A new approach to improve acoustic trapping effectiveness for *Aedes aegypti* (Diptera: Culicidae)

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ABSTRACT: Monitoring mosquito populations is essential to designing and implementing control strategies. Recent strategies based on releasing biologically modified mosquitoes have increased the need to effectively monitor mosquito abundance. Unfortunately, existing surveillance traps are of limited value due to their high cost and low capture rates. Here, we report the results of experiments designed to evaluate the effectiveness of an acoustic trap prototype. Stimuli synthesized from recordings of *Ae. aegypti* wingbeat signals and pure tones were evaluated as attractants to males in indoor and semi-field conditions. Overall, the acoustic trap´s efficacy differed significantly between indoor and semi-field conditions. After two hours of indoor recapture, ~69% of males were collected from acoustic traps broadcasting pure tones while ~78% of males were collected using synthesized wingbeat signals. Under semi-field conditions, however, acoustic traps collected less than ~1.7% of the males released. Increasing the intensity of the signals up to 90 dB (SPL re. 20 uPa at 1 m from the trap) did not improve the capture rate under semi-field conditions. Overall, our results indicate that acoustic signals synthesized from recordings of wingbeats can be used to enhance capture of male *Ae. aegypti*. ***Journal of Vector Ecology* 44 (2): 216-222. 2019.**

Keyword Index: Mosquito, surveillance, sound bait, wing beat, acoustic lure, dengue.

INTRODUCTION

Worldwide, *Aedes aegypti* mosquitoes are the main vectors of dengue, chikungunya, and Zika viruses (Mayer et al. 2017). As there are no effective treatments for these mosquito-borne diseases, prevention of their transmission depends mainly on controlling vector populations. Traditional control strategies, however, exhibit serious difficulties (WHO 2014). As a consequence, hindering reproduction has been one of the main reasons for developing new biologically-based strategies like radiated, transgenic or symbiont-based approaches (Bourtzis et al. 2016). Novel methods such as the use of the sterile insect technique and incompatible insect technique, or a combination of these two, illustrate recent approaches that are being evaluated to control *Ae. aegypti* both in laboratory and field conditions (Alphey et al. 2010, Bourtzis et al. 2014, Kittayapong et al. 2018). Implementation of such strategies introduces new challenges, like the need for accurate monitoring of males and females in the population. As current adult surveillance tools are designed to attract females only, have low efficacy, and tend to have high operational costs, it is crucial to find new mosquito attractants that improve the capture rates of traditional traps or develop new trapping strategies (Sivagnaname and Gunasekaran 2012). Given that these trapping methods rely on reproductive behaviors, development of attractants based on courtship features like visual cues (Diabate and Tripet 2015), pheromones (Fawaz et al. 2014), or sounds (Johnson and Ritchie 2015) are commonly explored.

Acoustic signals, in particular, play an important role in mosquito behavior prior to mating (Gibson and Russell 2006, Cator et al. 2009). It has been proposed that acoustic cues are also involved in *Ae. aegypti* swarm formation (Fawaz et al. 2014). Even though *Ae. aegypti* does not form large mating swarms like other mosquito species, the presence of a host or the onset of the sun elicit small aggregations of males with specific flight patterns (Cabrera and Jaffe 2007, Fawaz et al. 2014). Within such swarms, *Ae. aegypti* perform acoustic behavior for courtship (Cator et al. 2011). As males track females by the sound produced by their wingbeats, several studies have explored the possibility of using sound as an attractant (Ikeshoji and Yap 1990, Stone et al. 2013, Johnson and Ritchie 2015). Indeed, acoustic traps have successfully captured males using, for instance, pure tones that mimic the fundamental frequency of a female wingbeat (Johnson and Ritchie 2015, Johnson et al. 2018). However, these devices have not yet been employed as surveillance tools, probably due to their low effectiveness at collecting female mosquitoes and the discomfort that pure tones, within the audible range, produce to humans. Here we present a description of the prototype of an acoustic trap and the results of investigations to determine the effectiveness of signals synthesized from mosquito wingbeat recordings. To do so, we evaluate different acoustic lures in indoor conditions that mimic household settings. In addition, we compare the performance of our prototype with the commercial trap BG Sentinel under semi-field conditions. We aimed to establish the effectiveness of both the design of the prototype trap and a variety of acoustic attraction signals. In particular, we were interested in finding signals aimed at minimizing discomfort to humans by modulating sound frequency and intensity.

MATERIALS AND METHODS

Attraction signals

Mosquitoes flying individually or in pairs (male-female) were recorded following previously developed methods (Cator et al. 2009) and later used to synthesize pure tones and complex signals aimed to avoid habituation of mosquitoes to signals. In the first case, pure tones were synthetized by using the fundamental frequency of recordings of tethered females flying at 25 ± 1° C. Based on the fundamental frequency of 80 females with fundamental frequencies ranging between 450 and 550 Hz, we synthesized 10 s pure tones with the fundamental frequency of the wingbeat of females. These 80 pure tones (483.97 ± 5.22 [mean ± SEM]) were broadcast for 10 s periods, alternating between two speakers (Figure 1a), at a continuous intensity of ~10 dB SPL re. 20 μPa above noise floor at 1 m from the source. Sound intensity of the stimuli was changed in a particular experiment to test the role of this acoustic feature. The specific values used for each stimulus are indicated in the description of this particular experiment below. In contrast, complex signals were synthetized by using over 200 clips (10 to 30 s length) extracted from 240 recordings of male and female mosquitoes flying alone or in pairs. For the synthesis, clips were pasted one after another without any silent gaps. Using a random sequence of the clips allowed us to avoid generating periodical patterns and therefore, habituation of the mosquitoes to the signals. Thus, each speaker played simultaneously flight tones of individual mosquitoes or a male and female interaction (Figure 1b). Complex signals were broadcast for one min at a high sound intensity (~10 dB SPL re. 20 μPa above noise floor at 1 m from the source) followed by 9 min of low intensity (~2 dB SPL re. 20 μPa above noise floor at 1 m from the source). Complex signals were synthetized by using a sinusoidal representation of mosquito flight-tones (George 1997). From the short-time Fourier transform (Fast Fourier Transform length of 4,096 points, hamming window of 80 ms and 50% overlapping), we extracted the amplitude, frequency, and phase of the sine waves from the fundamental frequency and upper three/four harmonics of the flight tones. Using these characteristics, we produced signals that preserved the original wave shape of the flight tones and eliminated the background noise. Thus, this method allowed us to mix the sound produced by different mosquitoes without generating distortion or increasing the noise.

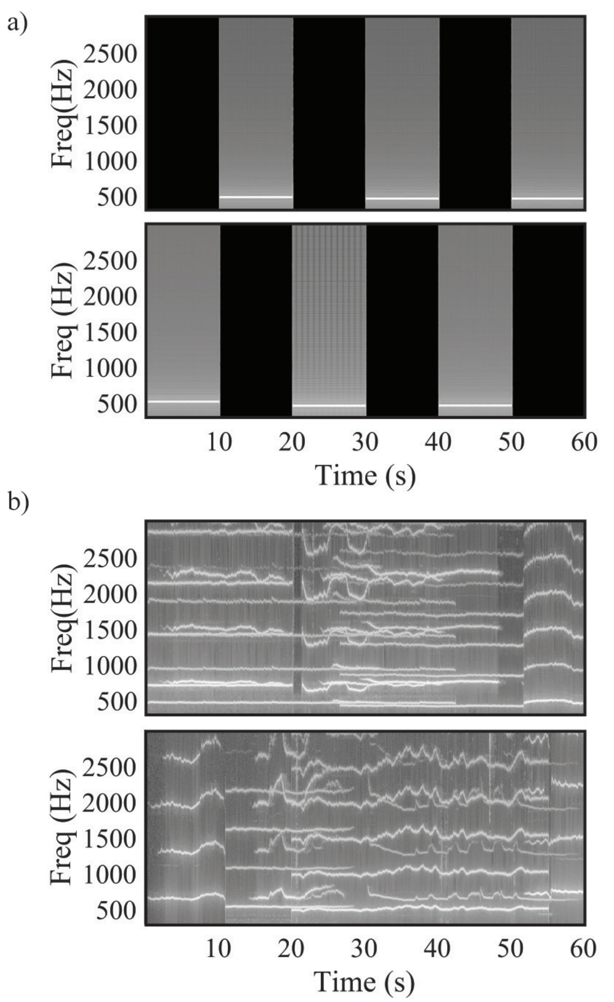

Figure 1. Representative spectrograms (one-minute segments) of attraction signals used in the experiment. Spectrograms of the left (L) and right (R) channels displaying (a) pure tones and (b) complex signals.

Acoustic trap prototype

Our prototype trap was designed to be used under indoor conditions. It was composed of an acrylic structure that holds an acoustic stereo system to broadcast the acoustic lure and a vacuum fan that captured mosquitoes in a collection bag (Figure 2a). The base of the trap was a 15 cm high isosceles trapezoid with short and long bases of 20 cm and 12 cm, respectively. The frontal face was 31 cm in length, and had a 11x14.5 cm^2 black and white area to increase the orientation of mosquitoes toward the suction fan. We used the SOMO II (4D systems) player module and two small speakers (Grove-speaker, Seed) to broadcast the attraction signals. All electronic components worked with a rechargeable 3.7 V power supply.

Acoustic trap performance under indoor conditions

Experimental site. This experiment was conducted at the University of Antioquia in Medellin, Colombia. In particular, the experiments were performed in medium-sized rooms (~4 m x 2 m x 2.5 m high) that simulated standard household conditions in which we controlled temperature (25 ± 1° C), humidity (60% RH), and light intensity (artificial light from

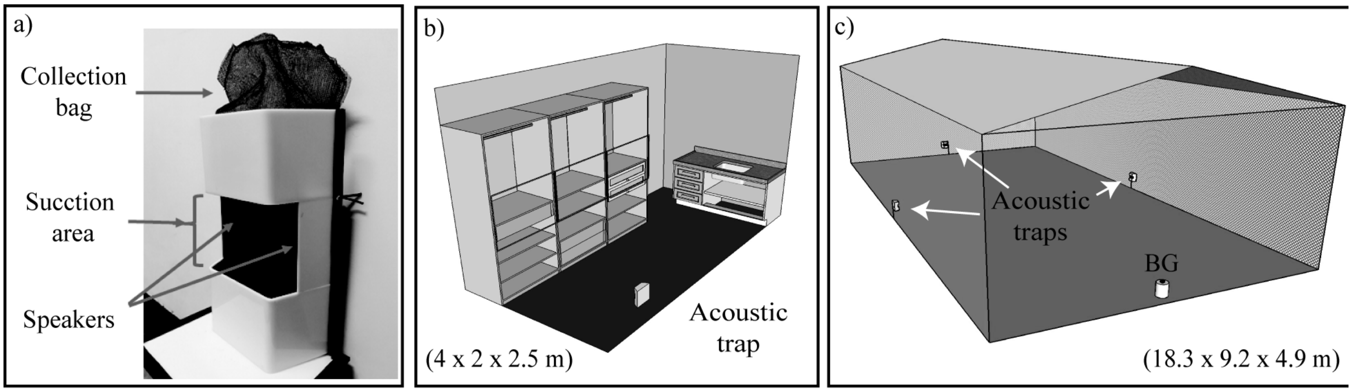

Figure 2. Prototype of the acoustic trap developed in this study (a) and sketches of the experimental set-up used to test the acoustic traps under indoor (b) and semi-field (c) conditions. The indoor room contained built-in furniture to mimic a kitchen with potential distractors. The acoustic trap was set on the wall in front to the furniture, 1 m above the ground. In contrast, in the experiments under semi-field conditions, the acoustic traps and BG-sentinel trap were located in four different locations inside the enclosure, and were rotated to the right at every replicate. Each trap was thus placed at a given location at least one time during the experiment.

standard fluorescent bulbs Philips F32T8/Super 84, 32 W). We also kept the distribution of furniture constant during and between experiments and prevented the use of the room by human visitors (Figure 2b). Several boxes, tables, and a sink in the room performed as distractors similar to those likely to be found in kitchens. Background noise intensity in the room was relatively high (77.6 ± 0.8 dB re. 20 µPa at the center of the room).

Mosquitoes. An *Ae. aegypti* colony was established with field-collected eggs from different locations in Medellin, Colombia. In the laboratory, the eggs brought from the field were placed in trays filled with 500 ml of unconditioned tap water. Forty (F1) wild type virgin mosquitoes were used for each replicate. To obtain virgin mosquitoes, pupae were separated individually into vials where they emerged without encountering other individuals. Recently emerged males were thus kept individually in small cylindrical containers (~20 cm x ~10 cm diameter) with mesh lids and maintained at constant environmental conditions (29±2° C, 80±5 RH). For the experiments, we used mosquitoes between three and seven-days-old.

Experimental design. The recapture rate of 40 mosquitoes originally released in the room at the beginning of each experiment was assessed every 30 min for a period of two h. We compared the capture rate of traps broadcasting no sound, pure tones, and complex signals. All experiments were performed between 16:00-18:00. We replicated each treatment seven times.

Data analysis. A one-way ANOVA followed by a Fisher's LSD *post hoc* test were used to compare the capture rate between treatments. Residuals of the model were tested for normality, homogeneity of variance, and independence using Shapiro-Wilks, Barlett, and Durbin-Watson tests, respectively. Analyses were performed using R (R Core Team 2018) and, in particular, the following packages: *dplyr*, *lmtest*, *nortest*, and *car*.

Acoustic trap performance under semi-field conditions

Experimental site. We examined the performance of the trap under semi-field conditions to evaluate its general performance under more complex conditions. This experiment was performed in large enclosures (18.3 m x 9.2 m x 4.9 m high) at the United States Department of Agriculture, Mosquito and Fly Research Unit at Gainesville, FL (Figure 2c). During the experiments, temperature, relative humidity, wind velocity, cloud cover, and ultraviolet (UV) index were recorded from a weather station (WRL 25, Texas Weather Instrument, located 700 m from the enclosures). Background noise intensity was measured from the center of the enclosure pointing to the corners and ranged between 63 and 70 dB re. 20 µPa.

Mosquitoes. Two to seven-day-old *Ae. aegypti* mosquitoes from the 1952 Orlando strain colony at the USDA Mosquito and Fly Research Unit (Gainesville, FL) were used for this experiment. Males were separated from females 1 h before each experiment to evaluate 4,800 males in eight replications of 600 males each.

Experimental design. To assess the performance of the acoustic trap under semi-field conditions, we determined the trapping effectiveness between 09:00 and 10:00. Six hundred individuals were released into the enclosure. Experiments followed a 4x4 Latin square design (Figure 2c) with three acoustic traps broadcasting different acoustic stimuli and, for comparison, a commercial trap BG-Sentinel trap (Biogents, Regensburg, Germany) using a Biogents (Regensburg, Germany) BG-Lure cartridge. We established the percentage of recaptured individuals for every trap after a period of six h. Two experiments were performed following the same methodology but varying the stimuli used for the three acoustic traps used in addition to the BG Sentinel. First, to evaluate the effect of sound intensity on the capture rate of males, experiment 1 used pure tones at two intensity levels. Three acoustic traps were used. One broadcasted pure tones at high intensity (90 dB SPL re. 20 µPa at 1 m from the trap), another trap broadcasted the same pure tones with a 10 min fading of intensity (90-65 dB SPL re. 20 μPa at 1 m from the trap), and a third (control) trap from which no sound was played. A second experiment was performed to examine trap performance when using different acoustic lures and evaluate the potential benefit of pairing sound with chemical cues. In this experiment, one trap broadcasted pure tones, a second one used the same pure tones plus a commercially available odorant cue (Biogents®, skin odor lure), and the third trap played complex signals with no additional cues.

Data analysis. We used a Generalized Linear Model (GLM) to evaluate differences among treatments. We also included the position of the trap and the environmental variables of the day of the experiments as random variables. We removed humidity and cloud cover from the analysis, as these variables were correlated with other variables included in the model. Temperature was highly correlated with humidity (r=-0.81, P=0.01) and cloud cover was also linearly related to UV index (r=-0.71, P=0.04). We thus included only temperature, wind velocity, and UV index in the analyses. During the experiments, mean temperature ranged between 21.67 and 28.33° C, wind velocity between 8 and 12.67 mph, and UV index between 3.33 and 8.67.

Residuals of the model were tested for normality, homogeneity of variance, and independence using Shapiro-Wilks, Barlett, and Durbin-Watson tests, respectively. Finally, to further examine the effect of environmental variables on the experiments we used a correlation analysis and multiple linear regressions. Analyses were performed using R (R Core Team, 2018) running the following packages: *lmtest, nortest,* and *car*.

RESULTS

Indoor acoustic tests

During the two h in which the traps were used, the acoustic signals broadcast in this study varied significantly in their ability to capture mosquitoes ($F_{2,18}$=30.44, P<0.001). When male mosquitoes were released into the room, the traps broadcasting pure tones (69.2 ± 2.1% [Mean ± SEM]) and complex signals (77.7 ± 2.9%) recaptured a significantly (P<0.01) higher number of individuals than the control (52.1 ± 3.1%). There was, however, no significant difference between the performance of the traps playing those two types of acoustic signals (P=0.064). Acoustic traps recaptured the majority of male mosquitoes (>60%) during the first hour (Figure 3a) and after this time, recapture rate decreased considerably.

Semi-field acoustic tests

During the experiment designed to test the effect of sound intensity on the capture rate, both environmental factors and treatments affected the number of mosquitoes captured (GLM, $F_{9,11}$=4.04, P=0.02). While location of the trap ($F_{3,11}$=1.13, P=0.43), wind velocity ($F_{1,11}$=0.49, P=0.5), and temperature ($F_{1,11}$=0.11, P=0.74) did not affect capture rates, UV index had a significant effect on the number of mosquitoes that were trapped ($F_{1,11}$=5.02, P=0.04). Despite the effect of this environmental factor, there was a significant difference in capture rates among treatments ($F_{3,11}$=3.95, P=0.03). The BG sentinel trap achieved the highest capture rate (3.92± 1.63%). In contrast, the acoustic traps had significant lower capture rates as follows: pure tones fading on intensity (1.02 ± 0.51%, LSD test P=0.03), high intensity pure tones (0.66± 0.24%, LSD test P=0.02), and no sound (0.25 ± 0.16%, LSD test P=0.01). There were no differences on the capture rate among acoustic

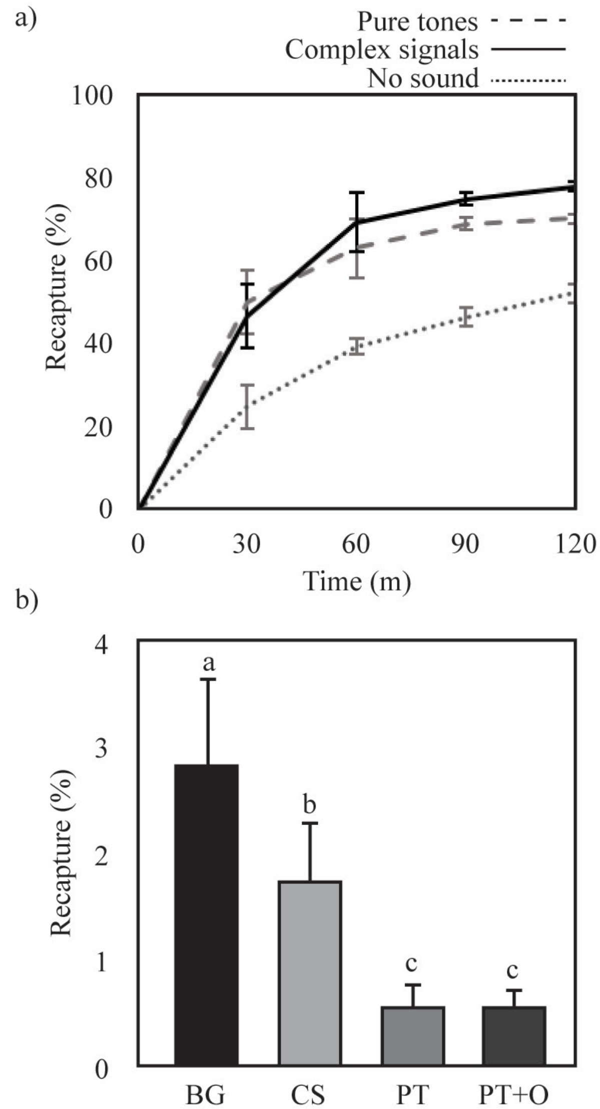

Figure 3. Capture effectiveness of the acoustic traps at attracting male mosquitoes under (a) indoor and (b) outdoor conditions. The traps used indoors broadcast no sound (dotted light gray), pure tones (dashed gray) or complex signals (continuous black). Mean and SEM are shown for the average number of mosquito recapture at 30, 60, 90 and 120 min. The lower panel shows the capture effectiveness under semi-field conditions of BG sentinel trap (BG) and acoustic traps broadcasting complex signals (CS), pure tones (PT) and pure tones plus odor cue (PT+O). Mean and SEM are shown for each treatment. Letters upon the bars indicate statistically different groups (p<0.05 [Fisher's LSD]).

traps (LSD test P>0.1 for every comparison).

When we evaluated the effectiveness of the acoustic stimuli and odor as lures for trapping males, only the type of stimuli associated with the trap influenced capture rates (GLM, $F_{9,11}$=4.33, P=0.04). Location of the trap ($F_{3,11}$=1.13, P=0.43), wind velocity ($F_{1,11}$=0.49, P=0.5), temperature ($F_{1,11}$=0.11, P=0.74), and UV index did not affect the experiment ($F_{1,11}$=5.02, P=0.04). Instead, the performance of the traps differed among treatments (GLM, $F_{3,11}$=8.25, P=0.003). Even though capture rates were low, the BG Sentinel trap (2.85 ± 0.69%) captured the highest number of males (Figure 3b). Among the acoustic traps, however, those broadcasting complex signals (1.75 ± 0.23%) recaptured a significantly higher number of mosquitoes than the traps with pure tones alone (0.56 ± 0.34%) or combined with the odor lure (0.57± 0.28%).

Overall, combining all experiments to analyze the effect of the environmental variables on the capture rates of the BG Sentinel trap, it was possible to determine that this trap was strongly affected by the intensity of ultraviolet light (UV index ; r=0.73, R^2=0.53, P=0.03 [Linear Regression]).

DISCUSSION

Our results showed that signals synthetized from recordings of individuals flying independently and male-female interactions were effective at attracting males under indoor conditions. Our findings also showed differences in attraction to lures depending on the specific acoustic features of the stimuli and the conditions of the place where the trap was set. This work contributes to the body of recent literature that is actively exploring venues to increase trapping efficiency of *Ae. aegypti*.

Under indoor conditions, complex signals and pure tones successfully increased capture rates of the traps. Under these conditions, our complex signals captured as many males as traps broadcasting pure tones. In accordance with a previous study showing that signals with multiple harmonics and frequency sweeps are more attractive for *Ae. aegypti* (Jakhete et al. 2017), our results also suggested that increased complexity of the signals did not deleteriously affect the capture rate. Indeed, our results show that it is possible to include male and female harmonics and frequency modulations to increase capture rates. Broadcasting just one min of high intensity complex signals every ten min was sufficient to capture ~80% of the mosquitoes released in the room. Using low intensities and exploring the synthesis of signals aimed to minimize discomfort to humans is critical due to the necessity of using traps for *Ae. aegypti* within houses. The presence of humans constrains the use of prolonged high intensity pure tones. Our study provides the first steps towards an exciting venue to further explore stimuli that integrate complex sounds and low playback rates without compromising capture rates.

The use of stimuli that mimic natural sounds also helps to overcome limitations of traps that need to be placed in environments, like houses, where visual and chemical distractors are common. Our indoor experiments were performed in a room full of objects with a broad variety of shapes and colors and still achieved capture rates similar to those from previous studies performed using acoustic lures in small, empty, indoor enclosures (Balestrino et al. 2016). Given that trapping effectiveness is strongly related to the capacity of mosquitoes to locate the lures among visual distractors (Ball and Ritchie 2010), our results show that acoustic signals with naturally-inspired lures can be rapidly located (~1 h) by mosquitoes even in the presence of abundant distractors.

Under semi-field conditions, stimuli consisting of high-intensity (90 dB) or intensity-fading (90-65 dB) pure tones performed poorly at attracting males. One factor that might have affected capture rates using these stimuli is the location of the traps. Acoustic traps have proved to be effective when positioned in dark areas, sheltered from wind, sunlight, and rain (Jakhete et al. 2017, Johnson et al. 2018), and our results are consistent with those from previous studies performed under open-field conditions (Stone et al. 2013, Balestrino et al. 2016). Thus, these findings support the observations that the conditions of the location where the trap is set are critical to reach acceptable capture rates of males. Studies that further examine the use of artificial shade and other types of shelters around the traps could provide further insights about whether the use of acoustic traps outdoors has potential.

The use of an odor bait to improve attraction for males under semi-field conditions was not successful. We did use, however, a commercially available lure (Biogents® skin odor) designed to attract females. Given that complex signals as the ones we broadcast in this study result from mosquito aggregations, hormonal attractants that mimic pheromones naturally produced in this type of context (e.g., Fawaz et al. 2014, Cabrera and Jaffe 2007) could increase capture rates. Further studies combining naturally produced acoustic and chemical signals are necessary to investigate the potentially additive effects of using lures that integrate multiple sensory modalities.

Our prototype trap had higher trapping effectiveness under indoor conditions. This higher performance in a closed room is probably due to the behavior of the males once they approach the trap. We observed males performing stereotypical flight patterns near the suction area of the trap, in response to both pure tones and complex signals. This behavior increases the capture probability as they keep flying near the suction area until they are captured. Under indoor conditions, it was possible to clearly observe this behavior by turning off the suction fan of the trap. This behavior, however, was not observed under semi-field experiments. We propose that differences between indoor and outdoor conditions, like the presence of wind and sunlight, prevented male mosquitoes from flying close to the suction area for long enough to be captured, which resulted in low capture rates. While these findings are not surprising given that it is well recognized that environmental factors can strongly influence mosquito capture rates (Lebl et al. 2013), the role of such variables at affecting swarm-like flying behavior are less understood.

The role of abiotic factors in affecting capture traps of mosquitoes is recognized across a variety of traps that use different types of lures and capturing mechanisms. For instance, field tests have shown that gravid *Aedes* traps (GAT) equipped with speakers broadcasting pure tones are able to catch the same number of males than BG Sentinel traps, when they are sheltered from wind, sunlight, and rain (Johnson et al. 2018). Our data also reveal a strong effect of UV light on the capture rate of BG Sentinel traps in particular. As *Ae. aegypti* has limited capacity to differentiate hue, they rely on contrast to locate and orient towards cues (Muir et al. 1992). An increase in UV index increases the conspicuousness of the trap funnels, which results in higher capture rate. This result highlights the relevance of visual cues on the effectiveness of traps. Future designs that combine visual, odorant, and sound attractants are likely to result in improved capture rates of traps.

Overall, our results suggest that complex acoustic stimuli that resemble mating signals can be used to design new surveillance tools for monitoring *Ae. aegypti* males or improve traditional strategies. There are, however, details that deserve further attention to enhance the performance of the traps. Sound pressure level and amplitude modulation of the signals should be investigated in further studies to design signals that maximize attraction while minimizing discomfort of residents. Combinations of long and short distance attractants using different sensory modalities will likely enhance attraction to the traps and result in more effective trapping strategies.

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