The Impact of Temperature and Body Size on Fundamental Flight Tone Variation in the Mosquito Vector Aedes aegypti (Diptera: Culicidae): Implications for Acoustic Lures

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The Impact of Temperature and Body Size on Fundamental Flight Tone Variation in the Mosquito Vector *Aedes aegypti* (Diptera: Culicidae): Implications for Acoustic Lures

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Abstract

Aedes aegypti (L.) males use female flight tone as a means of mate localization. By playing the sound of a flying female, males can be attracted to a trap to monitor mosquito populations and the progress of transgenic male releases. However, the female flight tone used to attract males needs to be optimized to maximize trap effectiveness. The fundamental frequency of female flight tone could be influenced by both body size and ambient temperature. However, no analysis yet has considered both the effect of body size and temperature on female flight tone of *Ae. aegypti*. Here, we present results for both these factors by recording the sounds of free-flying and tethered females across multiple temperature environments and with females reared for small, medium, and large body sizes. We demonstrate that female fundamental frequency is highly dependent on the environmental temperature, increasing ∼8–13 Hz with each °C gain. Body size and whether a female was tethered or free-flying did not impact the relationship between frequency and temperature, although further analysis is warranted. Our study highlights the importance of understanding the relationship between flight tone and temperature, and will inform the design of male mosquito traps.

Key words: wingbeat frequency, premating behavior, acoustic communication, sound trap

Mosquito-borne diseases claim millions of lives annually despite significant control efforts. In particular, *Aedes aegypti* (L.), the primary vector of dengue, chikungunya, yellow fever, and Zika viruses (Gubler 2002, Bhatt et al. 2013, Kendrick et al. 2014, Montero 2015, Meaney-Delman et al. 2016), is an especially difficult mosquito to control. New approaches that rely on mosquito behavior are needed to reduce global infections transmitted by this important disease vector.

Many surveillance strategies rely on trapping mosquitoes; to date, most mosquito traps use cues that attract and capture females. Males are considered unimportant, as they lack a role in disease transmission. However, as control strategies begin relying on releases of genetically modified males (Carvalho et al. 2015, Ernst et al. 2015) or *Wolbachia*-infected males/SIT (Ritchie et al. 2015), a reliable trapping mechanism for male mosquitoes must be designed to efficiently monitor released males and the spread of the trait in male progeny. Male *Ae. aegypti* possess CO_2 receptors and are attracted to human hosts, which serve as their mate encounter site

(Grant and O'Connell 2010); however, they are not readily captured in traps that rely on female behavioral cues. By focusing on male behavioral cues in addition to host-seeking cues, we can design optimal traps for male *Ae. aegypti* mosquitoes.

Male *Ae. aegypti*, along with males of many other medically important mosquito species, use their plumose antennae and Johnston's organ to detect sounds produced by female mosquitoes beating their wings in flight (Clements 1999). This sound plays an important role in mate localization and courtship behavior (Vigoder et al. 2013). During courtship, both males and females are able to adjust their flight tones to synchronize harmonically before copulation (Gibson and Russell 2006; Cator et al. 2009, 2010; Warren et al. 2009; Pennetier et al. 2010).

Although it has long been known that mosquitoes produce sound, and that males of multiple species of mosquitoes are attracted to certain frequencies, little focus has been placed on this information since the mid-20th century (Roth 1948, Kahn and Offenhauser 1949, Wishart and Riordan 1959). Recently, interest in

Table 1. Summary of female *Aedes aegypti* flight tone by temperature data to date

Reference	Temp(°C)	Fundamental frequency (Hz)	Tethered or free flight	Lab or field
Göpfert et al. 1999	22–23	445–475	Tethered	Lab
Arthur et al. 2014	23	511 ± 46	Tethered	Lab
Brogdon 1994	25	460	Free flight	Lab
Duhrkopf and Hartberg 1992	26	350–550	Free flight	Lab
Cator and Harrington 2011	33	664	Free flight	Field

this behavior has resurged, as the need to capture males in genetic mosquito control programs has become apparent. Both Stone et al. (2013) and Johnson and Ritchie (2016) have recently designed sound traps for male *Ae. aegypti*, as have Diabate and Tripet (2015) for *Anopheles* mosquitoes. However, the *Ae. aegypti* traps rely on previously recorded data of female fundamental frequencies that may not be ideal. To optimize a sound-baited trap, it is important to understand the variables affecting female flight tone, and to account for these external variables in deployed traps. This information also has significant implications for traps using optical wingbeat classification and other automated approaches (Ouyang et al. 2015, Potamitis and Rigakis 2015, Silva et al. 2015).

Wingbeat frequency of many insect species changes with ambient temperature, although the relationship varies greatly between different taxa (Sotavalta 1947, Unwin and Corbet 1984). For example, small insects exhibit a stronger relationship between wingbeat frequency and ambient temperature compared with large insects, because body temperature can deviate substantially from air temperature with larger mass (Unwin and Corbet 1984). Sotavalta (1947) was among the first to demonstrate that the fundamental frequency of female flight increased with ambient temperature in various fly species (*Drosophila melanogaster* (Meigen), *Drosophila funebris* (F.), *Drosophila repleta* (Wollaston), *Musca domestica* (L.), and *Musca autumnalis* (De Geer)), including an unnamed mosquito species. Later, Unwin and Corbet (1984) showed a linear increase in frequency, with ambient air temperature from 13.0°C to 29.5°C for three fly species (*Calliphora vicina* (Robineau-desvoidy), *D. melanogaster*, and *M. domestica*). The only study to assess the relationship between female fundamental frequency and changing ambient temperature in *Ae. aegypti* was with tethered females of an unknown strain described in an unpublished 1974 thesis; however, the author did not control for body size (Costello 1974).

Previous studies have reported the fundamental frequency of female *Ae. aegypti* flight in the range of 350–664 Hz, at single temperatures with no measurement of body size (Table 1). These studies roughly suggest that *Ae. aegypti* fundamental frequency increases with temperature, following the pattern Sotavalta described in other fly species and Costello's previous work with *Ae. aegypti*. However, these studies cannot be directly compared owing to varying experimental conditions. The relationship between fundamental frequency and changing ambient temperature, controlled for size, requires description.

In addition, few studies have fully explored the effect of tethering on fundamental frequency. Those studies that do exist are contradictory (Table 1), but suggest that tethering mosquitoes may alter flight tone frequency in an unnatural way. Some authors report a decrease in frequency (Arthur et al. 2014), whereas others suggest that tethering increases the load on the flight mechanism and increases fundamental frequency (Chadwick 1953, Belton and Costello 1979). Therefore, in our present study, we address the impact of tethering on female flight tone in addition to temperature and body size.

Our goal was to determine the relationship between the fundamental frequency of female *Ae. aegypti* flight tone and ambient temperature. We also compared, for the first time with *Ae. aegypti*, the effect of temperature across females of different body size classes. Our results will aid design of temperature-sensitive traps that optimally capture the greatest number of male *Ae. aegypti* mosquitoes.

Materials and Methods

Mosquito Rearing and Maintenance

The *Ae. aegypti* used in this experiment originated from Bangkok, Thailand (15.7193° N, 101.752° E), in 2011, and were supplemented with eggs from the same site in 2012 and 2014. The colony was held at 28°C, 80% relative humidity (RH), and a photoperiod of 10:10 (L:D) h, with 2-h simulated dusk and dawn. Mosquitoes were reared to obtain uniform small, medium, or large body-sized adults representing the range of sizes encountered in nature. Different body-sized females were obtained following the methods of Ponlawat and Harrington (2009). Briefly, one day after eggs hatched, larvae were counted (350 per tray for small, 200 per tray for medium, and 75 per tray for large) and placed in 28- by 22- by 8-cm plastic trays covered with mesh lids and containing 1 liter distilled water and four fish food pellets (Hikari Cichlid Gold, Hayward, CA; small body-sized females for this experiment were reared similarly, except with 350 larvae per tray). Once pupated, mosquitoes were separated visually by size and individual female pupae were placed in vials to ensure virginity. After confirming sex, eclosed females were transferred to 8-liter buckets upon eclosion and held at 28°C, 50% RH, and a photoperiod of 10:10 (L:D) h (2-h dusk or dawn) until experiments commenced. All females used were 2–4-d-old virgins. After experiment conclusion, one wing was removed from each individual and measured to confirm body size, following the methods of Nasci (1990).

Free Flight Assay

Female flight tone in response to temperature was recorded in free flight in two separate experiments. In the first experiment, one medium body-sized female was transferred into a 15- by 8- by 10-cm clear plastic box containing a particle velocity microphone (NR-21358; Knowles, Itasca, IL). The microphone was placed ~5 cm above the bottom of the box, and was connected to a custom built amplifier (as specified in Arthur et al. 2013; Fig. 1) and connected to a computer.

The box was placed in one of the four temperature environments—three separate incubators set to 18°C, 24°C, or 31°C, or a small (6.5 by 4 by 2.5 m) room set to 28°C. Individuals were left for 10 min to allow temperature acclimation. After 10 min, sound was recorded for 5 min. When the females were placed in an incubator, the incubator was turned off to reduce ambient noise, and a light source was added to encourage flight. To further encourage flight, the box was manually agitated every 30 s. For recordings in the small room, the apparatus was placed on a counter, and the female
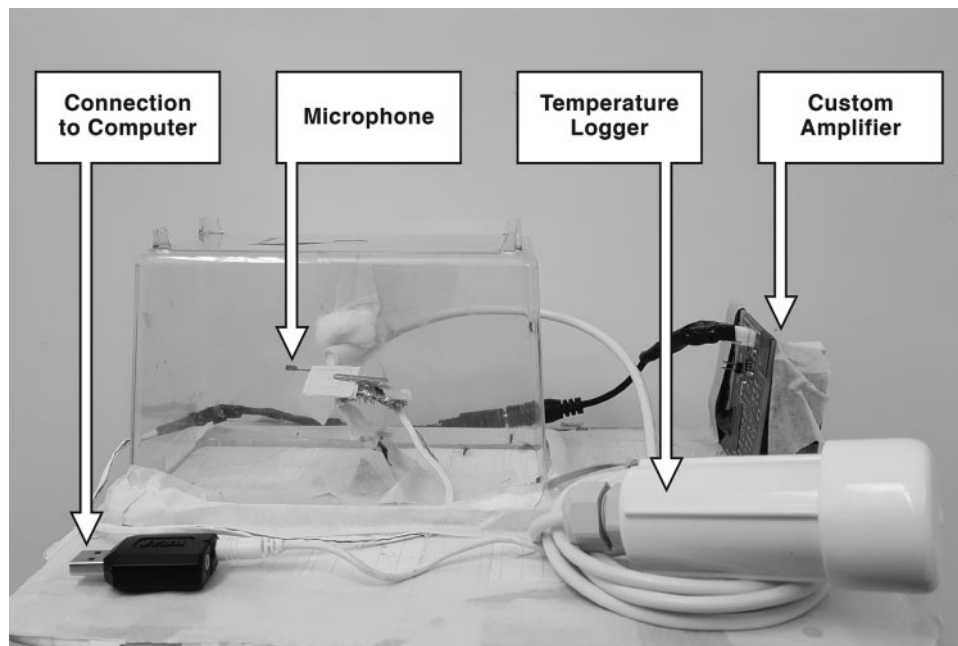
Fig. 1. Acoustic measuring apparatus for all free-flying *Ae. aegypti* female assays.

was monitored and stimulated to fly by agitation of the box for the duration of the recording. After recording in a temperature environment for 5 min, the process was repeated for the remaining temperature environments in a randomized order. Each trial was repeated to obtain a total of 10 individual females from three cohorts across all four temperatures. Data loggers set to read at 30-s intervals were placed inside the box and chamber to confirm temperature conditions (HOBO U23 Pro v2 Temperature and Relative Humidity Data Logger, Onset, Bourne, MA).

In the second experiment, flight tone from small (wing length $(\mu \pm stdev) = 2.70 \pm 0.09$ mm), medium (2.93 ± 0.06 mm), and large (3.02 ± 0.10 mm) body-sized females was measured in response to temperature. The same methods were followed as in experiment one above, with the exception that groups of 10 females were simultaneously monitored instead of one. Grouped females represents a more natural situation, as more than one female can often be observed around a host, which is the mating encounter site for *Ae. aegypti*, and grouping 10 females at a time allowed us to maximize recorded flight. This approach was repeated for three different female sizes (small, medium, and large) over two different replicates, to obtain a total of four group recordings for each body size across four temperatures.

Tethering Assay

To determine the relationship between free flight and tethering on the fundamental frequency of flight, medium body-sized females were tethered once they completed either the individual or group temperature trials. Tethering was achieved following the methods of Cator and Harrington (2011). Briefly, a female was suspended 2–5 cm above the microphone from a human hair attached to the anterior region of the female's thorax with Nailene glue (Pacific World Corporation, Aliso Viejo, CA), which was then attached to an insect pin and held within an alligator clip. Females that flew consistently after tethering were placed in either 18 °C or 31 °C and acclimated for 5 min. The fundamental frequency of female flight was then

recorded for 2 min. The same female was acclimated and measured in the other temperature, 18 °C or 31 °C. This was repeated for three females from the individual free-flying assay. For the group-held assay, two medium body-sized females from each replicate group were tethered and their flight tone averaged to obtain a single value for each replicate at each temperature. Therefore, there were a total of 14 tethered female trials (two females per group) for seven paired data points recorded at 18 °C and 31 °C.

Sound Analysis

All flight tones were recorded using Audacity 2.1.2 Cross-Platform Sound Editor (http://www.audacityteam.org/, accessed 2 April 2017) and exported as a 16 bit .wav file. Frequency analysis was completed in Raven Pro 1.5 (Bioacoustics Research Program, Cornell Laboratory of Ornithology, Ithaca, NY). Female fundamental frequency was visualized (Hann window; window length = 3,000 samples, 50% overlap), and then a selection spectrum was created for each recorded female flight (Hann window; window length = 7,800 samples, 50% overlap). For each recorded flight within a sound file, the peak frequency of the female's fundamental (usually between 300–600 Hz) was recorded and then averaged to produce a single, average fundamental frequency per recording.

Statistical Analysis

All analyses were performed using SPSS (Version 22.0, IBM Corp., Armonk, NY).

For all free flight and tethered experiments, the relationship between female fundamental frequency and temperature was analyzed using a linear mixed model (LMM), with a random intercept and slope at the subject level. Temperature was added to the analysis in two separate models as a continuous variable or a discrete variable with four levels (18°, 24°, 28°, 32°; Bonferroni corrected for multiple comparisons). For free flight experiment one with individual females, variation in female body size was controlled for by dividing female fundamental flight tone by wing length. For free flight
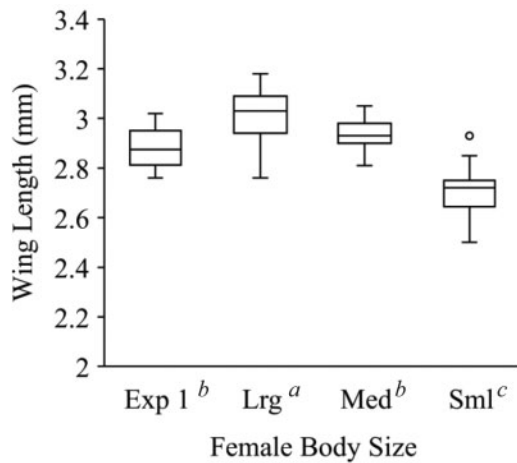
Fig. 2. Female *Ae. aegypti* wing length for reared cohorts of small, medium, and large body-sized females, as well as females from the first experiment (Exp 1). Lower case letters indicate pair-wise significance (Tukey HSD).

Fig. 3. Flight tone of *Ae. aegypti* recorded from individual females. Female fundamental frequency increases with temperature. Gray dots—individual data; Outlined circles $\pm$ stdev—average data for each of the four set temperatures ($n = 10$ each); Dotted line—GLM-derived relationship between frequency and temperature.

experiment two, female size was added as a discrete factor to the analysis.

The relationship between free-flying and tethered females was analyzed via a paired *t*-test, with the same female recorded at both 18°C and 32°C. Because temperature varied slightly between free flight and tethering, a separated paired *t*-test was performed on data adjusted for temperature. The flight tone adjustment was calculated by finding the difference between the two recorded temperatures for each female. Then, using the equation derived from the LMM in experiment one, the difference in temperature was multiplied by the slope of the equation and the recorded frequency was adjusted up or down based on the positive or negative values of the derived adjustment.

## Results

Confirmation of Body Size

To confirm that females reared for small, medium, or large body size exhibited statistically significant differences in wing length, a one-way ANOVA was performed, including the wing lengths of medium-sized females from the individual recordings. Female wing length significantly differed between large, medium, and small body-sized females (Fig. 2; one-way ANOVA; $F = 80.42$; df = 3, 112; $P < 0.001$). Although there was overlap between the groups, pairwise comparisons indicate statistical separation between all groups in experiment two (Tukey HSD $P < 0.05$), and females of medium body size for experiment one and two did not differ in wing length ($P > 0.05$).

Individual Flights

Medium-sized females, when recorded individually, exhibit a significant effect of temperature on their fundamental frequency, with frequency increasing with temperature (Fig. 3; LMM: *Frequency* = 293.71 + 8.24 × *Temperature*; $F = 44.73$; df = 1, 9; $P < 0.001$). This holds true even when frequency was adjusted based on female size (*Frequency per Wing Length* = 102.07 + 2.85 ×*Temperature*; $F = 42.22$; df = 1, 9; $P < 0.001$). When temperature was treated discretely, a significant effect of temperature on female flight tone remained (LMM, Frequency unadjusted: $F = 32.76$; df = 3, 27; $P < 0.001$; LMM, Frequency adjusted: $F = 33.43$; df = 3,
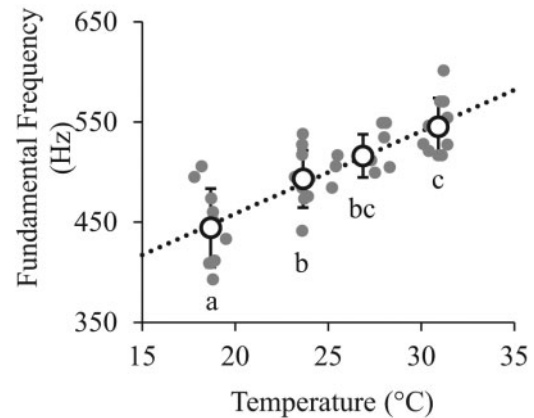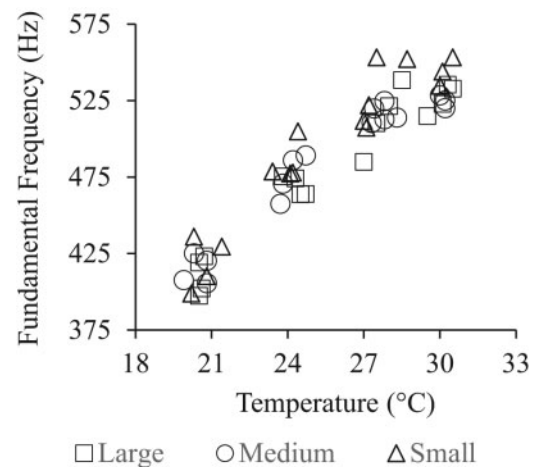
Fig. 4. Flight tone of *Ae. aegypti* recorded from grouped females based on body size. Female fundamental frequency increases with temperature, regardless of size. Shapes indicate small, medium, and large body size.

27; $P < 0.001$). Pairwise comparisons (Bonferroni corrected) are reported within Fig. 3. The slope of the regression line from these experiments with medium-sized females was used to estimate the increase in frequency for every increase in 1°C as 8–13 Hz.

Group Flights

Temperature remained a highly significant factor for female flight tone in the group recording experiments (Fig. 4; LMM: $F = 325.47$; df = 1, 35; $P < 0.001$). However, size had no significant effect on female fundamental frequency (Fig. 4; LMM: $F = 3.08$; df = 2, 3; $P = 0.096$). Whether temperature was treated as discrete or continuous had no effect on the overall significance of the parameters. There was no significant difference in the effect of temperature on fundamental frequency between medium-sized females in experiment one and experiment two (LMM: $F = 0.56$; df = 1, 12; $P = 0.470$).
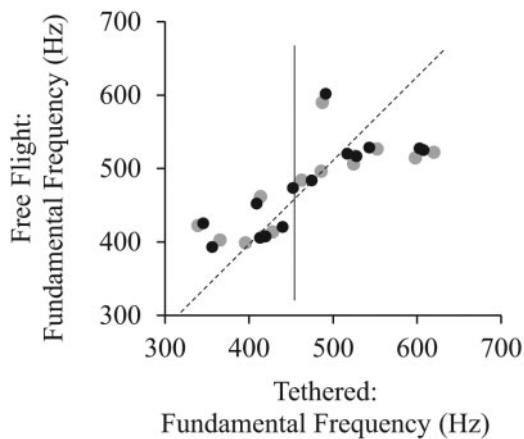
Fig. 5. Tethered versus free-flying female *Ae. aegypti* fundamental frequency. Each dot indicates a pair of recordings from free flight or tethered individuals or groups. A perfect one-to-one relationship would fall on the dotted line. The solid line separates the points between the two temperatures (left: 18 °C; right: 32 °C). Black dots—unadjusted pairs; Gray dots—pairs adjusted for minor differences in temperature.

Tethered Versus Free Flight

Female flight with and without tether was strongly correlated (Fig. 5; Pearson correlation; $r^2 = .77$; $P = 0.001$). Whether or not a female was tethered had no significant effect on her flight tone (paired *t*-test: $t = 0.425$; df = 13; $P = 0.678$; paired t-test, frequency adjusted: $t = 0.232$; df = 13; $P = 0.820$).

Discussion

We demonstrate an effect of temperature on female *Ae. aegypti* flight tone, regardless of female size or tethered-state. A relationship between flight tone and temperature is well demonstrated for a number of Diptera and other small-bodied insects (Sotavalta 1947, Unwin and Corbet 1984). This analysis is the first to systematically assess the effect of temperature and body size on wingbeat frequency in a mosquito, and has important implications for the development of novel acoustic traps to monitor male populations of *Ae. aegypti*.

Female fundamental flight tone frequency varies from ~300–600 Hz. When designing an acoustic trap, arbitrarily choosing a frequency within this range may not be specific enough to optimally attract male *Ae. aegypti*. Males are able to discriminate between females whose flight tone frequencies differ by a small margin of 75 Hz or less (Belton and Costello 1979), a range which only encompasses ~6–9 °C based on our present analysis. Additionally, Costello (1974) showed that *Ae. aegypti* males optimally responded to higher frequencies as ambient temperature increased. Integration of an adaptable female wingbeat frequency based on ambient temperature would improve the efficiency of traps currently being implemented to collect male *Ae. aegypti* (Stone et al. 2013, Johnson and Ritchie 2016).

Although we found a considerable effect of temperature on flight tone, female size, generated by adjusting rearing conditions (wing lengths of 2.5–3.2 mm), played only a minor role on female flight tone. However, the range of sizes we created for this experiment did not encompass the full range of female *Ae. aegypti* body sizes found in nature. Recorded sizes of wild *Ae. aegypti* females tend to be smaller (~2.46–2.64 mm; Nasci 1990, Chadee and Beier 1997, Scott et al. 2000). It is possible that if we generate a range of smaller-sized females, then we could observe a distinction between the small and

large cohorts, though no acoustic recordings from individuals this small have been done to date. At least for females with wing lengths in the range of 2.5–3.2 mm, size does not appear to be a factor.

In this study, we evaluated the effect of tethering and found no differences in female flight tone. In contrast, other studies (Chadwick 1953, Belton and Costello 1979, Arthur et al. 2014) have demonstrated that tethering affects female flight tone in a number of ways. Because there is no universal method for tethering mosquitoes, differences in the effect of tethering on female flight could be owing to differences in tethering techniques. For example, hover flight could be initiated by tethering and have different properties than forward flight. Unwin and Corbet (1984) proposed that the wingbeat frequency for forward flight is more correlated with temperature and may help cool small insects in warm environments, whereas hover flight merely allows the individual to stay aloft and is not correlated with temperature. Tethered females in the current study likely simulate forward flight, as wingbeat frequency increased with temperature. Arthur et al. (2014) also found the tethered females more closely simulated forward flight by observing the patterns of flight tone amplitude recorded around tethered females. However, other tethering techniques could initiate erratic or hover-like flight in mosquitoes and may contribute to the variation in the effect of tethering on female flight tone in these studies.

Recently, there has been considerable effort placed on understanding acoustic interactions between male and female *Ae. aegypti*, not just for surveillance strategies but also to explore the finer aspects of sound production in mosquitoes (Aldersley et al. 2016, Simoes et al. 2016). Potamitis and Rigakis (2016) found that tethering changed the amount of energy in the harmonics, but did not characterize any other changes. Because harmonic convergence is important for mating success, and tethering appears to alter the female's harmonics, future studies looking to optimize an acoustic trap for mosquitoes would be best served using female flight tone information recorded from free-flying females.

In summary, we have demonstrated that female *Ae. aegypti* flight tone frequency increases linearly with temperature, increasing ~8–13 Hz for each °C increase in temperature. We also demonstrated that female *Ae. aegypti* flight tone frequency is not affected by body size nor tethering within the parameters of these experiments. This information will support design of future male *Ae. aegypti* traps, and will improve surveillance and control of medically important mosquitoes.

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