142
	2	4	0			
	3	4	5			
	4	7	5			
320	**1**	0	1	4.922	3	0.178
	2	3	3			
	3	11	4			
	4	4	0			
360	**1**	2	0	0.713	3	0.870
	2	4	0			
	3	11	1			
	4	7	1			

Probe: 340 Hz

Mask (Hz)	Group size	RFM		G-test (Williams correction)		
		no	yes	G value	d.f.	p
370	**1**	3	0	1.603	3	0.659
	2	5	1			
	3	8	1			
	4	8	0			
390	**1**	2	0	-	3	1.000
	2	4	0			
	3	12	0			
	4	8	0			
440	**1**	2	0	1.051	3	0.789
	2	7	1			
	3	12	0			
	4	4	0			
500	**1**	2	0	0.734	3	0.865
	2	3	1			
	3	10	2			
	4	7	1			
550	**1**	1	0	2.598	2	0.273
	2	5	5			
	3	4	11			
600	**1**	0	2	1.118	2	0.572
	2	1	5			
	3	5	13			
700	**1**	0	2	1.450	3	0.694
	2	1	3			
	3	1	11			
	4	2	6			
800	**1**	0	1	0.284	2	0.868
	2	2	20			
	3	0	3			
1000	**1**	0	1	0.684	3	0.877
	2	2	6			
	3	1	8			
	4	1	7			

Probe: 400 Hz

Mask (Hz)	Group size	RFM		G-test (Williams correction)		
		no	yes	G value	d.f.	p
0	**1**	0	3	2.172	3	0.537
	2	2	8			
	3	2	7			
	4	0	4			
100	**1**	0	1	1.358	3	0.715
	2	0	6			
	3	2	13			
	4	1	3			
150	**2**	1	5	0.873	2	0.646
	3	1	11			
	4	2	6			
200	**1**	1	4	1.997	2	0.368
	2	6	12			
	3	0	3			
250	**1**	0	3	1.129	2	0.569
	2	3	11			
	3	2	7			
280	**1**	1	4	0.290	3	0.962
	2	2	6			
	3	3	6			
	4	1	3			
300	**1**	2	1	0.449	2	0.799
	2	10	10			
	3	2	1			
330	**1**	1	1	1.491	2	0.475
	2	6	6			
	3	9	3			
350	**1**	1	1	2.541	2	0.281
	2	11	1			
	3	8	4			
360	**2**	5	1	0.751	2	0.687
	3	8	4			
	4	5	3			
380	**1**	3	0	3.127	3	0.373
	2	5	1			
	3	9	0			
	4	6	2			

Probe: 400 Hz

Mask (Hz)	Group size	RFM		G-test (Williams correction)		
		no	yes	G value	d.f.	p
420	**1**	2	0	0.804	3	0.849
	2	2	0			
	3	16	2			
	4	4	0			
450	**1**	2	0	2.883	3	0.410
	2	7	1			
	3	11	1			
	4	2	2			
500	**1**	3	3	3.159	3	0.368
	2	5	1			
	3	4	2			
	4	3	5			
550	**2**	8	14	2.811	1	0.094
	4	0	4			
600	**1**	1	6	0.323	3	0.956
	2	1	5			
	3	1	8			
	4	1	3			
700	**1**	0	3	2.270	3	0.518
	2	0	4			
	3	1	2			
	4	3	13			
800	**1**	2	3	4.024	3	0.259
	2	2	8			
	3	1	2			
	4	0	8			
900	**1**	1	0	2.123	3	0.547
	2	2	6			
	3	2	7			
	4	2	6			
1000	**1**	2	2	2.202	2	0.332
	2	1	9			
	3	2	10			

SUPPLEMENTAL TABLE 3 (Continuation)

Probe: 450 Hz

Mask (Hz)	Group size	RFM		G-test (Williams correction)		
		no	yes	G value	d.f.	p
0	**1**	0	5	1.693	2	0.429
	2	3	15			
	3	0	3			
150	**1**	2	4	0.437	2	0.804
	2	3	13			
	4	1	3			
200	**1**	3	10	1.408	2	0.495
	2	1	9			
	3	0	3			
300	**1**	4	5	2.904	2	0.234
	2	3	11			
	3	0	3			
330	**1**	7	4	0.487	3	0.922
	2	4	4			
	3	2	1			
	4	2	2			
350	**1**	1	3	1.912	2	0.384
	2	10	8			
	4	3	1			
370	**1**	4	0	2.991	2	0.224
	2	15	3			
	4	2	2			
400	**1**	4	0	1.185	2	0.553
	2	13	3			
	3	5	1			
410	**1**	6	1	3.720	3	0.293
	2	10	2			
	3	1	2			
	4	2	2			

Probe: 450 Hz

Mask (Hz)	Group size	RFM		G-test (Williams correction)		
		no	yes	G value	d.f.	p
430	**1**	9	2	1.261	2	0.532
	2	9	3			
	3	3	0			
470	**1**	3	2	4.509	3	0.211
	2	9	5			
	3	3	0			
	4	1	3			
480	**1**	2	4	1.366	2	0.505
	2	8	6			
	3	2	4			
500	**1**	2	1	3.838	3	0.280
	2	4	12			
	3	1	2			
	4	0	4			
550	**1**	3	5	0.719	2	0.698
	2	3	9			
	3	1	5			
650	**1**	1	5	0.456	2	0.796
	2	1	13			
	3	1	5			
700	**1**	1	8	2.557	3	0.456
	2	3	7			
	3	1	2			
	4	0	4			
1000	**1**	1	2	1.979	3	0.577
	2	4	12			
	3	1	2			
	4	0	4			

SUPPLEMENTAL TABLE 4: Behavioural Experiment 2 – Group Size

Probe: 340 Hz

Mask (Hz)	Group size	RFM no	RFM yes	G-test (Williams correction) G value	d.f.	p
0	**1**	4	6	1.540	1	0.215
	2	4	18			
200	**1**	4	8	0.136	1	0.713
	2	8	12			
250	**1**	6	8	0.049	1	0.825
	2	7	11			
300	**1**	8	8	1.119	1	0.290
	2	5	11			
320	**1**	7	7	0.864	1	0.353
	2	12	6			
330	**1**	12	4	0.553	1	0.457
	2	10	6			
340	**1**	9	1	0.724	1	0.395
	2	17	5			
350	**1**	10	0	2.815	1	0.093
	2	18	4			
360	**1**	13	3	-	1	1.000
	2	13	3			
380	**1**	10	3	0.041	1	0.840
	2	14	5			
400	**1**	6	8	1.009	1	0.315
	2	11	7			
450	**1**	2	10	1.230	1	0.267
	2	7	13			
500	**1**	3	10	0.041	1	0.840
	2	5	14			
550	**1**	5	13	2.213	1	0.137
	2	1	13			

Probe: 400 Hz

Mask (Hz)	Group size	RFM no	RFM yes	G-test (Williams correction) G value	d.f.	P
0	**1**	2	6	0.232	1	0.630
	2	4	20			
200	**1**	2	6	0.048	1	0.826
	2	7	17			
250	**1**	4	3	2.412	1	0.120
	2	6	19			
300	**1**	1	7	1.325	1	0.250
	2	8	16			
320	**1**	2	2	0.016	1	0.900
	2	15	13			
340	**1**	4	4	0.158	1	0.691
	2	10	14			
350	**1**	6	3	0.528	1	0.467
	2	12	11			
360	**1**	7	1	0.899	1	0.343
	2	17	7			
380	**1**	4	0	2.815	1	0.093
	2	18	10			
390	**1**	5	1	0.019	1	0.892
	2	21	5			
400	**1**	3	1	0.817	1	0.366
	2	14	14			
410	**1**	5	5	3.069	1	0.080
	2	18	4			
420	**1**	1	5	1.377	1	0.241
	2	11	15			
430	**1**	3	1	0.817	1	0.366
	2	14	14			
450	**1**	2	3	0.103	1	0.749
	2	13	14			
470	**1**	3	2	0.817	1	0.366
	2	10	17			
500	**1**	0	2	0.728	1	0.393
	2	7	23			
550	**1**	1	1	0.586	1	0.444
	2	6	24			

Probe: 450 Hz

Mask (Hz)	Group size	RFM no	RFM yes	G-test (Williams correction) G value	d.f.	p
0	**1**	3	5	2.946	1	0.086
	2	2	22			
200	**1**	2	8	0.028	1	0.868
	2	5	17			
250	**1**	4	4	0.649	1	0.420
	2	8	16			
280	**1**	5	6	0.153	1	0.696
	2	8	13			
300	**1**	4	4	0.039	1	0.843
	2	11	13			
350	**1**	3	1	0.431	1	0.512
	2	16	12			
380	**1**	3	4	0.003	1	0.959
	2	11	14			
400	**1**	10	3	2.755	1	0.097
	2	9	10			
430	**1**	4	6	0.474	1	0.491
	2	6	16			
440	**1**	5	5	0.217	1	0.641
	2	9	13			
450	**1**	6	4	2.121	1	0.145
	2	7	15			
460	**1**	4	5	0.163	1	0.686
	2	8	14			
470	**1**	3	7	0.117	1	0.732
	2	8	14			
500	**1**	4	6	1.540	1	0.215
	2	4	18			
550	**1**	0	6	2.406	1	0.121
	2	6	20			

SUPPLEMENTAL TABLE 5. ANOVA results for the male Wing Beat Frequency measured during simultaneous acoustic stimulation with probe and masking tone.

Source	***d .f.***	***SS***	***F***	***P***
Probe tone	2	50895	14.26	<0.0001*
Mask tone	20	53946	1.51	0.07
Probe x Mask	21	59233	1.58	0.05
Error	1364	2433534		

Probe tone: 350 Hz, 400 Hz, 450 Hz; Mask tone: see Table 2. Asterisk denotes statistical significance.