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Courtship duet between the female and the male *Aedes aegypti queenslandensis* (Theobald) (Diptera: Culicidae) under laboratory conditions

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ABSTRACT

This study aimed to determine the wing beat frequencies and spherical spreading between the female and the male *Aedes aegypti queenslandensis* during courtship under laboratory conditions. The field-collected larvae and pupae were reared into adult mosquitoes and were morphologically identified. Male and female individuals were coupled. Second generation of live adult mosquitoes were tethered to a stainless wire in their upright position and flight tones produced by their wings were recorded using pressure-gradient microphones. Results showed that the fundamental wing beat frequencies of male mosquitoes (607-1,037Hz) were higher than those of the female (487-660Hz). The different distances between male and female mosquitoes did not influence their wing beat frequencies ($p>0.05$). Wing beat frequencies of male mosquitoes differed significantly when paired with the female, in all distances between them ($p<0.05$), whereas those of females, did not differ ($p>0.05$). Thus, the male *Ae. aegypti queenslandensis* adjusted and converged with the female's flight tone. Convergence was restricted to the fundamental frequency for all distances except at the 19-cm distance between them, where convergence happened in the harmonics. Analysis on the spherical spreading on their wing beat frequencies did not differ significantly ($p>0.05$) in six locations of the microphones relative to the mosquitoes, thus, mosquitoes' flight tone moved in a spherical manner and that courtship could happen in different directions. Results are relevant for mosquito control by developing an acoustical device to disrupt their courtship.

Keywords: *Aedes aegypti queenslandensis*, dengue, wing beat frequency, spherical spreading, phase difference

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Aedes aegypti (Linneaus) (Diptera: Culcidae) is the primary mosquito vector of dengue (DENV) and Zika (ZIKV) viruses. These flaviruses can be acquired from the blood of infected human and passed on to another human through an infected female *Ae. aegypti* (WHO 2015), although, ZIKV can also be transmitted sexually (WHO 2016). Among the ten Association of Southeast Asian Nations (ASEAN), Philippines ranked fourth in the number of dengue cases (Shepard et al 2013, WHO-WPRO 2013). Dengue fever and Zika infections are on the rise (Benelli & Mehlhorn 2016). Moreover, the recent tetravalent dengue vaccine (Dengvaxia) has an efficacy rate of only 56% for first time patient (Capeding et al 2014). Recently, the Philippine Department of Health (DOH 2017) received information from Sanofi Pasteur that Dengvaxia has shown consistent and sustained benefit for those who were previously infected with DENV. In the longer term, severe cases may occur following a subsequent dengue infection among those who were not previously infected.

Ae. aegypti queenslandensis (Theobald) is the very pale form and the most domestic of the three intraspecific forms of *Ae. aegypti*, breeds, and rests very close to humans, and co-occurs with the *type* form or *Ae. aegypti* sensu stricto (Mattingly 1957). Mattingly gave this form only a varietal rank (*Ae. aegypti* var. *queenslandensis*). Recently, Rasić et al (2016) reported that the *queenslandensis* and the *type* form are genomically indistinguishable implying that these forms freely interbreed at least in Australia and Singapore. Both sexes of *Ae. aegypti* in Cebu city, Philippines (Edillo et al 2015) and those of the pale and dark forms of *Ae. aegypti* in Bangkok, Thailand (Thungrungkiat et al 2012) showed natural vertical and transovarial transmissions of DENVs, respectively.

Female and male *Ae. aegypti* engage in acoustic manipulations as they encounter each other in mid-air that bring their flight tones (wing beat frequencies) in tune with each other (Cator et al 2009, Warren et al 2009). Their flight tone has their own unique type of vibration or standing wave pattern and are more than a mere byproduct of locomotion but are critical communication signals (Charlwood & Jones 1979, Gopfert et al 1999). Moreover, flight tones produce a continuous sinusoidal tone at the frequency of wing movement, although higher harmonics (ie, the pattern produced with precise vibration frequencies and any frequency other than it results to an uneven, non-repeating medium disruption) may dominate in some insects (Sotavalta 1947, Williams & Galambos 1950, Webb et al 1976). The relationship between two or more sound waves, when the mosquitoes meet in time and space, can be referred to as phase relationship. In-phase and outphase waves both have two waves with same frequency and are produced simultaneously and non-synonymously, respectively (Warren et al 2009, Wishart et al 1962, Tischner 1953, Tischner & Schieff 1855, Belton 1974).

Long-standing questions remain about the sensory modalities and behavioral mechanisms that mediate mate finding and recognition. Thus, examining further the two-way communication between the male and the female *Ae. aegypti queenslandensis* may lead to discovering novel ways to control them and diseases they cause, particularly that there has been no study yet about this mosquito variety in the country.

MATERIALS AND METHODS

1. Collection, rearing, and coupling of Ae. aegypti queenslandensis

Ae. aegypti larvae and pupae were collected in Nasipit, Talamban, Cebu City, which had dengue cases (Cebu CHO 2016) and accessible to the researchers. Basins filled with mineral water and lined with filter papers at the side, which served as egg-laying substrates, were used to collect the mosquito eggs. Rearing of *Ae. aegypti* subadults was modeled after Clemons et al (2010) and Edillo et al (2012), and was carried out inside the mosquito laboratory maintained at 23°C and 78 ± 7% relative humidity (RH). Flakes of fish food (Sakura, All Aquarium Co Ltd Thailand) were used to feed the larvae (Figure 1). Newly emerged *Ae. aegypti* adults were allowed to become sexually mature for 3-4 days (Clemons et al 2010) and were sorted out morphologically by sex. Both male and female *Ae. aegypti queenslandensis* (see identification below) were placed in another coupling jar (Figure 2). Female mosquitoes were starved for a day and were fed by sucking the warm chicken blood placed inside an inverted microcentrifuge tubes covered with parafilm and were put on top of the fine mesh cloth. Female mosquitoes need blood to have the protein needed to synthesize the yolk and develop eggs for survival and reproduction (Nikbakhtzadeh et al 2016, Leal 2014). Meanwhile, cotton balls soaked in 10% sucrose solution were placed on top of each coupling jar to feed the male mosquitoes. A filter paper was placed at the bottom of the coupling jar for the collection of mosquito eggs. The first generation (F1)of *Ae. aegypti queenslandensis* eggs were obtained from the filter paper and were flushed with distilled water (DW) for hatching in another jar covered with fine-mesh cloth. Continuous rearing of mosquito subadults was done to ensure the supply of adult mosquitoes for the measurement of their wing beat frequencies.

Figure 1. A glass jar with *Aedes* larvae being reared in the laboratory

Figure 2. Coupling jar with a male and a female mosquitoes, a cotton ball on top soaked with 10% sucrose solution, and a filter paper that served as the substrate at the bottom for collection of mosquito eggs

2. *Identification of Ae. aegypti queenslandensis*

Newly emerged adults of *Ae. aegypti* were exposed inside the coupling jar with a cotton ball soaked with ethylene acetate for 10-15 sec. While the mosquitoes were still unconscious, they were placed under a dissectoscope for morphological identification following Mattingly (1957) and Kalra et al (1985). Male mosquitoes have a pair of more plumose antennae than those of females (Arthur et al 2014). Females are larger than male mosquitoes (Nicbakhtzadeh et al 2016). Briefly, *Ae. aegypti queenslandensis* has bleached dark scales, ranging from mid-brown to various shades of buff to almost white, found on the mesonotum as a distinct factor. Intrusion of pale, basal bands were also found on the abdominal tergite and on the tips of the succeeding segments (Figure 3A). Species identification was based on Thungrungkiat et al (2010) (Figure 3B-C).

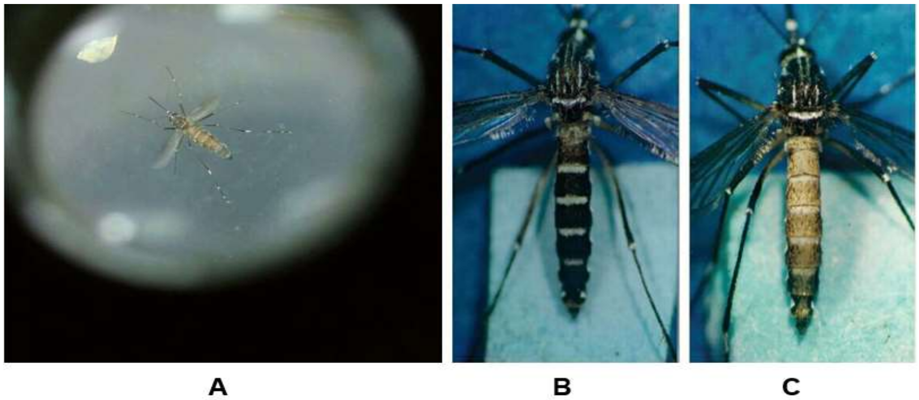

Figure 3A-C. *Ae. aegypti queenslandensis* in the current study (A); *Ae. aegypti formosus* (common form)(B) and *Ae. aegypti queenslandensis* (C) by Thungrungkiat et al (2010)

3. Measurement of the Wing Beat Frequencies

3.1. Preparation of the microphones

Measurement microphones of National Instruments Integrated Electronic Piezo Electric (NI-IEPE) (National Instruments Corporation, Austin, TX, USA) were used to capture the analog signal from the sound source. Calibration was performed in non-echoic laboratory conditions following Arthur et al (2010). *In situ* stimuli intensities were measured before recording sessions using NI-EPE measurement microphones. The microphone was positioned to where the mosquito was placed in parallel to the wave front coming from the distant speaker. To determine the sensitivity and dynamic range of the speaker, different tone frequencies and intensities were played, adjusted via a computer. Laboratory conditions were measured to check for changes in low-frequency sensitivity.

Figure 4. Tethered *Ae. aegypti queenslandensis* on its dorsal prothorax

3.2.Wing beat frequencies

Tethering mosquitoes in studying their flight kinematics is widespread. Consistent with Aldersley et al (2016), the simpler tethered experiments were performed because of the challenging nature of free flight experiments and at least some aspects of free flight behavior are preserved in tethered individuals. Each live mosquito (3- to 5-day old) was tethered on its dorsal prothorax with instant glue (Pioneer Mighty Bond, Philippines) to a stiff stainless wire (Figure 5); the latter was held for 5 sec with the mosquito's upright flight position. It was allowed to acclimate by resting in a non-echoic laboratory for ~5min. A pair of calibrated pressure-gradient microphones was positioned on opposite sides of individual male (n=10 per trial) and female mosquitoes (n=10 per trial), simultaneously measuring the sound radiating from their wings in three successive trials. For the control group (Figure 5A), the wing beat frequencies of solo male and female mosquitoes were recorded with a 1-cm distance from the microphone. For the duets (Figure 5B), the distances between a pair of male and female mosquitoes (n=10 pairs per trial) were varied (1cm, 7cm, 13cm, and 19cm) (Arthur et al 2013), with three trials per distance.

Figure 5A-B. Pressure-gradient microphones placed at a 1-cm distance from the tethered mosquito (A) and at varied distances (1cm, 7cm, 13cm, and 19cm) for the duet group (B)

4. Analysis of Wing Beat Frequencies

4.1.Harmonicity and phase relationship

Spectrograms were created using Fourier-transformed data on overlapped segments of time. Frequency modulation of its fundamental and harmonics were quantified using the estimated phase change that occurred in the Fourier coefficients if the time segment was shifted by one sample (Charpentier 1986, Brown & Puckette 1993). Amplitudes of the fundamental and their harmonics at an instant in time were determined by averaging Fourier coefficients across a few successive segments that exhibited minimal frequency modulation, interpolating the magnitude spectrum at the set of integer-related frequencies closest to the peaks, and correcting with the microphone calibration data.

Decrease in amplitude with distance was adjusted with a power function, taking into account the 1-mm distance between the front of the microphone and the sensing diaphragm. Polyspectra were calculated on a line corresponding to the harmonic ratios of interest rather than over the full space (Brillinger 1965, Mendel 1991). Complex Fourier coefficients were conjugated as appropriate before plotting the logarithmic magnitude of their product (Kim & Powers 1979).

4.2. Spherical spreading

Spherical spreading refers to the decrease of sound level generated by the mosquito as it propagates away from a source. From the center location of a source, crests and troughs are established as spheres. This estimate expresses the uniformity of sound propagation in all directions. Six locations of the microphones relative to the mosquitoes were done, namely, (1) in front of their head, (2) behind the last abdominal tergite, (3) left wing, (4) right wing, (5) above, and (6) below in order for spherical spreading of their flight tones become evident. In each location, three trials were performed.

Between recordings, mosquito flight was inhibited by allowing it to grab on a piece of paper (5cm x 5cm) and was initiated by removing the paper and when necessary, a gentle air puff was applied unto the mosquitoes. Data were acquired in a computer after digitizing (National Instrument USB Digitizer) by using custom software written in Laboratory Virtual Instrument Engineering Workbench (LabVIEW, National Instruments Corporation, Austin, TX, USA). A 10,000-Hz sample rate was permitted for 5 sec of the flight tone for each trial.

4.3. Statistical analysis

Dependent variables (wing beat frequencies, harmonics, and phase relationships) of the above trials were analyzed by determining first the descriptive statistics. Student's T-test was used to determine whether there was a significant change between the mosquito's flight tones when in solo and in courtship. One-way analysis of variance (ANOVA) was used to determine whether distance affected the wing beat frequencies of the mosquitoes during courtship by using Minitab 17 statistical software (Pennsylvania, PA, USA).

RESULTS AND DISCUSSION

1. Wing Beat Frequencies of Ae. aegypti queenslandensis

1.1. Single mosquitoes

The male fundamental wing beat frequencies (range: 607-1,037 Hz; mean: 824 ± 72 Hz) of *Ae. aegypti queenslandensis* were higher than those of the female (range: 487-660 Hz; mean: 570 ± 39 Hz) (Figure 6A-B). Both sexes differed in their wing beat frequencies significantly (*p*=0.00).

Similar to the previous reports (Cator et al 2009, Gŏpfert et al 1999), tethered male *Ae. aegypti* fly at a higher fundamental frequency than female mosquitoes (691.2 Hz vs. 479.8 Hz, respectively). Current results showed that the wing beat frequencies of tethered *Ae. aegypti queenslandensis* were slightly higher than those reported by Göpfert et al (1999) and Cator et al (2009). However, free flight of *Ae. aegypti* (Cator et al 2011) shows higher fundamental frequencies than in this study (982 Hz vs. 824 Hz for males; 664 Hz vs. 570 Hz for females, respectively). Evidences showed that tethering results in a decrease in mosquito wing beat frequency compared with free flight (Arthur et al 2014, Cator et al 2009, Cator et al 2011).

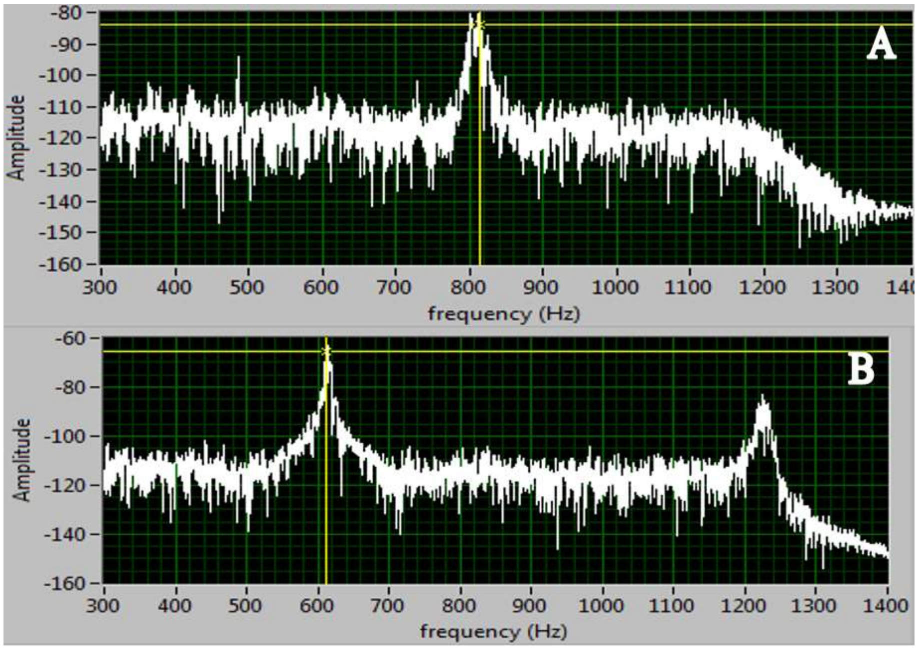

Figure 6A-B. Fast Fourier-transformed wing beat frequencies of isolated male (A) and female (B) *Ae. aegypti queenslandensis* under laboratory conditions

1.2.*Duet of Male and Female Mosquitoes*

During the duet between the male and the female *Ae. aegypti queenslandensis*, the wing beat frequencies of male mosquitoes ranged from 615 Hz to 665 Hz (mean: 638 ± 89 Hz), whereas those of female frequencies ranged from 544 Hz to 585 Hz (mean: 558 ± 38 Hz) (Figures 7-10). Student's t-test analysis (Table 1) showed that wing beat frequencies of male mosquitoes differed significantly ($p<0.01$) during courtship when paired with female mosquitoes in all distances between them. Those of female mosquitoes remained steady when in solo flight and when paired with male mosquitoes in all distances ($p>0.05$, $df=3$, $F=2.36$). Consistent with Charlwood et al (2002), once a potential mate is located, male mosquitoes orient themselves according to flight tones produced by females.

Table 1. Results of T-test analyses between isolated and paired male and female *Ae. aegypti queenslandensis*

Distance between male and female (cm)	*P*-value (male)	*P*-value (female)
1	0.002	0.599
7	0.007	0.491
13	0.004	0.455
19	0.003	0.299

Courtship duet between the female and the male

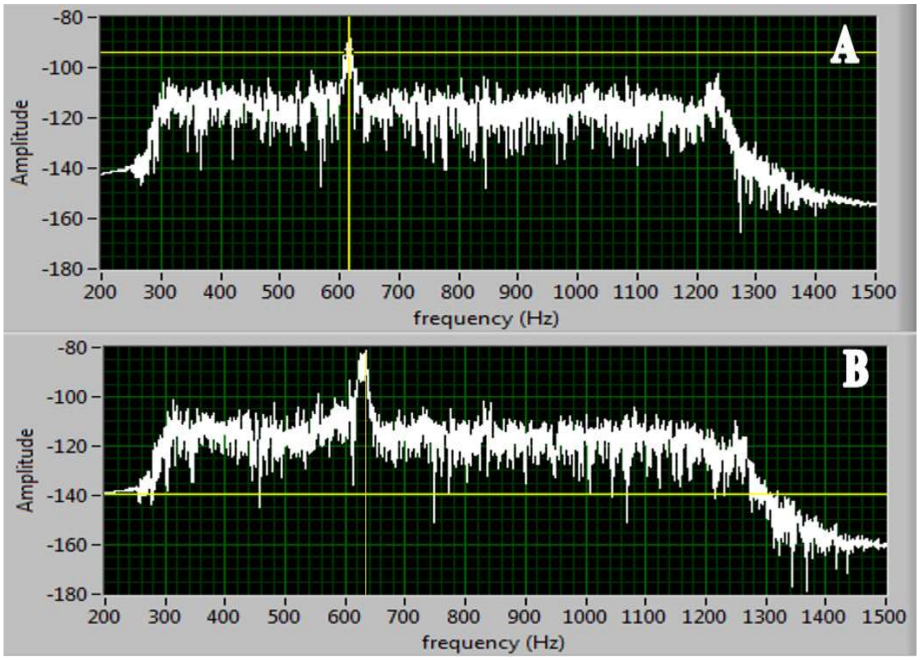

Figure 7A-B. Fast Fourier-transformed wing beat frequencies of male (A) and female (B) *Ae. aegypti queenslandensis* during courtship at 1-cm distance from each other under laboratory conditions

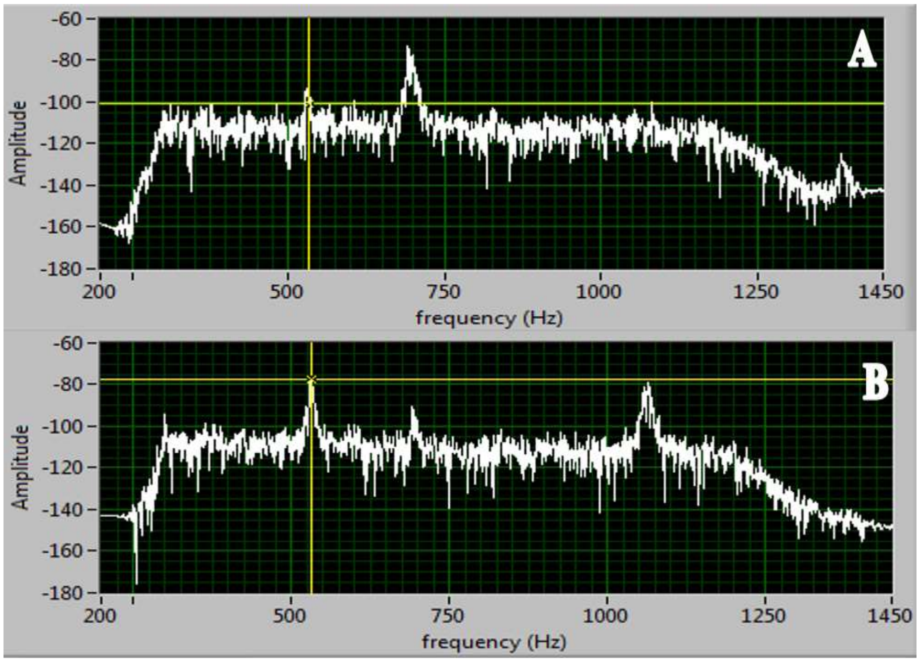

Figure 8A-B. Fast Fourier-transformed wing beat frequencies of male (A) and female (B) *Ae. aegypti queenslandensis* during courtship at 7-cm distance from each other under laboratory conditions

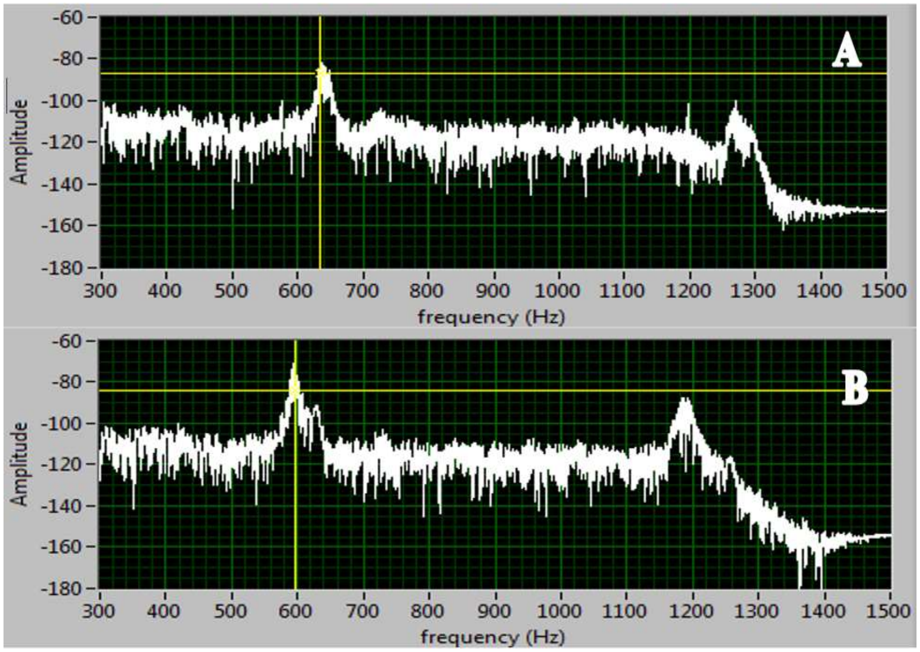

Figure 9A-B. Fast Fourier-transformed wing beat frequencies of male (A) and female (B) *Ae. aegypti queenslandensis* during courtship at 13-cm distance from each other under laboratory conditions

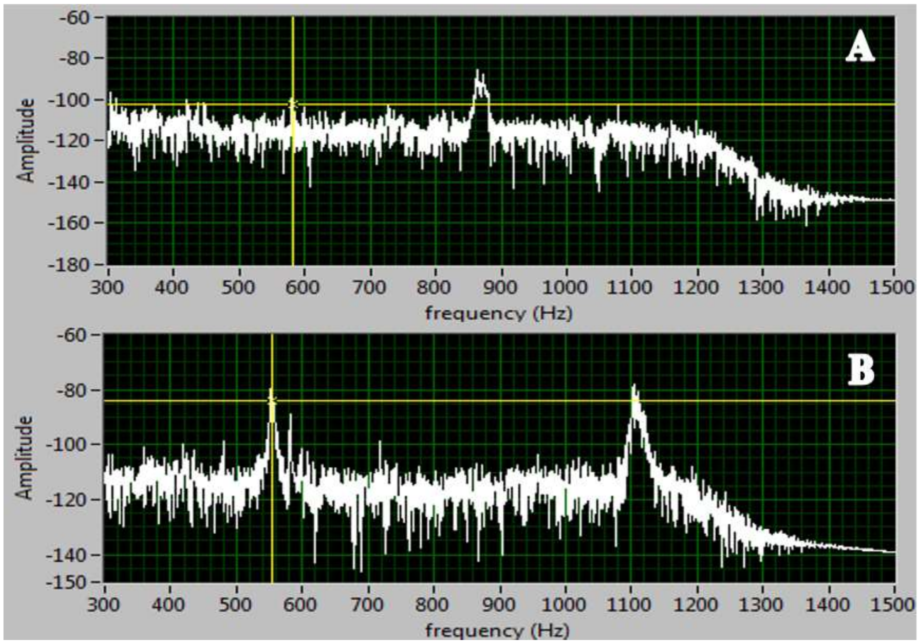

Figure 10A-B. Fast Fourier-transformed wing beat frequencies of male (A) and female (B) *Ae. aegypti queenslandensis* during courtship at 19-cm distance from each other under laboratory conditions

Courtship duet between the female and the male

All ten pairs of male and female mosquitoes at different distances from each other for three trials demonstrated an apparent effort to converge their wing beats (Figures 11-14) to a common frequency (Table 2). These results were consistent with other mosquitoes, namely, *Toxorhynchites brevipalpis* (Gibson & Rusell 2006), *Culex quinquefasciatus* (Warren et al 2009), *Ae. aegypti* (Cator et al 2009), and *Anopheles gambiae* (Pennetier et al 2010). Although the sound generated by the female wings is complex, only the fundamental frequency of their wing beat is essential to attract the males (Wishart & Riordan 1959); this was confirmed in the present study. In comparison with other insects, which advertise species and sex via time-based pulse patterns (Gerhardt & Huber 2002), *Ae. aegypti queenslandensis* can vary only their carrier frequencies of wave signal implying that these mosquitoes could change the wave signals of their wings to convey specific information.

Table 2. Convergence frequencies of the wing beat frequencies of male and female *Ae. aegypti queenslandensis* during courtship under laboratory conditions

Distances between male and female (cm)	Average convergence frequency (Hz)	Average convergence at harmonics (Hz)
1	561 ± 66	
7	575 ± 21	
13	578 ± 40	
19	577 ± 8	1,022 ± 6

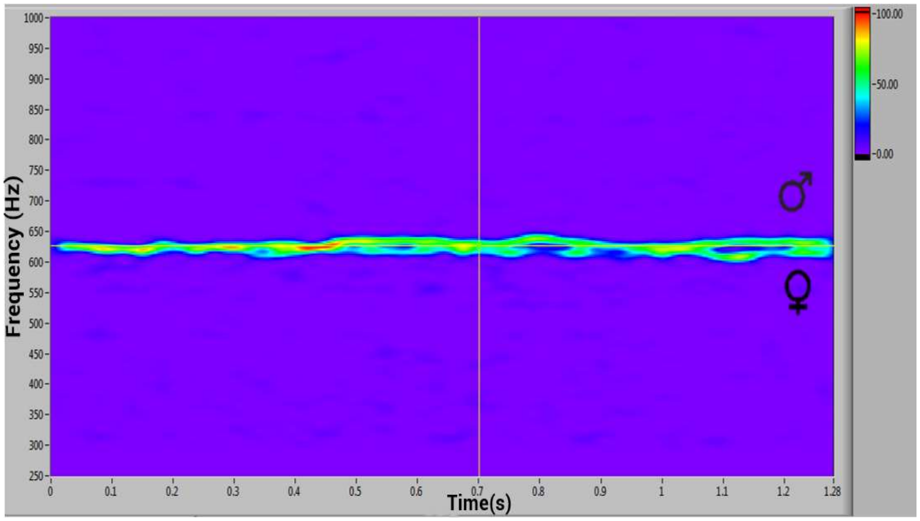

Figure 11. Spectrogram showing convergence frequencies of male and female *Ae. aegypti queenslandensis* wing beat frequencies at 1-cm distance from each other during courtship under laboratory conditions

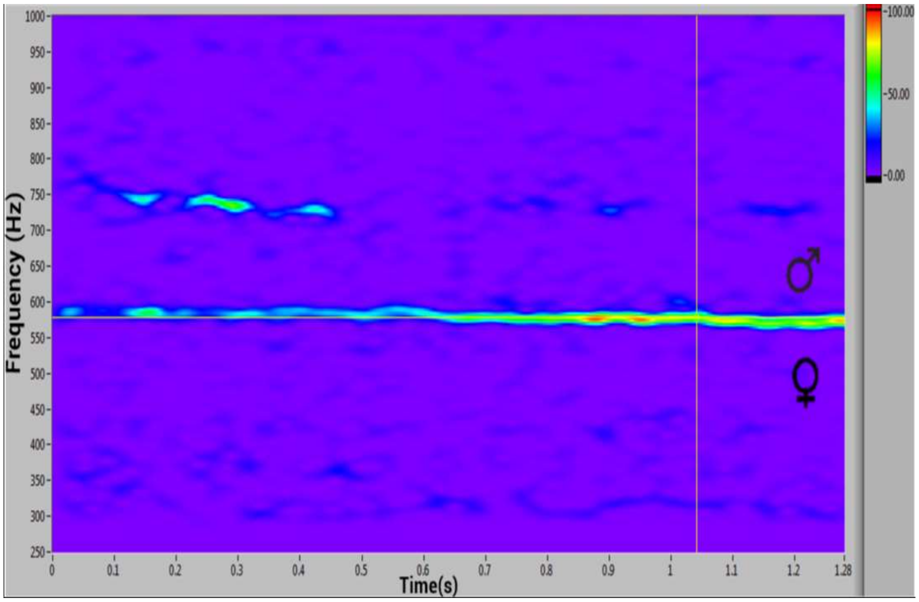

Figure 12. Spectrogram showing convergence frequencies of male and female *Ae. aegypti queenslandensis* wing beat frequencies at 7-cm distance from each other during courtship under laboratory conditions

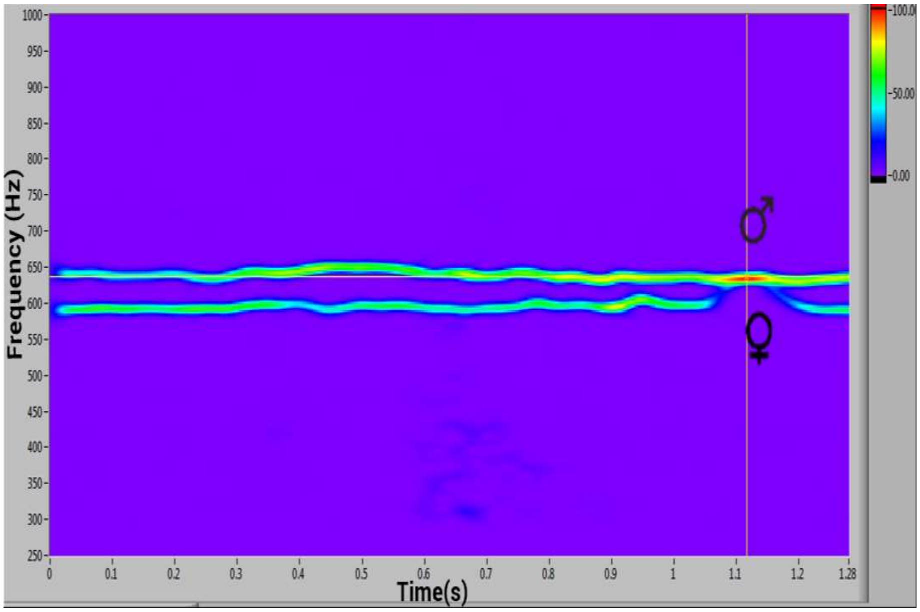

Figure 13. Spectrogram showing convergence frequencies of male and female *Ae. aegypti queenslandensis* wing beat frequencies at 13-cm distance from each other during courtship under laboratory conditions

Courtship duet between the female and the male

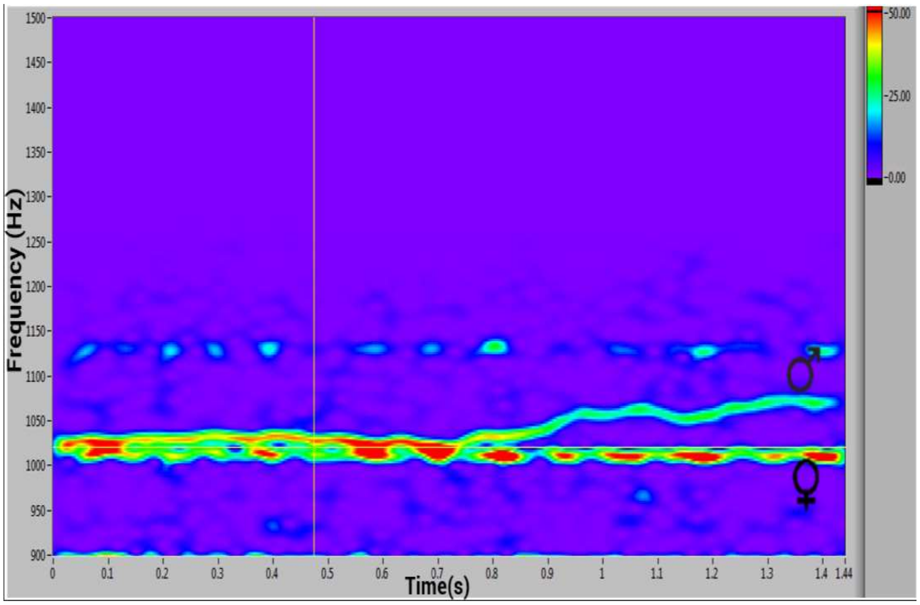

Figure 14. Spectrogram showing convergence frequencies of male and female *Ae. aegypti queenslandensis* wing beat frequencies at 19-cm distance from each other during courtship under laboratory conditions

1.3.*Harmonicity*

In the current study, all analyses of the wing beat frequencies of *Ae. aegypti queenslandensis* were restricted to the fundamental frequency, except for the pairs with a 19-cm distance between them (Table 2). This implies that there was convergence of wing beat frequencies at a 19-cm distance between pairs of male and female mosquitoes. Results on the harmonic convergence of mosquito pairs were consistent with the findings of Cator et al (2009). Gibson and Russel (2006) also reported that all pairs of male and female *Ae. aegypti* converge at their fundamental frequency, within the tuning range of their antennae. Hence, Aldersley et al (2016) noted that harmonic convergence is an active phenomenon and does not occur by chance.

2. *Phase Relationships*

When two mosquitoes are flapping their wings together (converging in the same frequency), trying to converge at a specific frequency, one mosquito is flapping ahead or behind from the other, as reflected by their phase difference. Table 3 shows the phase difference between the two wave signals for each pair of mosquitoes in a duet, with wing beat frequencies in convergence. When this phase difference between the two waves is 0°, these signals are produced simultaneously which imply that both mosquitoes are flapping their wings together at the same time (Crummett & Western 1994).

Table 3. Phase difference of the wing beat frequencies of the male and female *Ae. aegypti queenslandensis* during courtship under laboratory conditions

Distances (cm) between male and female	Phase difference (°)
1	0 - 75
7	0 - 55
13	0 - 75
19	18 - 64

3. *Spherical Spreading of Male and Female Ae. aegypti queenslandensis*

The mean frequencies of flight tones of male mosquitoes (mean: 987 ± 18 Hz) were higher than those of female mosquitoes (519 ± 42 Hz) that were recorded in six major locations of the microphone relative to the tethered mosquitoes (Table 4). Results imply that the sound produced by the flapping of wings of *Ae. aegypti queenslandensis* spread away spherically, consistent with that of *Ae. aegypti* (Arthur et al 2014).

The loudest flight tones of male *Ae. aegypti queenslandensis* were recorded at their back; those of the female, at their left side (Table 4). The quietest flight tones of male mosquitoes were recorded at their right; those of the female, at their upper side (Table 4). Although, these flight tones did not differ significantly (*p*>0.05) in six locations of the microphones relative to the mosquitoes, this may require further studies on the differences of flight tones at different parts of the male and female mosquitoes. Arthur et al (2014) reported that the loudest flight tones are recorded ahead and behind of *Ae. aegypti*, the quietest to their right and left.

Table 4. The mean wing beat frequencies (Hz) of tethered male and female *Ae. aegypti queenslandensis'* flight tone relative to six locations of the microphone

Locations of microphone relative to mosquitoes	Male	Female
Back	1188.18	517.36
Down	949.38	526.53
Front	937.87	512.59
Left	933.43	538.10
Right	931.06	526.45
Up	983.95	494.20
Mean ± SD	987±18	519±42

Air, as a fluid medium for sound propagation, has a specific acoustic resistance. To have a good match of the sound source to the medium, the specific acoustic resistance of the source, which depends on its size and configuration (Olson 1957), should match that of the fluid medium (Olson 1957, Bennet-Clark 1995) for effective spreading of sound waves. For some insects, sound spreads as a spherical-wave in the near field or at short ranges from the sound source. In the far field (ie, many wavelengths away), sound propagates as a plane-wave (Bennet-Clark 1998). Sound intensity decreases with both distance and harmonic number.

The current study analyzed several aspects of the flight tone of tethered *Ae. aegypti queenslandensis* under laboratory conditions in order to better understand the acoustics of these courtship signals. Moreover, both the optimal temperature and RH for adult mosquitoes' longevity and biting activity are well established, but there were no available data on the optimal conditions for flight activity. Temperature, unlike RH, markedly influences the flight ability of *Ae. aegypti*. Rowley and Graham (1967) noted that RH (30% to 90%) has a very little effect on the flight activity of the virgin female *Ae. aegypti*, except at 32ºC with 30% RH, in which flight depression can occur. If temperature conditions are suitable, mosquitoes may fly at any RH, therefore, their performance at any given temperature is independent of the RH (Rowley & Graham 1967).

Since the early 1900s, acoustic recording and playback technologies have already been employed for both insect detection and monitoring (Mankin et al 2011). Acoustic devices attract and trap insects (Walker 1988, Walker 1996), manipulate their behavior (Gwynne 1995), and interrupt intraspecific communication (Samarra et al 2009). These devices do not just capture, sterilize, or kill but also collect the live specimens for biological studies (Fowler 1988, Campbell & Shipp 1974) and control programs (Frank 1994, Frank & Walker 2006), and monitor insect diversity (Riede 1998, Chesmore & Ohya 2004), population levels (Forrest 1988, Mankin 1994, Raman et al 2007), and their geographic distributions (Cooley et al 2011).

Several studies attempted to test the effectiveness of sonic pest devices but most such as those for ants (Huang et al 2002), cockroaches (Schreck et al 1984, Gold et al 1984, Koehler et al 1986), and bed bugs (Yturralde & Hofstetter 2012) were ineffective. Interestingly, some devices are able to attract, repel, and increase *Ae. aegypti*'s bite rate by as much as 50% (Andrade & Cabrini 2010). Greenlee (1970) described that commercial mosquito "repellers" are sine waves (Kutz 1974, Singleton 1977) or fundamental frequencies.

A few studies (Roth 1948, Belton & Costello 1979) reported that a female mosquito is repelled by the same sound frequency that attracts the male. The sound that attracts the male mosquito is generated by the female wings. In the present study, the fundamental frequency of the female, at which the male *Ae. aegypti queenslandensis* converged, was at 544 Hz to 585 Hz (mean: 558 ± 38 Hz). For this type of mosquito control, the focus is not only on females but also males because they play a role in courtship. Thus, developing an acoustic device that produces within the range of frequencies can disrupt the wing beat frequencies of both sexes from converging and can prevent their courtship and subsequently their mating. This device can help in the control of *Ae. aegypti queenslandensis* without using harmful chemicals. Although there are devices that produce such frequencies and have been tested on other insects, these frequencies acted only as attractants rather than repellants on *Ae. aegypti* or *Culex pipiens* (Greenlee 1970). Construction of these devices requires thorough research considering temperature and humidity of the environment as well.

CONCLUSION AND RECOMMENDATIONS

In conclusion, the male *Ae. aegypti queenslandensis*' wing beat frequency converged with the female's frequency at their fundamental frequency, with a range of phase difference for each of the distances tested between these mosquito pairs. Sound produced by the flapping of wings, spread away from the mosquito spherically, which is true for both the *type* form (Mattingly 1957) and *Ae. aegypti queenslandensis.*

For further studies, we recommend to determine the following: (1) the wing beat frequency of *Ae. aegypti queenslandensis* and *Ae. albopictus* in free flight, (2) differences of flight tones between male and female dengue mosquitoes, (3) if different levels of temperature and relative humidity affect the mosquitoes' flight tones, and (4) if different wing sizes affect the mosquito's wing beat frequency.

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