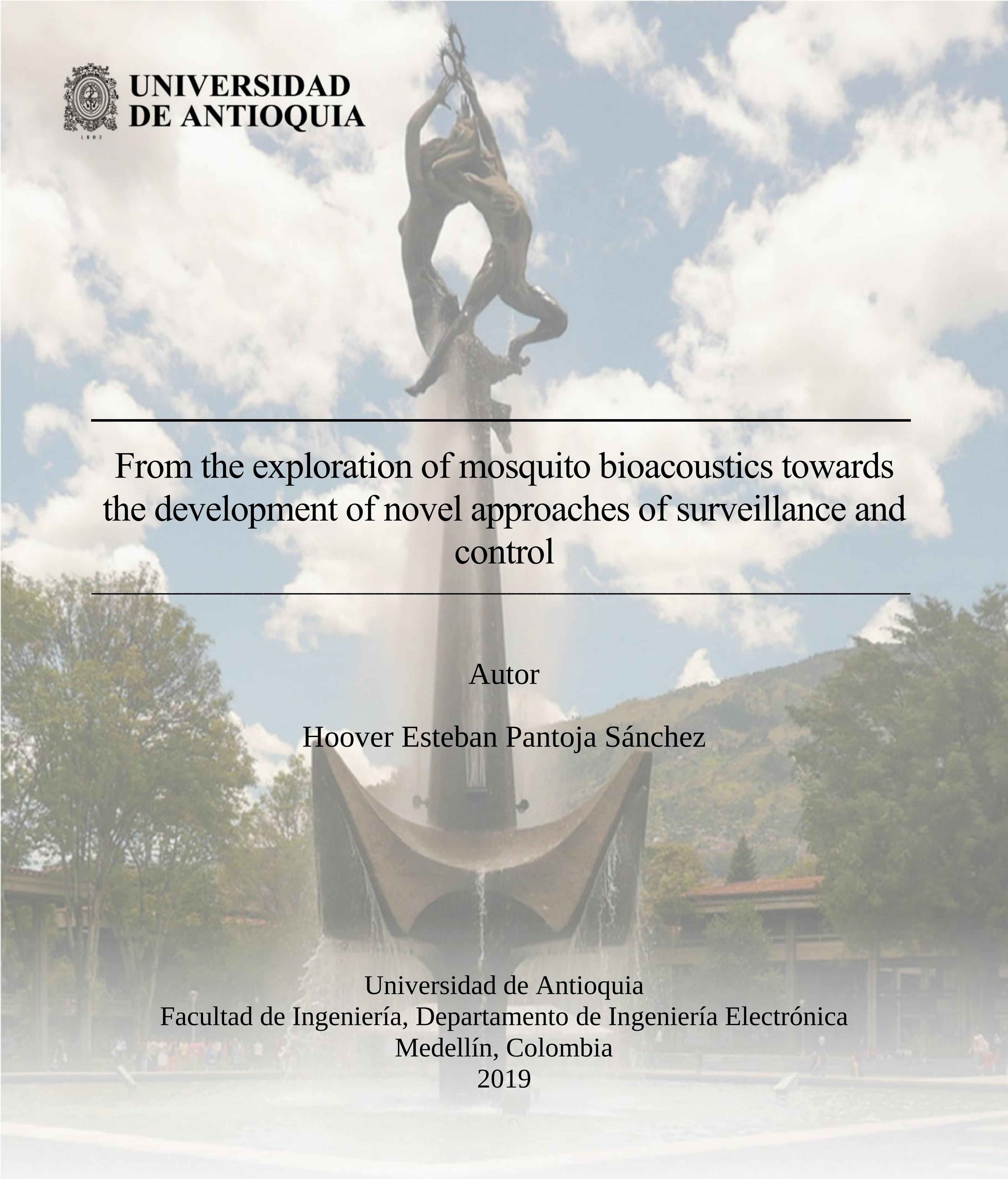

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From the exploration of mosquito bioacoustics towards the development of novel approaches of surveillance and control

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Trabajo de grado como requisito para optar al titulo de
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2019

Declaration

I declare that the thesis has been composed by myself and that the work has not be submitted for any other degree or professional qualification. I confirm that the work submitted is my own, except where work which has formed part of jointly authored publications has been included. My contribution is explicitly indicated below. I confirm that appropriate credit has been given within this thesis where reference has been made to the work of others.

The work presented in Chapter 2 was performed at PHYSIS laboratory of the Faculty of Medicine of University of Antioquia. Professor Juan Camilo Calderón Vélez actively participated in the standardization of the methods, the design of the experiments, analysis and discussion of results.

The work presented in Chapter 4 was previously published in *Parasites and Vectors* as *Precopulatory acoustic interactions of the New World malaria vector Anopheles albimanus (Diptera: Culicidae)* by *Pantoja-Sánchez, H., S. Gomez, V. Velez, F. W. Avila, and C. Alfonso-Parra*. This study was conceived by all of the authors. My contribution is indicated in the on-line version. (https://doi.org/10.1186/s13071-019-3648-8)

The work presented in Chapter 5 will be published in *The Journal of Vector Ecology (Vol 44 No 2)* as *A new approach to improve acoustic trapping effectiveness for Aedes aegypti (Diptera: Culicidae)* by *Pantoja-Sánchez, H., J. Vargas, F. Ruiz-Lopez, G. Rua-Uribe, V. Vélez, D. L. Kline, and X. E. Bernal*. This study was conceived by all the authors. As the first author, I participated in the design and performance of experiments, the analysis and discussion of results and the elaboration of the manuscript.

Hoover Esteban Pantoja Sánchez

10th October 2019

Abstract

Diseases transmitted by mosquitoes cause public-health problems worldwide. In the absence of cost-effective treatments, the impact of these diseases is mitigated mainly by controlling mosquito populations. As current control methods exhibit serious difficulties, optimized and sustainable solutions are required. Due to the relevance of acoustic signals in mosquito reproduction, acoustic-based surveillance and control strategies are promising. In this thesis, three possible research approaches are explored: 1) Studying mosquito bioacoustics. 2) Designing acoustic traps. 3) Recognizing mosquitoes by their flight-tones.

First, the study of mosquito bioacoustics provides valuable information to improve control strategies that rely on reproduction. Here, we study *Aedes aegypti* and *Anopheles (Nyssorhynchus) albimanus* bioacoustics from the receiver and the emitter perspective. By investigating audition from the receiver perspective, we describe general characteristics of the auditory organs of mosquitoes and evaluate the particular auditory response of each one of the studied species. Moreover, we establish similarities and differences between the species and address questions regarding mosquito sound reception. From the emitter perspective, we analyse the acoustic signals produced by tethered and free-flying mosquitoes, describing the effect of the recording protocol on the characteristics of flight-tones. Lastly, we use tethered and free-flying *An. albimanus* mosquitoes to uncover sex-specific acoustic behaviours related to reproduction and to establish a relationship between flight-trajectories and flight-tones.

Second, the use of traps to collect mosquitoes is one of the operational foundations of mosquito control programs. Here, we use the inherent ability of male mosquitoes to locate a sound source in order to develop a novel acoustic trap prototype. By using acoustic attractants synthesized from mosquito flight-tone recordings, promising capture rates are obtained under indoor and semi-field conditions.

Finally, using flight-tones to recognize mosquito species has proven to be a promising surveillance technic. In this thesis, we evaluate classification algorithms to differentiate species that share flight-tones with comparable frequency characteristics. Our results demonstrate that, by using the spectral information of flight-tones, it is possible to obtain a classification accuracy higher than 70 %, when classifying species with similar wingbeat frequency distributions.

Overall, this thesis combines basic and applied research oriented towards the study of mosquito bioacoustics. Our results contribute to the literature that is actively studying mosquito biology and constitute a step forward in the fight against mosquito-borne diseases. We hope the methods and the technology developed during the execution of this research will be used in the future to accomplish innovative research projects.

Keywords: mosquito, bioacoustics, flight-tone, audition, trap, classification

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Publications associated with this research

Pantoja-Sánchez, H., J. Vargas, F. Ruiz-Lopez, G. Rua-Uribe, V. Vélez, D. L. Kline, and X. E. Bernal. **2019**. A new approach to improve acoustic trapping effectiveness for Aedes aegypti (Diptera: Culicidae). J. Vect. Ecol. 44:2. Pages 216-222. DOI: https://doi.org/10.1111/jvec.12352

Pantoja-Sánchez, H., S. Gomez, V. Velez, F. W. Avila, and C. Alfonso-Parra. **2019**. Precopulatory acoustic interactions of the New World malaria vector Anopheles albimanus (Diptera: Culicidae). Parasit. Vectors. 12: 386. DOI: https://doi.org/10.1186/s13071-019-3648-8

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A mi Mona, porque volver al hogar y verte sonreír justifica cualquier esfuerzo.

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A mis profesores, porque me llevo una parte de su pensamiento, espero enorgullecerlos.

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A Camilo, porque mi creatividad es un reflejo de la tuya.

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List of abbreviations

ANN: Artificial Neural Network

MFCC: Mel Frequency Cepstral Coefficients

SVM: Support Vector Machine

SPL: Sound Pressure Level, re. 20 uPa above noise floor at 1 meter from the source.

WBF: Wingbeat Frequency

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Chapter 1: Introduction

Mosquitoes are one of the deadliest animals in the world. Genera *Aedes*, *Anopheles* and *Culex* are responsible for transmitting around 17 % of all infectious diseases, causing more than 700,000 deaths annually(WHO 2014). As there are no cost-effective treatments to eliminate the pathogens, the prevention of their transmission depends mainly on monitoring and controlling mosquito populations (WHO 2014). Despite the implementation of various control methods, which include insecticide spraying and the use of insecticide-impregnated bed nets, these interventions have not been sufficient in controlling mosquito-borne diseases (Paixão et al. 2018). As current surveillance and control strategies exhibit serious difficulties, more sustainable, effective and biologically based solutions are needed. Reproduction processes are one of the main targets to develop new mosquito surveillance and control strategies (Lees et al. 2014, Diabate and Tripet 2015, Childs et al. 2016). Releasing sterile mosquitoes into the wild to reduce the population size is an example of a control strategy that relies on the reproductive behaviour of mosquitoes (Alphey et al. 2010). In addition, reproduction-based lures have been used to develop novel surveillance tools (Diabate and Tripet 2015). Pheromones, visual and acoustic cues used by mosquitoes in different stages of reproduction are commonly used to create new traps and improve current ones (Bidlingmayer 1994, Pitts et al. 2013, Jakhete et al. 2017, Johnson et al. 2018). However, the success of such strategies is directly related to the available knowledge about species-specific mosquito reproductive behaviour.

In order to copulate, males and females use olfactory (Pitts et al. 2013, Fawaz et al. 2014) and acoustic signals (Robert 2009). Acoustic signals, in particular, play a major role in their reproductive behaviour. The sound produced by a mosquito wingbeat has been called flight-tone and it is used by mosquitoes to communicate with their conspecifics in different scenarios. A flight-tone is characterized by a fundamental frequency and several harmonics — multiple integers of the fundamental— up to at least 12 kHz (Arthur et al. 2014). As flight-tones are generated by the motion of the wings, the fundamental frequency matches the wingbeat frequency (WBF). Mosquitoes use flight-tones in different stages of reproduction like localization (Jackson and Robert 2006), coordination of their movement and mating (Aldersley and Cator 2019).

Reproductive behaviour is initiated by males swarming and acoustically interacting in active search for females (Assogba et al. 2013, Fawaz et al. 2014). As soon as a female reaches the swarm, a male orientates towards the female by using its flight-tone (Simões et al. 2016). Once they are close to each other, both perform different acoustic behaviours that influence the mating outcome (Simões et al. 2016, Aldersley and Cator 2019). Since mosquito mating behaviour is modulated by bioacoustics, sound plays a major role in their reproductive biology.

Due to the relevance of sound in mosquito reproductive biology, acoustic-based surveillance strategies have been developed. Several studies have explored the possibility of using sound as an attractant (Ikeshoji and Yap 1990, Stone et al. 2013, Johnson and Ritchie 2015, Balestrino et al. 2016, Jakhete et al. 2017, Johnson et al. 2018). Acoustic traps have been successfully used to capture males using pure-tones that mimic the WBF of females (Johnson et al. 2018). These devices, however, have not been extensively employed as a surveillance tool due to their low effectiveness at collecting female mosquitoes and the discomfort that pure-tones might produce to humans. Recognizing mosquitoes by their flight-tones is an additional approach now being implemented as a surveillance strategy (Reed et al. 2019). This technological advance constitutes the first step toward the development of novel automatic entomological surveillance tools (Potamitis and Rigakis 2016, Mukundarajan et al. 2017). One of the main challenges of acoustic-based species recognition is to accurately differentiate species with similar flight-tones. Developing acoustic tools for mosquito surveillance present several challenges described in the last two sections of this study. We believe, however, that a deeper understanding of the insights of the mosquito acoustic behaviour can lead to overcoming some of these challenges.

The present research integrates a fundamental component oriented to investigate mosquito bioacoustics and an applied component, centred on the development of novel acoustic approaches for mosquito control and surveillance. We decided to use species *Ae. aegypti* and *An. albimanus*, mainly for two reasons. First, both species are responsible for transmitting pathogens that cause severe public health issues around the world. Second, while *Ae. aegypti* is one of the most studied species worldwide, there is a scarcity of information on *An. albimanus* biology. In this thesis, therefore, *Ae. aegypti* was used as a reference to validate our methods and *An. albimanus* was used to generate fresh knowledge about mosquito bioacoustics. By combining engineering and biology, our intention is to expand the horizon of questions regarding mosquito bioacoustics.

[redacted]Background

1.1.1 Diseases transmitted by *Ae. aegypti* and *An. albimanus*

Female mosquitoes feed on blood to obtain the nutrients to complete egg development (Zhou et al. 2007). Consequently, mosquito-borne diseases are transmitted by the female’s bite. Among them, dengue fever and malaria are two of the most relevant diseases for public health. Due to the absence of reliable and cost-efficient vaccines, control programs concentrate their efforts on limiting the contact between humans and mosquitoes (WHO 2014). In the Americas, an average of USD $ 2.1 billion was expended on control and prevention of mosquito-borne diseases from 2000 to 2007 (Halasa et al. 2011). Specifically in Colombia, the institutional costs generated by the preventative actions to control dengue and malaria exceeded USD $ 300 million during the last decade (Padilla et al. 2010).

Aedes aegypti is the main vector of dengue, Zika and chikungunya viruses on the American continent. Dengue virus incidence has dramatically increased over the last decade, becoming one of the most important mosquito-borne diseases. An estimate of 50 to 100 million humans are infected annually and half of the world´s population is at risk by living in endemic countries. In addition, recent outbreaks of Zika and chikungunya affected more than 100,000 people in the Americas, increasing the relevance of this species (Paixão et al. 2018). In Colombia, *Ae. aegypti* is distributed in different regions situated below 2,302 m.a.s.l., inhabiting every department (Figure 1-1 a). Colombia recorded its largest dengue outbreak in 2010, generating more than 150,000 confirmed cases and 217 mortalities (Villar et al. 2015). Zika and chikungunya have recently emerged in Colombia. The first autochthonous chikungunya case was reported in September 2014 and the first case of Zika was reported in October 2015 (INS 2018). Since then, numerous cases of both viruses have been reported and the country is constantly at risk of new epidemic outbreaks.

Anopheles albimanus is one of the main vector of malaria throughout Central America, the northern portion of South America, including Colombia, and the Caribbean

(Montoya-Lerma et al. 2011). Despite its status as an important vector in the Americas, there exists a large gap in our knowledge about its behaviour, ecology and biology, turning the implementation of control strategies complicated. Malaria is caused by *Plasmodium* parasites that are transmitted through the bites of infected *Anopheles* females. Approximately half of the world's population is at risk of contracting malaria (WHO 2017), with children under five years old accounting for over two-thirds of malaria-related deaths. Although most lethal malaria cases are concentrated in Sub-Saharan Africa (Hay et al. 2004), numerous cases occur in South America (Recht et al. 2017). An estimated 12 million people live in malaria-endemic areas and are at risk of infection. In Colombia, *An. albimanus* is distributed mainly in different regions in areas near the Atlantic and Pacific coasts (Figure 1-1 b). In 2017, ~53,000 cases of malaria were reported in these regions.

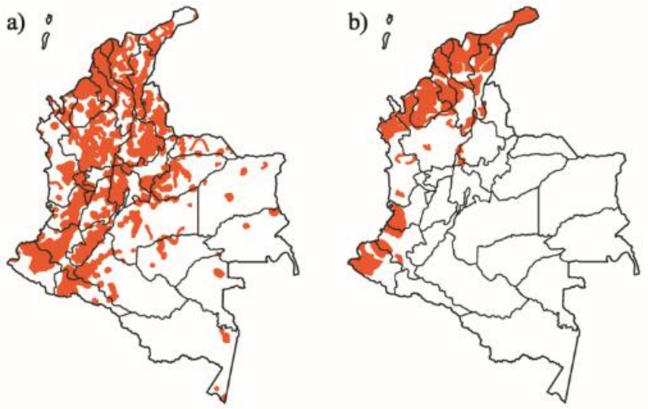

Figure 1-1. Distribution of (a) *Ae. aegypti* [35] and (b) *An. albimanus* [36] in Colombia. Orange marks in the map indicate zones inhabited by each one of the species.

1.1.2 Mosquito surveillance and control limitations

Mosquito-borne diseases control programs in Colombia are based on two control initiatives: Integrated Management Strategy (PAHO 2018) and Integrated Vector Management (WHO 2017). These strategies incorporate social mobilization at the community level as part of a wider strategy to control mosquito-borne diseases. In areas with active transmission, spraying insecticide to reduce the mosquito population is the main strategy to prevent transmission. However, this strategy is expensive, limited to specific regions of the country and promotes resistance when the same insecticide is frequently used (Recht et al. 2017). Insecticide control programs have not been cost-effective since 1960 when DDT programs supposedly eradicated *Ae. aegypti* from the Americas for 10 years (Recht et al. 2017).

Two alternative strategies are currently emerging to reduce *Ae. aegypti* and *An. albimanus* disease transmission. 1) The incorporation of the bacteria *Wolbachia pipientis* in *Ae. aegypti* stops the transmission of Zika, dengue and chikungunya viruses (Caragata et al. 2016). When *Wolbachia*-infected mosquitoes mate with wild-type mosquitoes, new infected generations are incapable of transmitting the pathogens. 2) Genetically modified males *Ae. aegypti* and *An. albimanus* produce non-viable offsprings impeded to survive adulthood (Alphey et al. 2010). Both strategies show great promise but their performance is directly related to the reproductive behaviour of mosquitoes and subsequently to mosquito bioacoustics.

In order to assess the performance of control and surveillance strategies, effective and cost-efficient tools are required to monitor and understand the dynamics of mosquito populations. Currently, trapping and the active search of adults are the predominant methods to estimate mosquito populations. These methods, however, have not sufficiently cost-effective (Sivagnaname and Gunasekaran 2012, Pepin et al. 2013, Osório et al. 2014, Petrić et al. 2014). Finding sustainable collection methods is one of the main challenges for countries affected by mosquito-borne diseases.

1.1.3 Targeting mosquito bioacoustics to develop new surveillance and control strategies

We identified three possible research areas to improve mosquito acoustic-based surveillance and control methods. 1) Understanding mosquito bioacoustics. 2) Designing acoustic traps. 3) Recognizing mosquitoes by their flight-tones.

1.1.3.1 Understanding mosquito bioacoustics: During courtship, different stereotypic acoustic behaviours have been proved to influence mating success. Yet, the influence of these behaviours on mate selection is not completely understood. Moreover, acoustic cues are also involved in male-male interactions and appear to be associated with *swarm formation* (Fawaz et al. 2014). The male-specific acoustic behaviour has been barely *explored in the context of control and surveillance, probably because males are not directly responsible for the transmission of pathogens. Understanding how females assess male fitness to select a mate and how males interact within a swarm, would constitute important steps towa*rds improving control strategies based on the releasing of biologically modified mosquitoes in the wild.

1.1.3.2 Designing acoustic traps: One of the operational foundations of vector control programs is the use of capture devices in order to monitor, in time and space, mosquito populations. Unfortunately, most traps use light as attractant, which renders them useless for diurnal species. Chemical baits (e.g. CO2) are used to increase capture rates, but the costs of implementing these traps increase substantially (Sivagnaname and Gunasekaran 2012). Therefore, in most cases, vector control organizations design their strategies using indicators based on the collection of immature stages of the vector (eggs, larvae and pupae). The contradiction between larval indicators and disease transmission, however, reveals the need for more accurate indexes that are representative of vector populations and virus transmission (Sivagnaname and Gunasekaran 2012, Pepin et al. 2013, Osório et al. 2014, Petrić et al. 2014).

A promising capture technique uses the innate attraction of males to the sound emitted by females (Johnson et al. 2018). Even though capturing males is preponderant for mosquito surveillance, acoustic traps have not been employed widely as a surveillance tool, probably due to their low effectiveness at collecting females. The low effectiveness at attracting females is surprising given their high hearing sensitivity, which is comparable to that of males (Su et al. 2018). Exploring the insights of mosquito bioacoustics might yield new ideas to design novel acoustic attractants for both males and females.

1.1.3.3 Recognizing mosquitoes by their flight-tones: Several studies have demonstrated that it is possible to recognise mosquitoes by their flight-tones (Chen et al. 2014, Potamitis et al. 2015, Mukundarajan et al. 2017, Kiskin et al. 2018, Sedda et al. 2019). Novel sound-based mosquito surveillance tools are now under-development (Reed et al. 2019). One of the main challenges, however, is to successfully differentiate species with similar flight-tones. Extracting new characteristics from flight-tones and generating new classification approaches is necessary to improve the performance of this kind of tools.

[redacted]Aim and objectives

1.2.1 Aim

To explore different targets of mosquito bioacoustics to improve surveillance and control strategies

1.2.2 Objectives

To characterize the auditory response of *Ae. aegypti* and *An. albimanus*

To characterize the acoustic behaviour of *Ae. aegypti* and *An. albimanus*

To design and evaluate acoustic attractants for males and females synthesized form flight-tone recordings

To determine the accuracy of the classification between species with similar flight-tones

[redacted]Document structure

The remainder of this work is divided into six sections. In Chapters 2, 3 and 4 we present an exploration of mosquito bioacoustics from the receiver (audition) and the emitter (flight-tone) perspective. In Chapters 5 and 6 we examine acoustic applications, such as traps, designed to improve mosquito surveillance strategies. Chapter 7 comprises a final conclusion and an analysis of the implications of this study to future mosquito surveillance and control strategies.

Chapter 2: Receiver perspective. Mosquito hearing and the challenges of understanding the role of sound in mosquito biology

[redacted] Introduction

Insects ears have generally been considered simple compared to those of vertebrates. Although anatomically appear to be simple, insect ears are remarkably elaborated (Fullard and Yack 1993, Hoy 1998, Albert and Kozlov 2016, Göpfert and Hennig 2016). Because of their small size, the time it takes for a sound wave to pass by an insect is extremely short. To compute the information in such a short-time scale, an extremely fast functioning of the neural system is required (Hoy and Robert 1996, Michelsen 1998). These facts constrain the design of insect auditory systems and therefore, innovative hearing designs have evolved in insects, in response to the physical challenges generated by their small size (Fullard and Yack 1993).

Morphologically, there are two basic forms of insect's ears: tympanal and flagellar. While tympanal ears are well known as they sense pressure waves like humans (Hoy and Robert 1996), flagellar ears are less understood (Schneider and Römer 2016, Shamble et al. 2016). The antennae of insects are a common example of flagellar ears, where sound-induced vibrations are transduced by mechanosensory cells situated at the base of the antenna (Johnston 1855), within the Johnston's Organ (JO). Although more than 95% of insect species have JOs (Kristensen 1981), specialization of this organ for hearing is remarkable among Diptera, in particular in the families Culicidae (Arthur et al. 2010, Pennetier et al. 2010, Simões et al. 2016, Lapshin and Vorontsov 2017), Chironomidae (Lapshin 2015) and Corethrellidae (Bernal et al. 2006, Bernal and de Silva 2015). The JO of culicid mosquitoes, for instance, contains around 15,000 mechanosensory cells (Boo and Richards 1975), a number comparable to the number of hair cells in the human cochlea. This characteristic illustrates the complexity of mosquito audition and the relevance of sound as part of their biology.

Sound reception and neural processing play a major role in the life of mosquitos. Acoustic signals are, for instance, at the core of their reproductive biology. Males swarm to attract females while sound mediates species recognition and mate choice. In these mating aggregations, a few to hundreds of males coordinate their flying patterns avoiding acoustic interference. Thus, males hear females in the proximity of the swarm. As soon as a female is detected, males track and chase the female until they intercept her. This behaviour is characteristic of many dipterans, including families of Culicidae (Gibson 1985, Fawaz et al. 2014, Hassan et al. 2014), Chironomidae (Ogawa and Sato 1993) and Choretrellidae (de Silva et al. 2015). While it has long been recognized that male mosquitos use acoustic signals to attract females (Johnston 1855), it was recently revealed that males and females interact acoustically displaying courtship-like behaviours, which ultimately are used by females to assess males (Cator and Harrington 2011a, Aldersley and Cator 2019). When a male and a female acoustically interact at a local scale, both modulate their wingbeat frequencies (WBFs) in response to the flight-tone of the other, finally converging in a shared harmonic (Cator et al. 2009, Warren et al. 2009). This reciprocal tuning has been called harmonic convergence and it seems to match specific characteristics of the mosquito audition system of mosquitoes (Simões et al. 2016, Aldersley and Cator 2019).

Although several species of mosquitoes use their antennae to sense and process acoustic signals associated with reproductive behaviours, some species of mosquitoes (Borkent and Belton 2006, Bartlett-Healy et al. 2008) and midges (Bernal et al. 2006) have evolved the ability to find and feed on anurans by using the frogs' mating call as a cue. Thus, in mosquitoes hearing obeys two fundamental purposes: foraging and communicating with conspecifics. These purposes entail several different auditory challenges such as recognizing or locating a sound source.

In this chapter, we review current knowledge about mosquito ears and analyse some of the physical challenges of their audition. Particularly we explore the sound source localization problem and the ability of mosquitos to sense an acoustic stimulus far from the source. Finally, we describe a method used to analyse the electrophysiological response of the JO and, by studying *Ae. aegypti* and *An. albimanus* audition, we discuss the relevance of understanding the insights of this exceptional sensory system.

[redacted] Johnston's organ (JO) and mosquitoes' active, non-linear audition

In 1855 Cristopher Johnston described for the first time a sensory organ in the second segment of the antenna (Johnston 1855). Since then, it has been accepted that the Johnston's organ is the key structure of the mosquito antennal ears. The antenna is responsible for transmitting sound-induced vibrations to the radially arranged mechanosensory neural units of the JO. Nanoscale vibrations are transduced by the JO into electrophysiological signals conducted by the antennal nerve to the brain (Göpfert et al. 1999, Göpfert and Robert 2001, Arthur et al. 2010, Su et al. 2018). Given the electrophysiological signals generated by the ear are transmitted through the antennal nerve by non-spiking mechanisms (Lapshin and Vorontsov 2017), it is possible to record continuous extracellular and intracellular electrophysiological signals from the nerve to analyse the frequency response of the JO. The existence of narrow-tuned auditory units in the mosquito JO and the brain (Lapshin and Vorontsov 2017) suggest they are capable of analyzing the spectral components of an acoustic stimulus.

Each mosquito JO contains a surprisingly high number of primary sensory neurons (~15000 in males and ~7000 in females), comparable to the number of hair cells of the human cochlea but in a volume ~100,000 times smaller (Boo and Richards 1975). The JO also contains efferent neurons driving the flagellum to enhance mosquito audition (Su et al. 2018). Males and females show an active power gain of specific frequency bands (Göpfert and Robert 2001, Lapshin and Vorontsov 2017, Windmill et al. 2018). Amplification is greater in males through spontaneous oscillations generated by the JO. In the absence of an acoustic stimulus, autonomous vibrations with a frequency close to the female's wingbeat frequency are detected in the antenna (Göpfert and Robert 2001, Lapshin and Vorontsov 2017). The autonomous vibration disappears in the presence of an acoustic stimulus with similar frequency. Behaving as a powerful narrowband amplifier, the autonomous vibration might be used to detect the faint female flight-tones.

Different studies of mosquito hearing have highlighted the non-linearity of the mosquito auditory system as a fundamental characteristic (Göpfert et al. 1999, Warren et al. 2009, Arthur et al. 2010, Lapshin and Vorontsov 2017, Windmill et al. 2018). During courtship, for instance, the male's antennae vibrate in response to simultaneous acoustic stimulation from its own flight-tone and those produced by the female. The interaction between both signals generates strongly amplified intermodulation distortion products. To better understand this phenomenon, here we simplify the mosquito hearing as a quadratic time-dependent system. Hence, the electrophysiological response of the antenna $y(t)$ is defined by Equation 1 where $x(t)$ is the input acoustic signal, α and β are constants that define the linear and nonlinear components of the system's and $q(t)$ is additive Gaussian white noise.

$$y(t) = \alpha x^2(t) + \beta x(t) + q(t) \text{ [Eq 1]}$$

If we analyse the mosquito response during a male-female interaction by only considering the fundamental frequency, the input signal $x(t)$ is defined by Equation 2 as the sum of two pure-tones where S is the amplitude, f is the fundamental frequency and ϕ is the phase of each one of the pure-tones. The fundamental frequency of the male and the female can be represented by sinusoids with frequency f_1 and f_2 respectively.

$$x(t) = S_1 \sin(2\pi f_1 t + \phi_1) + S_2 \sin(2\pi f_2 t + \phi_2) \text{ [Eq 2]}$$

In this scenario, the response of the system is defined by Equation 3 where C is considered a sustained deflection, normally observed in the extracellular response of the mosquito JO.

$$\begin{gathered} y(t) = C + q(t) + \alpha S_1 \sin(2\pi f_1 t + \phi_1) + \alpha S_2 \sin(2\pi f_2 t + \phi_2) \\ -\beta \frac{S_1^2}{2} \cos(2\pi(2f_1)t + 2\phi_1) - \beta \frac{S_2^2}{2} \cos(2\pi(2f_2)t + 2\phi_2) \\ +\beta S_1 S_2 \sin(2\pi(f_1 - f_2)t + (\phi_1 - \phi_2)) + \beta S_1 S_2 \sin(2\pi(f_1 + f_2)t + (\phi_1 + \phi_2)) \text{ [Eq 3]} \end{gathered}$$

If we analyse the components of the response, it is possible to differentiate three different sections. First, the linear response (y_l(t)), associated with the male and the female fundamental frequency (Equation 4).

$$y_l(t) = \alpha S_1 \sin(2\pi f_1 t + \phi_1) + \alpha S_2 \sin(2\pi f_2 t + \phi_2) \text{ [Eq 4]}$$

Second, harmonics (y_h(t)) associated with the fundamental frequency of the male and the female (Equation 5).

$$y_h(t) = -\beta \frac{S_1^2}{2} \cos(2\pi(2f_1)t + 2\phi_1) - \beta \frac{S_2^2}{2} \cos(2\pi(2f_2)t + 2\phi_2) \text{ [Eq 5]}$$

And third, intermodulation products (yi(t)) generated by the interaction between the two tones (Equation 6).

$$\begin{aligned} y_i(t) = {} & \beta S_1 S_2 \sin(2\pi(f_1 - f_2)t + (\phi_1 - \phi_2)) \\ & + \beta S_1 S_2 \sin(2\pi(f_1 + f_2)t + (\phi_1 + \phi_2)) \end{aligned}$$
[Eq 6]

By modelling the system as a quadratic polynomial sequence, second-order intermodulation products are produced. Several studies have shown that male's JO is tuned to intermodulation products (f_1-f_2 and f_1+f_2) rather than to the female tone (f_2) by itself (Simões et al. 2016). It has been proposed that the JO autonomous vibrations would allow a resting mosquito to keep, at least partially, the advantages of the two frequency acoustic signal processing (Lapshin and Vorontsov 2017).

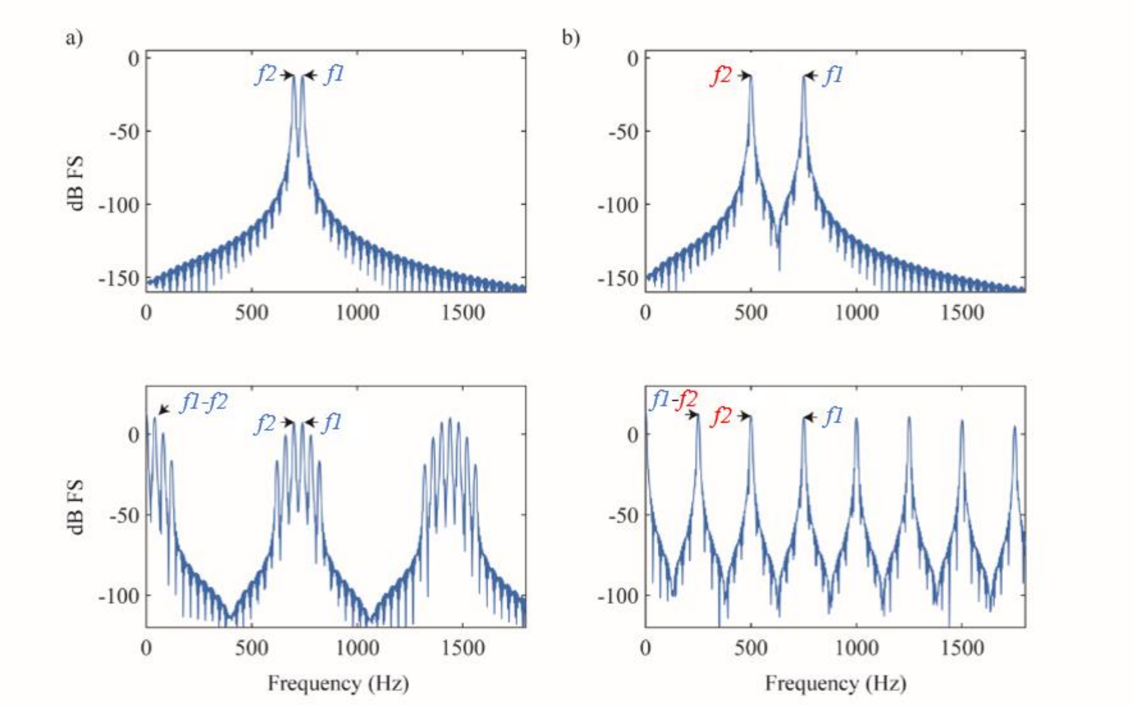

Figure 2-1. Five order polynomial simulation of the JO response. Top panels show the input of the system during a (a) male-male and a (b) male-female interaction. Bottom panels show the non-linear response of the JO. Arrows in panels indicate pure-tones (*f1* and *f2*) and one intermodulation product (*f1* - *f2*). Red and blue letters in the figure indicate female and male tones, respectively. Simulations concord with experimental data reported by Arthur et al. (2010) and Simões et al. (2016).

Increasing the complexity of the model by including higher-order terms generates a good approximation to the electrophysiological response of the JO. A comparison of the modeled neural response to flight-tones produced during male-male (Figure 2-1a) and male-female (Figure 2-1b) interactions, shows that intermodulation produces major changes in the response due to the magnitude of the frequency difference between tones. In a similar way, when mosquitoes fly, the sound broadcast by their own wingbeat generates intermodulation products in response to additional acoustic stimuli. Mosquitoes hearing organs, however, should have evolved to avoid interference generated by their own flight-tones. Despite the obvious challenge of hearing while producing sound, little is known about how the role of intermodulation products in the auditory organs of mosquitoes. Increasing our knowledge about mosquito non-linear audition is essential to understand the role of flight tones as communication signals.

[redacted]Near-field and Far-field reception

In *Ae. aegypti*, their hearing organs are capable of perceiving sound as far away as 10 m from the sound source (Menda et al. 2019). The hearing organs of both males and females can sense intensities as low as 31- and 43-dB SPL, respectively. This finding demonstrates that an antenna is sensitive enough to detect the vibrating air particles far from the source where the intensity is highly attenuated. The nanoscale sound-induced motion of the air particles drives the mosquito antenna oscillation, which generates the electrophysiological response of the JO.

The thinner the sensory structure is the more sensitive to acoustic stimulus. For instance, thin structures such as a spider silk, effectively detect acoustic vibrations in far-field as its motion is governed by the forces of the air (Zhou and Miles 2017). Although the physic characteristics governing the motion of the mosquito antenna are still unknown, the evidence of males and females detecting acoustic signals in far-field generate new questions about the role of sound in mosquito biology. If mosquitoes hear acoustic cues far away from the source, they may use audition in less-known scenarios. Human-biting mosquitos, for instance, could use the human voice to detect their hosts and have a blood-meal (Menda et al. 2019). Studying the role of hearing in relation to host-location or predator-avoidance will likely provide valuable insights regarding mosquito auditory organs and their natural history.

[redacted]Locating a sound-source

Two different contexts have been described in which mosquitoes need to orientate towards a sound source. First, male mosquitoes and midges of numerous species pursue females using their flight-tones(Robert 2009). Second, females of some species recognize and orientate towards anurans by using their calls (Bernal et al. 2006, Bartlett-Healy et al. 2008). Using this knowledge, it has been possible to develop acoustic traps to attract and capture males or females of several mosquito species (Bartlett-Healy et al. 2008, Johnson et al. 2018). Both scenarios present the same question. How do mosquitoes and midges use their antennal hearing to locate the source of the sound? Although at first glance, it is a simple question, the small size of mosquitoes creates physical constrains that turn the solution complicated (Lapshin 2015).

To determine the direction of an incoming soundwave, animals use binaural audition. By interpreting amplitude or time differences between the signals detected by both ears, animals are able of locating a sound source. These differences decrease proportionally to the size of the head of the animal and therefore, locating a sound source is particularly challenging for small animals (Michelsen 1998). A wave passing across any animal generates two kinds of Interaural Differences: time (Interaural Time Difference ITD) and intensity (Interaural Intensity Difference IID). Both types of cues decrease proportionally to the size of the head of the animal.

A thought exercise illustrates the acoustic challenge confronted by mosquitoes when locating sounds. Human ears are placed ~18 cm apart from each other. Because of this distance, when a sound source is located at one side (90° angle from the midline), the time it takes for a sound wave to pass from one ear to the other is ~500×10^{-6} s (maximal ITD). Compared to the maximal ITD, our neural system can binaurally resolve ITDs in the range of 4×10^{-6} to 8×10^{-6} s. This range represents a deviation by the sound source of 1 - 2° from the midline (Ricketts 2000). If we use the same approach to analyse mosquito audition, due to the distance between the pair of antennae (~0.05 cm), the maximal ITD generated is 1.5×10^{-6} s. Even if mosquitoes compute the information as fast as humans do, it is highly unlikely that their neural system can solve such short ITDs. Similarly, in mosquitoes, the IID is nearly zero as no mass absorb the sound energy between the antennae. Assuming the reception of a 500 Hz pure tone, a comparison between the ITDs detected by the ears of mosquitoes and humans reveals the difference. While in the case of humans the ITD is high compared to the period of the soundwave (~500×10^{-6} s compared to 2000×10^{-6} s, Figure 2-2a), in mosquitoes, this difference is minimal (1.5×10^{-6}s compared to 2000×10^{-6}s, Figure 2-2b).

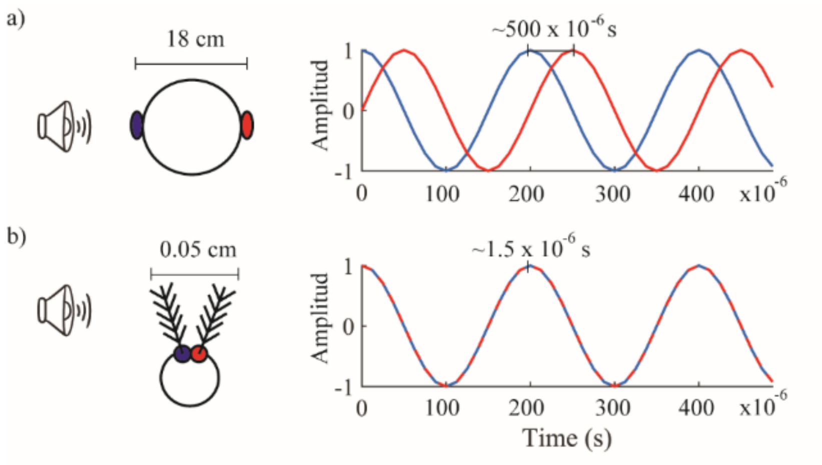

Figure 2-2. Sketch of the maximum Interaural Time Difference (ITD) generated by a 500 Hz pure-tone broadcast from one side (90°) of (a) a human and (b) a mosquito head. While the time difference between the signals (right panel) received by the left (blue) and the right (red) ear is significant for a human, it is not discernable by a mosquito.

The radial symmetry of the JO is considered inherently directional (Lapshin and Vorontsov 2017). Recent findings, however, have suggested that the distribution of sensory units in the JO might be insufficient to provide the required accuracy for determining the angular coordinates of a sound source (Lapshin and Vorontsov 2019). Consequently, the question about how mosquitoes locate a sound source remains unsolved.

[redacted] Studying mosquito audition: the auditory response of *Ae. aegypti* and *An. albimanus*

To date, most of the morphological, physiological and behavioural information about mosquito audition has been obtained from species of genera *Toxorhynchites*, *Aedes*, *Culex and Anopheles*. There is a scarcity of information, however, about species of the New World like *An. albimanus*. In this study, we briefly explore the audition of *Ae. aegypti* and *An. albimanus* by implementing a methodology to record the electrophysiological response of different JO sensory neurons. Due to the large amount of information available about *Ae. aegypti* audition, we use this species as a reference to standardize the methods used here. We use the same method to explore the JO response of *An. albimanus*. Here we describe the range of frequency that males and females can detect, analyse the non-linear characteristics of the JO and compare the characteristics of the response between the two studied species.

2.5.1 Mosquito preparation

Mosquitoes were obtained from the colonies maintained at Programa de Estudios y Control de Enfermedades Tropicales (PECET) —Universidad de Antioquia, Medellín, Colombia. The specimens used for experimentation were reared and maintained in a walk-in climate chamber at 27°C and 80% relative humidity (RH) under a 12:12 light: dark photoperiod.

An *Ae. aegypti* colony was established with field-collected eggs from different locations in Medellín, Colombia. In the laboratory, the eggs brought from the field were placed in trays filled with 500 ml of unconditioned tap water. Mosquitoes *Anopheles albimanus* were obtained from a colony of mosquitoes originally collected in Santa Rosa, Bolivar, Colombia in 1995. Eggs were collected in 473 ml cups filled with dH_2O. Upon hatching, larvae were individually transferred to trays (~60 per tray) containing 500 ml of dH_2O and fed daily with 20 mg of fish food (Tetracolor, Tetra). Pupae were individually transferred to 15 ml vials to ensure virginity. Upon eclosion, adults were separated by sex, transferred to 4 l plastic cages, and held in same-sex groups until experiments commenced. Mosquitoes had access to 20% sucrose *ad libitum*. We used 4-7-day-old adults for experiments.

2.5.2 Neural response recording protocol

Recordings were obtained at PHYSIS Laboratory of the Faculty of Medicine at University of Antioquia (Medellín, Colombia). Following Lapshin and Vorontsov (2013), we used a puller machine (Pul-1000, WPIInc) to develop $\sim 14M\Omega$ electrodes from glass capillaries (19100F-4, WPIInc). Table 2-1 indicates the protocol used to generate the specific resistance.

Table 2-1. *Pulling protocol*

Step	Heat	Force	Distance	Delay
1	550	200	1.70	10
2	350	120	2.00	0

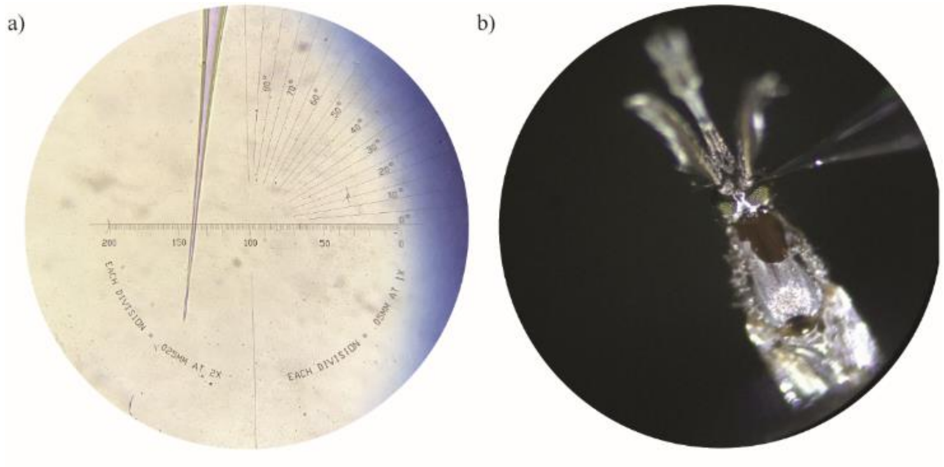

Figure 2-3. (a) Sharp glass electrodes are introduced in the antennal nerve by (b) puncturing the soft cuticle below the pedicel.

Sharp glass electrodes (Figure 2-3a) were filled with 0.15 M sodium chloride to transmit the extracellular response and to minimize the damage caused by the solution flowing out from the electrode out to the neurons of the antennal nerve. The electrode was operated by a micromanipulator (MHW-3, Narishige) until the tip reached the soft cuticle at the lower edge of the pedicel (Figure 2-3b). The electrical activity was pre-amplified by a head stage (CV-7B, Molecular Devices), using a toothless alligator clip as the electrical reference (Figure 2-4a). An AC microelectrode amplifier (MultiClamp 700b, Molecular Devices) was used to amplify the analogue signal obtained from the head stage. Signals

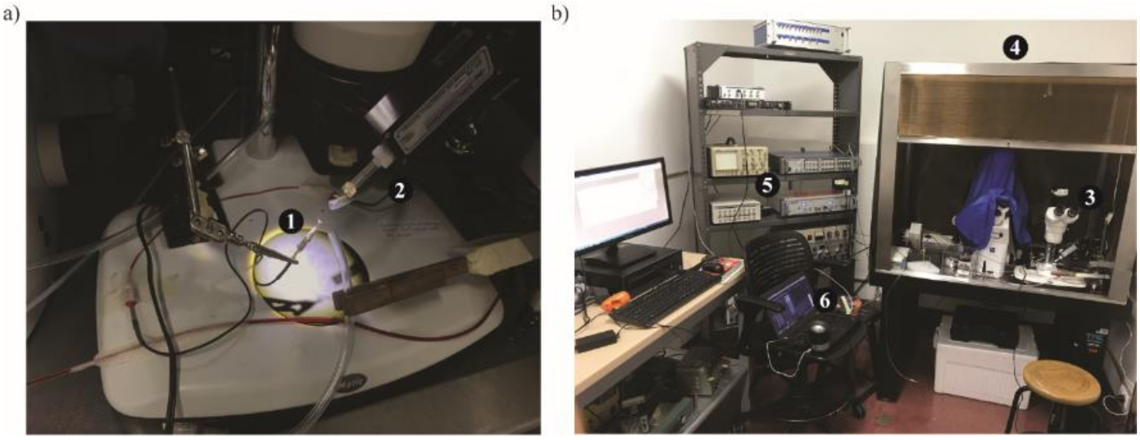

Figure 2-4. Electrophysiological recording setup. Sharp (a) glass electrodes are introduced in the (b) antennal nerve of the mosquito to record the electrophysiological response of the JO. The electrode motion is monitored with a (c) stereomicroscope until the tip of the electrode reaches the puncture spot. Recordings are performed inside a (d) Faraday's cage placed upon a vibration-isolating air table. (1) Mosquito immobilized by the wings with a toothless alligator clip. (2) Preamplifier head stage. (3) Stereoscope within the Faraday's cage. (4) Faraday's cage and vibration-isolating air table. (5) Amplifier and digitizer. (6) Computers to visually monitor the time and frequency response of the JO and play acoustic stimuli.

were amplified by using a feedback resistor of 500 MΩ and filtered using a low pass filter (4 kHz). Finally, the amplified signals were digitalized (PowerLab 8/35, ADInstruments) at a frequency rate of 10 kHz. Recordings were performed inside a Faraday's Cage coupled to the electric ground of the building to reduce the electromagnetic noise (Figure 2-4b). A vibration-isolating air table was used to minimize vibrations from the building and external environment.

2.5.3 Characterization of the JO response

A speaker (Z50, Logitech) set 2 m away from the mosquito, broadcast the acoustic stimuli with an overall intensity of approximately 80 dB SPL. To find the antennal nerve response, pulses of 280 Hz were played every second during 0.34 s (Figure 2-5a). This frequency was used since it matches the wingbeat frequency difference between males and females and therefore, the response is maximized (Simões et al. 2016). The electrode was manipulated until we reached the antennal nerve. From the onset of the electrical contact of the electrode with the cuticle, we recorded a "traditional" response of the JO with an emphasized second harmonic (Figure 2-5b). By monitoring the spectrogram of the antennal nerve signal, we identified the first four harmonics (Figure 2-5c). During recordings, a velocity particle microphone was used to monitor the acoustic stimuli at 1 cm from the antenna. Recordings from the microphone were used to guarantee that the recorded response was generated exclusively by the JO. Once the response was identified, a 20 to 2000 Hz linear chirp was used to evaluate the frequency range of the JO response (Figure 2-5d). At the end of each experiment, the electrode was removed from the antennal nerve and the same stimuli were played to verify results were not an artefact produced by electromagnetic interference.

We recorded the electrophysiological response of the JO of seven males of each one of the studied species. In concordance with previous studies (Lapshin and Vorontsov 2013), we obtained signals with different characteristics when recording from nearby sites within the antennal nerve. Variations in the response were generated, presumably, by different principal sensory neurons. Males of both species exhibited two kinds of response distinguished by the presence or absence of autonomous vibrations. As we show previously, a non-linear response included several harmonics but in most of the recordings, it was possible to identify until the third harmonic.

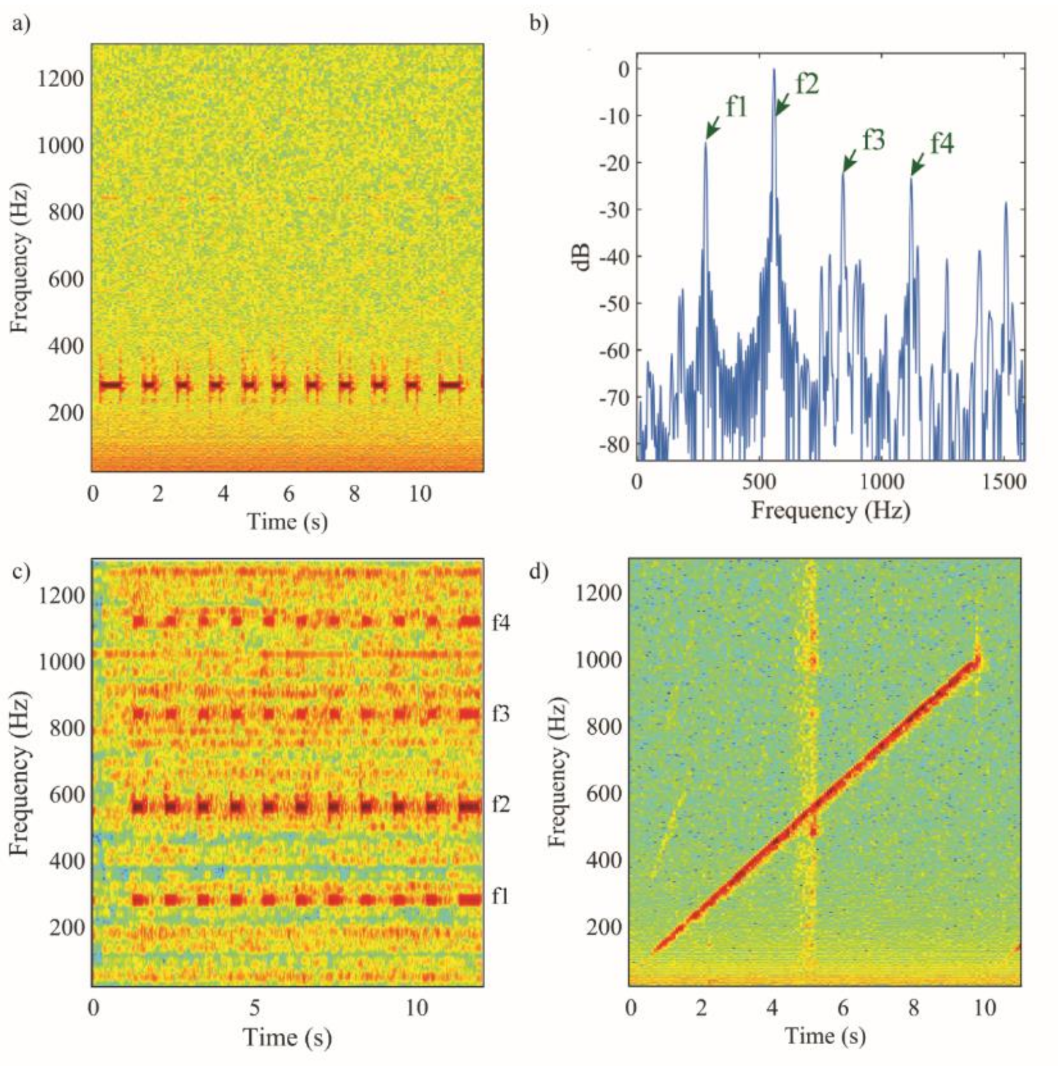

Figure 2-5. **(**a) Spectrograms of 280 Hz pulses used to search the antennal nerve. (b) The power spectrum of response recorded from the antennal nerve reveals an emphasized second harmonic and (c) the spectrogram confirms the non-linear characteristics of the system. Besides the fundamental frequency (f1), higher harmonics (f2 tof4) appear in the recorded signal. c) Once the electrode is positioned in the right position, (d) a linear chirp (20 to 1000 Hz) is used to establish the frequency range of the JO response.

Five males *Ae. aegypti* and three males *An. albimanus* exhibited no autonomous vibrations. In the absence of autonomous vibrations, the JO of *Ae. aegypti* and *An. albimanus* responded to tones of 150 and 610 Hz (Figure 2-6a) and to tones of 150 and 500 Hz (Figure 2-6b), respectively. The presence of autonomous vibrations, however, revealed significant differences between the species. In *Ae. aegypti* we identified a narrow-band vibration in the range from 390 to 480 Hz (mean at 430 Hz) entrained by tones with similar frequencies (Figure 2-6a right panel). While the second harmonic produced by the autonomous vibration was evident, we could not detect harmonics associated to the stimulus in this frequency range. In presence of an autonomous vibration, the intermodulation products generated by the stimuli and the autonomous vibration were easily detected. On the other hand, *An. albimanus* exhibited a wide-band autonomous vibration (Figure 2-6b right panel). This band was approximately 250Hz wide and normally founded in a range between 450 and 750 Hz.

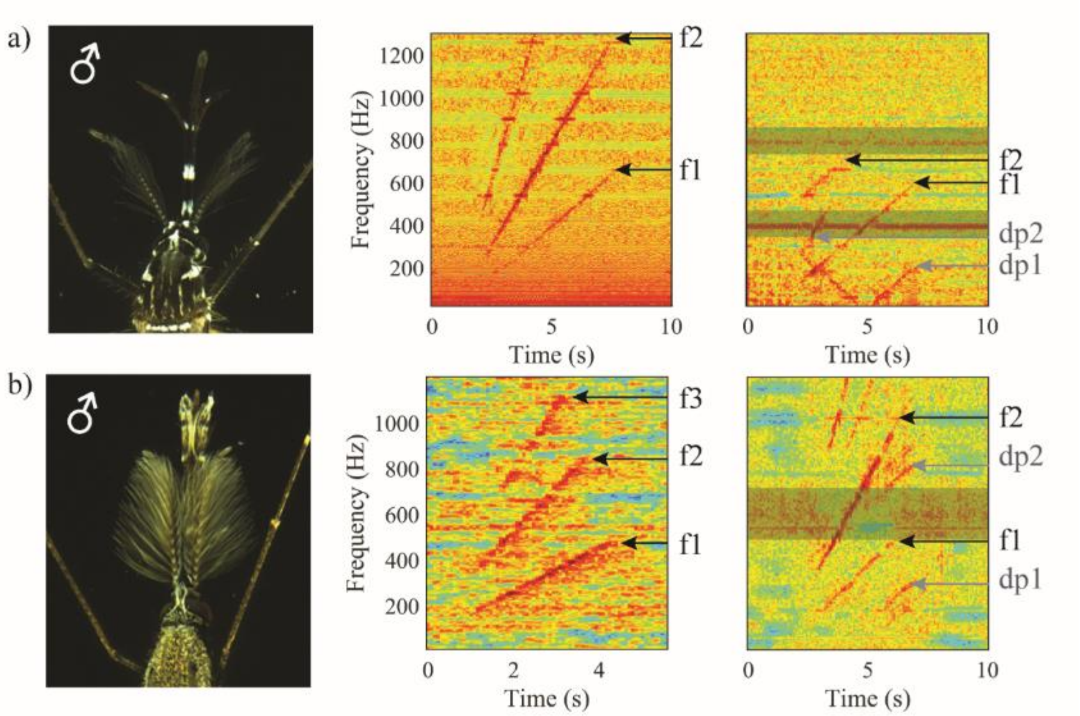

Figure 2-6. Comparison of the JO's response between males (a) *Ae. aegypti* and (b) *An. albimanus*. The left panel shows pictures of males' heads of both species. In the pictures, morphological differences between species can be observed. The middle panel shows the electrophysiological response of both species in the absence of autonomous vibrations. The response is characterized by a fundamental frequency (f1) and higher harmonics (f1-f3). The right panel shows an analysis of the response in the presence of autonomous vibrations (highlighted by green transparent rectangles). The harmonics of the response (f1-f2) and the distortion products (dp1-dp2) generated by the difference between the stimulus and the autonomous vibrations are indicated.

Autonomous vibrations considerably changed our perception of the response of the JO of *An. albimanus*. The second harmonic associated with the stimulus was amplified when it reached frequencies within the autonomous vibration band. On the contrary, the first harmonic associated with the stimulus disappeared when it reached frequencies within the autonomous vibration band. Intermodulation products generated by the autonomous vibration and the first harmonic appeared in the response. This mechanism might increase the auditory range of *An. albimanus* at least by 150 Hz.

The JO response of females was less intense and consequently it was more difficult to detect higher harmonics. Even though we did not detect autonomous vibrations in females, there are differences between species. From five *Ae. aegypti* females recorded, we detected responses to acoustic stimuli between 150 and 570 Hz (Figure 2-7a). While *Ae. aegypti* females exhibited a "traditional" JO response with an emphasized second harmonic, the response of *An. albimanus* female was different. In this species, from 6 females, we detected higher harmonics in response to low stimuli with frequencies between 180 and 250 Hz (Figure 2-7b). At higher frequencies, approximately until 550 Hz, the response was linear and matched the stimulus.

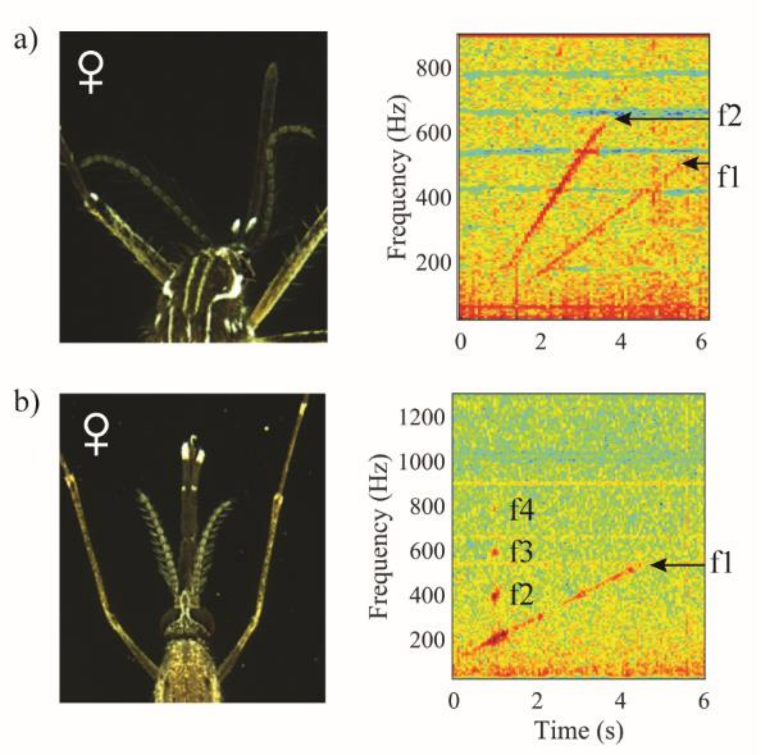

Figure 2-7. Comparison of the JO's response between females (a) *Ae. aegypti* and (b) *An. albimanus*. The left panel shows pictures of the head of each species. In the pictures, morphological differences between the species can be observed. The right panel shows the electrophysiological response of both species. The response is characterized by a fundamental frequency (f1) and higher harmonics (f1-f3).

[redacted]Discussion and contribution to the research field

Even though mosquito audition has been widely studied, describing the JO response is challenging due to the high complexity of the cell structure of the organ. In this study, we aimed to briefly characterize the range of frequency that generates an electrophysiological response of males and females of *Ae. aegypti* and *An. albimanus*. By using glass electrodes, here we describe the audition range and analyse nonlinearities exhibited by the JO of males and females. Despite the similar audition range, we found substantial differences in the JO response between species. While results regarding *Ae.*

aegypti are in concordance with previous findings, to our knowledge, this is the first study addressing *An. albimanus* audition. Although additional experiments are necessary to understand the implications of the characteristics of the auditory response of *An. albimanus*, we hope this study and the methods standardized here will lay the groundwork to perform new experiments and keep expanding the knowledge about this species.

In agreement with previous studies, we found the JO of *Ae. aegypti* males and females responds to tones between 150 and 600 Hz. (Menda et al. 2019). In males, we also found autonomous vibrations similar to those that have been described in other mosquito species (Arthur et al. 2010, Lapshin and Vorontsov 2017). These vibrations are generated by efferent neurons and they seem to work as a narrow band amplifier of specific frequencies (Su et al. 2018). It has been proposed that autonomous vibrations modulate the reception of female flight-tones while males are flying and resting. Here, we demonstrate that in the presence of autonomous vibrations, intermodulation products are generated by the interaction between stimuli and autonomous vibrations. This result suggests that, besides amplifying specific frequencies, autonomous vibrations might help males to retain the audition characteristics in the absence of their own flight-tone.

Even though *An. albimanus* exhibited an auditory frequency range comparable to *Ae. aegypti*, the characteristics of the response are less common. In this species, males exhibited a wide-band autonomous vibration. This vibration, however, amplified the second harmonic of the response. Once the first harmonic reached a frequency close to the autonomous vibration it was no longer distinguishable; distortion products appeared instead. Although the implications of this trait in *An. albimanus* audition need to be further explored, the result of this study suggests that the range of audition may increase by means of the distortion products generated by the interaction between stimuli and autonomous vibrations. The JO response of females was also different. Interestingly, we found that stimuli with frequencies higher than 250 Hz elicited a linear response. The consistency in the result among the replicates and the presence of higher harmonics in response to low frequency tones suggest that the response of *An. albimanus* females is different. Additional studies are required to uncover the particularities of the auditory organs of this species.

As the acoustic behaviour of mosquitoes is directly related to their mating success, their auditory system must have evolved to overcome the physical challenges produced by their small size and the interference generated by the sound produced by their own wingbeat. We know these limitations have shaped an antennal ear with singular characteristics. The active, continuous and non-linear response of mosquito ears are examples of such characteristics. Understanding mosquito audition is a key requirement to uncover the traits that modulate their acoustic behaviour.

In this chapter, we summarize relevant aspects of mosquito audition. We also present to the reader unanswered questions and describe the methods used to address the questions from a physiological perspective. Ultimately, we aim to provide an overview of the role of sound in mosquito biology from the receiver perspective. In the next chapter, we analyse, from the emitter perspective, the acoustic signals emitted by mosquitoes and evaluate the effect of the recording protocol in the characteristics of such signals.

Chapter 3: Emitter perspective. Flight-tone characterization and the effect of the recording protocol and mosquito size on the wingbeat frequency

[redacted] Introduction

Mosquito flight produces an easily identifiable acoustic signal known as buzz or flight-tone. The movement of the wings generates a tone at the frequency of the wingbeat. For mosquitoes, the sound emitted by their wingbeat is more than a by-product of locomotion; it is a communication signal. Several species of mosquitoes actively use their flight-tones to communicate with conspecifics during male-male and male-female interactions (see Chapter 4). Moreover, flight-tone differences among species may contribute to reproductive isolation and consequently, speciation (Pennetier et al. 2010).

The particular characteristics of flight-tones have been described in detail (Arthur et al. 2014). Flight-tones are composed by a fundamental frequency equivalent to the WBF and higher harmonics — integer multiples of the fundamental (Arthur et al. 2014). While the acoustic signals emitted from the left and the right of the mosquito are in phase, the signals emitted from the front and the back and from above and below are out of phase. The phase relationship among harmonics is complicated and no clear patterns have been detected. Due to the complexity of the phase characteristics of flight-tones, most of the information available relies on frequency.

In order to study mosquito communication from the emitter perspective, two different methods have been implemented to record flight-tones. 1) By recording tethered mosquitoes, it is possible to obtain high-quality recordings. The immobilization restricts mosquito movement and presumably leads to unnatural modifications in the acoustic behaviour. Although changes in the WBF have been attributed to the immobilization, the real effect of this procedure is not completely understood and contradictory results have been reported in different species (Simões et al. 2016, Villarreal et al. 2017). 2) Recording free-flying mosquitoes allows the acquisition of information in a more realistic context but the low intensity of flight-tones constrains this approach. Hence, in order to record free-flying mosquitoes, a sound-proof arena with limited dimensions is required.

Temperature and mosquito size also affect flight-tones, especially the WBF. While temperature increases the WBF consistently among species (Mukundarajan et al. 2017, Villarreal et al. 2017), the effect of size is not completely understood. Size affects the WBF of *An. gambiae* (Cator et al. 2010) but it does not affect the WBF of *Ae. aegypti* (Villarreal et al. 2017). In this chapter, we evaluate protocols used to record flight-tones of tethered and free mosquitoes. We also compare *Ae. aegypti* and *An. albimanus* flight-tones and discuss the effect of the recording protocol on their WBF. Finally, we estimate the effect of size on the WBF of both species.

[redacted] Methods

The colony of mosquitoes used in this experiment is described in Chapter 2 (Section 2.4.1.). Flight-tones were recorded inside a soundproof chamber made of acoustic neoprene, fibreglass and drywall. Two different protocols were standardized as follows.

3.2.1 Tethered mosquitoes

Mosquitoes were anaesthetized on ice and tethered to a human hair using a cyanoacrylate-based adhesive (Super Pega Infinita, Medellin, Colombia). The hair was attached on one end to an insect pin while the opposite end was attached to the pronotum (dorsal section of the thorax) as described by Cator and Harrington (2009a). Before recording, the position of the hair was checked to ensure natural wing movement **(Supp. Video 3-V1)**. To record flight-tones, mosquitoes were placed approximately 1 cm above a single particle velocity microphone (Figure 3-1a). When necessary, the flight was triggered by blowing or by gently moving the legs of the individual. To record flight-tones, a particle velocity microphone (NR-23158-000, Knowles, Itasca, IL, USA) was placed 1 cm below the mosquitoes (Figure 3-1 b). Microphone signals were amplified and digitalized by a USB audio interface (M-Track Quad Four-Channel Audio, M-Audio). Mosquito flight-tones were processed using Matlab® (2016a) at a sample rate of 11025 Hz/24 bits.

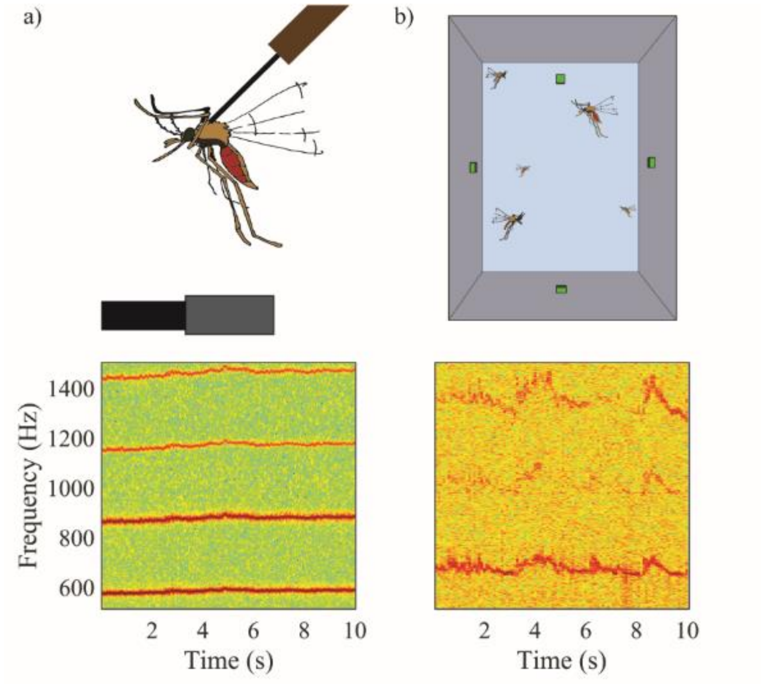

Figure 3-1. Recording protocol sketch (top) and spectrogram examples (bottom) of (a) tethered and (b) free-flying mosquitoes. Spectrograms exhibit the frequency components of male *An. albimanus* flight-tone.

We recorded 150 males and 150 females *Ae. aegypti* and 78 males and 116 females *An. albimanus.* Since flight-tones are harmonic sounds (Arthur et al. 2014), a sinusoidal-harmonic model was used to extract the WBF information of the recorded signals. From the spectrogram (Fast Fourier Transform (FFT) length of 4096 points, hamming window of 80 ms and 50% overlapping), we extracted the WBF by tracking the lower frequency peak of the spectrogram that persisted for at least 1 s and fulfilled two conditions: between consecutive windows, the frequency and power did not change more than ±30 Hz and ±10 dB FS, respectively.

3.2.2 Free-flying mosquitoes

Audio and video recordings of free-flying mosquitoes were performed in an experimental arena (20 × 20 × 25 cm^3 transparent plastic box). The experimental arena contained four electret microphones (Knowles FG-23329-C05), three placed on the side and back walls and one on the bottom of the box (Figure 1-1b). Microphone signals were amplified by an operational amplifier (INA128, Texas Instruments) and digitalized by a 16-channel digital-analogue converter (779676-01, National Instruments) at a sample rate of

11025/16 bits. For video recording, a webcam (Logitech HD Pro Webcam C920) was placed 30 cm in front of the arena to follow mosquito movement.

We recorded 30 individual males and females of each species flying within the experimental arena. Signals from each of the four microphones were used to extract frequency components of the flight-tones. By obtaining the cross-spectrum among the microphones (FFT length of 4096, window length 0.08 s, 50% overlapping) (Swami et al. 2001), we generated a high order spectrogram to analyse the signals regardless of the distance between mosquitoes and microphones. The WBF distribution and range of modulation was determined.

3.2.3. Statistical analysis

T-tests were used to compare frequency distributions. Multifactor ANOVAs were used to assess the effect of tethering and sex on the WBF. Residuals of t-tests and factorial models were tested for normality, homogeneity of variance and independence using Shapiro-Wilks, Bartlett and Durbin-Watson tests, respectively. Reports from statistical analyses include the test statistic, degrees of freedom and p value (e.g. Multifactor ANOVA, F_{df_effect,df_error}, p).

[redacted]Results

3.3.1 Effect of tethering on the WBF

There was a significant difference in the WBF between tethered and free mosquitoes (Table 3-1). In both species, males and females increase their frequency when flying free.

Table 3-1. *Comparison of size and WBF between tethered and free-flying Ae. aegypti and An. albimanus.*

		Wing length (mm)	**WBF (Hz)**		
			Tethered	**Free**	**t_test**
Ae. aegypti	♂	1.89 ± 0.02	684.27 ± 52.39	751.00 ± 50.59	$t_{1,178}$=-6.02, p<0.01
	♀	2.34 ± 0.02	500.63 ± 40.65	567.10 ± 58.96	$t_{1,178}$=-18.03, p<0.01
An. albimanus	♂	2.77 ± 0.02	524.11 ± 63.73	713.76 ± 3.16	$t_{1,106}$=-9.03, p<0.01
	♀	2.89± 0.02	368.91 ±3 4.28	430.34 ± 28.48	$t_{1,144}$=-15.00, p<0.01

Male: ♂. Female: ♀

The effect of the tethering, however, was dependent on the species. Males and females *Ae. aegypti* increased their WBF to a similar extent when flying free (Figure 3-2a). On the contrary, the effect of tethering was more evident in males *An. albimanus* than in females (Figure 3-2b).

The spectrograms of tethered and free-flying mosquitoes reveal differences in the extent of modulation of the WBF (Figure 3-1). In order to test the effect of tethering on the modulation range, we analysed the spectrogram of 30 tethered and 30 free-flying for males and females. We used therefore tethering and sex as factors. Males and females exhibited shorter WBF modulation ranges when tethered (Multifactor ANOVA, *Ae. aegypti*: $F_{1,116}=80.15$ $p<0.01$; *An. albimanus*: $F_{1,116}=32.13$ $p<0.01$). The effect of tethering was similar for both males and females *Ae. aegypti* and therefore, no effect of sex (Multifactor ANOVA, $F_{1,116}=0.15$ $p=0.69$) or an interaction between tethering and

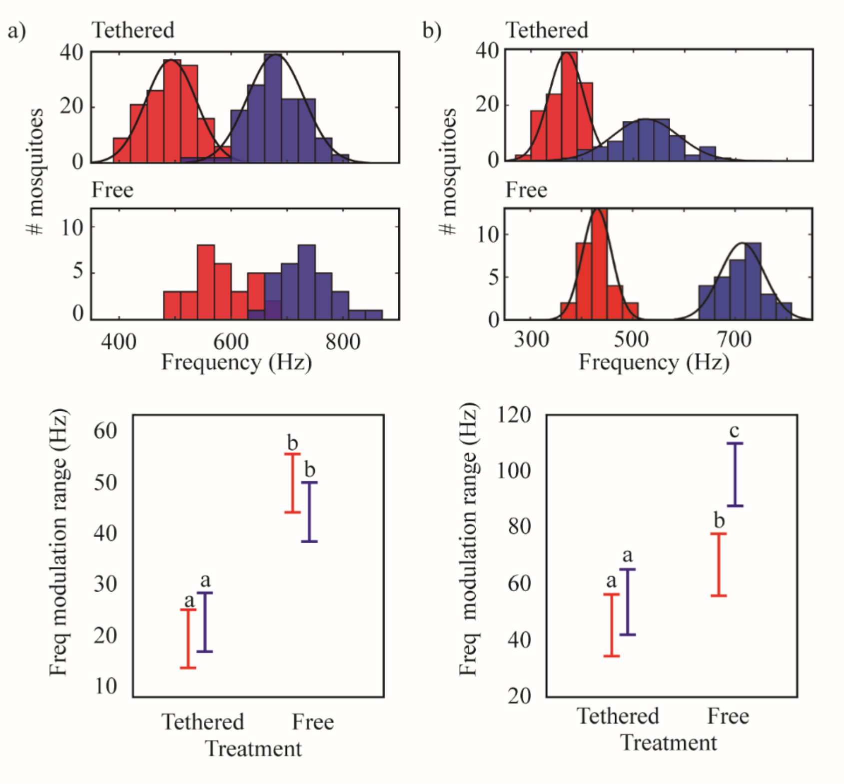

Figure 3-2. WBF distributions (top) and range of modulation (bottom) of (a) *Ae. aegypti* and (b) *An. albimanus*. Males and females are indicated by colours blue and red, respectively. Vertical bars denote 0.95 confidence intervals and letters indicate significant differences for a post hoc Tukey test with 0.95 confidence.

sex (Multifactor ANOVA, $F_{1,116}$=2.20 p=0.14) was detected on the modulation range (Figure 3-2a). In *An. albimanus*, however, there was a significant effect of sex (Multifactor ANOVA, $F_{1,116}$=13.79, p<0.01) and an interaction between sex and tethering (Multifactor ANOVA, $F_{1,116}$=5.31, p=0.02, Figure 3-2b).

3.3.2 Comparison between species

Males and females *Ae. aegypti* broadcast higher frequencies than *An. albimanus* in both conditions, tethered (t-test; ♂: $t_{1,223}$=-20.44, p<0.01; ♀: $t_{1,264}$=24.18 p<0.01) and free-flying (t-test, ♂: $t_{1,58}$=2.52, p=0.01, ♀: $t_{1,58}$=13.76 p<0.01). This difference is associated with the size difference between species (Cator and Zanti 2016). By measuring the wings of males and females, we determined that *Ae. aegypti* are, as expected, smaller than *An. albimanus* (Table 3-1).

3.3.3 Effect of size on the WBF

There was no intraspecific relationship between size and WBF of *Ae. aegypti* (Figure 3-3a) and *An. albimanus* (Figure 3-3b). In males, there is no association between and size in both species — *Ae. aegypti* (Linear regression, R^2<0.01, p=0.94) and *An. albimanus* (R^2<0.01, p=0.576). In females, although there is a significant relationship between size and WBF, the effect is week in both species — ♀*Ae. aegypti* (Linear regression, R^2<0.04, p<0.01) and ♀*An. albimanus* (R^2=0.03, p=0.03). Between species, as indicated in the previous section, the effect of size is significant (Figure 3-3c). Lower WBFs are associated with bigger interspecific sizes in both males and females.

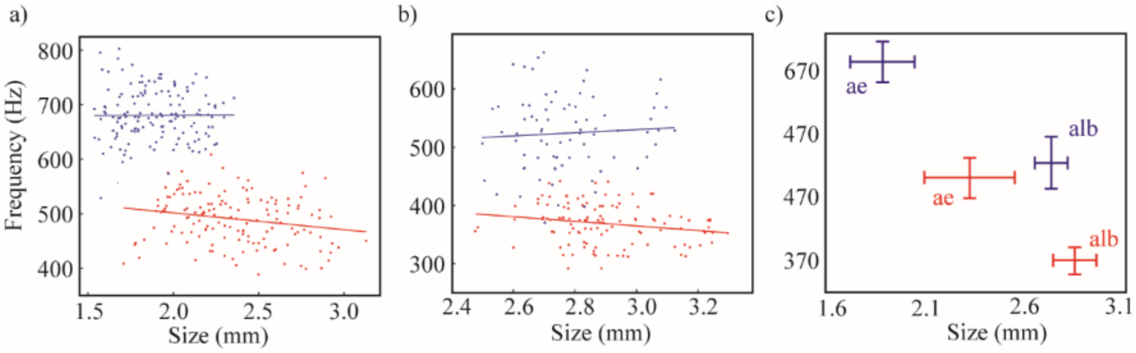

Figure 3-3. Relationship between size and WBF of (a) *Ae. aegypti* and (b) *An. albimanus*. Blue and red colours indicate males and females measurements, respectively. Points indicate an individual measurement and lines indicate the linear interpolation between size and WBF. There was no relationship between size and WBF. A (c) comparison between species shows the interspecific size is inversely related to the WBF. Vertical and horizontal bars indicate the WBF and size interquartile range of *Ae. aegypti* (ae) and *An. albimanus* (alb).

[redacted] Discussion of results and contribution to the research field

Here we demonstrate a significant effect of tethering on the flight-tones of two different mosquito species. Even though tethering generates lower WBFs in both *Ae. aegypti* and *An. albimanus*, the impact of immobilizing a mosquito depends on the species. On the contrary, the intraspecific size does not affect the WBF of tethered males nor females. Independently of the size, mosquitos broadcast a wide range of WBFs. Size differences among species, however, are associated with variations in WBF (Belton and Costello 1979). In this case, size and WBF are inversely related. Understanding the impact of these two characteristics, tethering and size, on mosquito flight-tones is essential to address new questions about their biology and to improve acoustic surveillance and control strategies.

Tethered insects have been used to study locomotion (Ribak et al. 2017), migration (Minter et al. 2018) and wing movement (Thomas et al. 2004), studies that have demonstrated that tethering leads to unnatural flight behaviours. One explanation could be that tethered individuals are restricted to a horizontal flight path and in addition, they do not support their own body mass. These factors may produce an inaccurate view of natural flight (Dudley 2018). As flight-tones are linked to motor function (Thomas et al. 2004), our results show that restricting motion alters two flight-tone characteristics: the WBF and the ability to modulate it. Although a decrease in the WBF linked to tethering has been reported for *Culex quinquefasciatus* (Simões et al. 2016), midges (de Silva et al. 2015) and locusts (Kutsch and Stevenson 1981), previous reports have shown there is no difference in WBF of tethered and free-flying *Ae. aegypti* females when assays were performed at the same temperature (Villarreal et al. 2017). On the contrary, although our assays were performed at the same temperature, we found that tethering impacted *Ae. aegypti* and *An. albimanus* WBF.

The impact of size on mating calls has been largely studied. In a wide range of species, these two characteristics are correlated and used by females to assess a mate. In mosquitoes, however, the relationship between flight-tones and size is not completely understood. Studies in tethered *An. gambiae*, have shown there is a direct relationship between size and WBF (Cator et al. 2010). Bigger males and females of *An. gambiae* broadcast higher WBFs. On the contrary, the impact of size in female *Ae. aegypti* WBF is minor during free-flight (Villarreal et al. 2017). Our results demonstrate size has no effect on tethered *Ae. aegypti* nor *An. albimanus* WBF, when recorded at the same temperature. Both males and females broadcast a wide range of frequencies without regard to the size of their bodies. Even though we found no relation between size and WBF of tethered mosquitoes, this relation deserves attention. As flight-tones are linked to motion (Thomas et al. 2004), studying the dynamics of flight-tones rather than the WBF as a single feature is an interesting approach.

While intraspecific size does not affect the WBF, differences in the size between species are linked to differences in the WBF (Belton and Costello 1979). Males and females of bigger species broadcast lower WBFs. This is common among species of genera *Aedes, Culex and Anopheles*. In our study, as *Ae. aegypti* is smaller than *An. Albimanus* and they, therefore, broadcast higher WBFs.

Overall, in this chapter, we contribute to uncover the effect of the recording protocol on mosquito flight-tones. Free-flying mosquitoes increase their WBF (~66- 190 Hz) depending on the sex or the species. We also provide evidence of no relationship between intraspecific size and WBF. Bigger species, however, broadcast lower WBFs. These results suggest both recording protocols can be used to comprehend different traits of mosquito acoustic behaviour. In the next chapter, we use the two protocols to characterize acoustic behaviours displayed during male-male and male-female interactions.

Chapter 4: Precopulatory acoustic behaviours of the New World malaria vector *An. albimanus* (Diptera: Culicidae)

[redacted]Introduction

Despite the status of *An. albimanus* as an important vector in the Americas, there exists a large gap in our knowledge of the behaviour, ecology and biology of this species, making the development and/or improvement of control methods difficult. Much of our understanding of anopheline reproduction is inferred from studies in *An. (Cellia) gambiae*, whose post-mating reproductive biology differs substantially from *An. (Nys.) albimanus* (Mitchell et al. 2015).

Mosquito capacity of transmitting disease is dependent on biological factors that include survival and population density, traits directly related to successful reproduction (Kramer and Ciota 2015). Thus, the processes of reproduction offer promising targets to control mosquito vectors and, by extension, the diseases they spread. Reproduction requires males and females to successfully locate each other, interact and mate. In several *Anopheles* species, reproduction begins when males form a swarm (Zawada et al. 2018). Although swarms have been characterized in African anopheline species (Charlwood et al. 2003, Manoukis et al. 2009, Diabaté et al. 2011, Hassan et al. 2014), swarming behaviour has not been thoroughly investigated for New World species. In African vectors such as *An. funestus*, *An. gambiae* and *An. arabiensis*, swarm formation begins when a few males begin to fly simultaneously; additional males rapidly join the flight, increasing the swarm size and density (Zawada et al. 2018). At the initiation of swarm formation, males fly in a non-specific circular motion before forming a more tightly patterned, cohesive group. Once formed, females penetrate the swarm to locate a suitable mate. Swarms are typically formed at dusk and at differing heights above the ground; swarm specific markers, on the ground or on the horizon, appear to determine swarm formation (Howell and Knols 2009). However, the nature of swarming in the wild, and female mate selection in this setting, are not completely understood.

Acoustic signals play a major role in *Aedes* (Cator et al. 2009, 2011), *Culex* (Warren et al. 2009) and *Anopheles* (Cator et al. 2010, Pennetier et al. 2010) species for the localization, recognition and attraction of opposite-sex individuals. However, male-male acoustic interactions during swarming have not been described for *Anopheles* species and, to the best of our knowledge, pre-copulation male-female interactions during courtship and copulation have not been examined in New World anopheline species. When measuring the WBF of two tethered, closely located *Ae. aegypti* males, both frequency convergence and frequency divergence behaviours are observed (Aldersley et al. 2016, 2017), showing how variable such interactions can be. Frequency divergence has also been reported between pairs of tethered *Culex quinquefasciatus* males (Warren et al. 2009). Recently, cohesive acoustic behaviour between multiple tethered *Ae. aegypti* males have been observed (Aldersley et al. 2017)—males in groups exhibit similar flight-tones, a phenomenon more evident in larger groups (Aldersley et al. 2017). Thus, characterization of male-male acoustic interactions may be key in understanding swarm formation and swarm cohesion, although additional study is required.

Studies describing acoustic interactions between opposite-sex mosquitoes are more numerous. In several species, female flight-tones act as a mating call that attracts males of the same species (Charlwood and Jones 1980, Belton 1994, Gibson et al. 2010). In experiments with tethered *Ae. aegypti*, *C. quinquefasciatus* (Warren et al. 2009), and *An. gambiae* (Cator et al. 2010, Pennetier et al. 2010), males and females modulate their WBF to match each other during courtship, a phenomenon known as harmonic convergence. Such convergence does not happen in the WBF of the flight-tones but instead in a shared harmonic. The ability of males to converge may indicate an indirect benefit for females, as the offspring resulting from converging pairs have increased mating success in *Ae. aegypti* (Cator et al. 2011b). Flight-tones and interactions of free-flying *Ae. aegypti* in the field have also been described (Cator et al. 2011a).

Here, we characterized the flight acoustics in *An. albimanus* and examined pre-copulatory acoustic interactions. During courtship in free-flight, we were unable to observe harmonic flight-tone convergence. However, we detected that during mate rejection, females increase their WBFs significantly faster than those that mate. Finally, through audio and visual analysis, we show that male groups in free-flight change flight trajectories and match flight-tones during a stereotypic, patterned flight, which could be suggestive of swarm-like behaviour in *An. albimanus*. Our results represent important first steps toward dissecting the subtle, pre-mating behavioural interactions that occur in this important malaria vector.

[redacted]Methods

The colony of mosquitoes used in this experiment is described in Chapter 2 (Section 2.4.1.). Virgin of 4-7 days old mosquitoes were used for the experiments. The sample size is indicated by letter “n” in each one of the experiments.

4.2.1 Tethered mosquitoes

4.2.1.1 Audio recording set up (see Chapter 3 section 3.2.1.).

4.2.1.2 Experimental procedures. Using tethered individuals, male-female interactions (couples) were recorded to determine if *An. albimanus* display the pre-copulatory behaviour of harmonic convergence (Cator et al. 2009). One mosquito was placed 1cm above one microphone and kept stationary. A tethered individual of the opposite sex, placed ~1 cm above a second microphone, was brought in and out of the near field of the first, stationary mosquito for intervals of 10 s with 5 s of rest between intervals. After repeating 3 times, the positions of the individuals were switched. Wing length of males and females were measured as previously described (Heuvel 1963) to estimate the individual size. All experiments were done at 26 ± 2°C. Tone analysis was performed using recordings that had >10 s of continuous flight.

4.2.1.3 Audio signal analysis. Since mosquito flight-tone has been described as a harmonic sound (Arthur et al., 2014), a sinusoidal-harmonic model was used to extract the WBF information of the recorded signals. From the spectrogram (Fast Fourier Transform (FFT) length of 4096 points, hamming window of 80 ms and 50% overlapping), we extracted the WBF by tracking the lower frequency peak of the spectrogram that persisted for at least 1 s and fulfilled two conditions: between consecutive windows, the frequency and power did not change more than ±20 Hz and ±10 dB FS, respectively. For individual flights, we analysed WBF mean and WBF range of both sexes. For male-female interactions, we identified harmonic convergence as in Aldersley et al (2016). Comparing the extent of frequency modulation and frequency distribution prior to and during interactions, we identified events with an active modulation of the WBF in response to the conspecific flight-tone. To label convergence events as positive or negative, we used a time-varying fundamental frequency ratio between females and males (♀WBF/♂WBF). For instance, a ratio of 0.667 means that the female's third harmonic is converging with the male's second harmonic. We set a positive convergence as an event of duration greater than 1 s, in which the convergence ratio was within an interval of tolerance ±1.5%. The time between individual introduction and the onset of convergence was noted as latency. The analysis was performed using Matlab® (2016a).

4.2.2 Free mosquitoes

4.2.2.1 Audio and visual recording set up (see Chapter 3 section 3.2.2.)

4.2.2.2 Experimental procedures. Three types of recordings were performed: 1) We recorded individual males and females inside the arena (n=30 for each sex). 2) Male-female acoustic interactions were recorded by introducing two males into the arena followed by one female. Flight-tones were continuously recorded until mating was observed or the individuals stopped flying. Mosquitoes were removed after a successful mating. Successful copulations were verified by dissecting females to determine insemination status (i.e. presence/absence of sperm in spermathecae). A mating attempt was counted as successful if a couple locked genitalia for more than 5 s and sperm was transferred to the female. An attempt was considered a rejection when the male and female genitalia did not come into contact or the female did not allow the male to lock genitalia. As females often rejected courting males, we recorded 167 total mating attempts from 42 different females. Recordings from nine females were discarded, as it was not possible to clearly identify the frequency components of each sex. 3) Acoustic interactions of male groups were recorded by introducing 8 mosquitoes into the arena 5 hours before recording started. Although 8 mosquitoes were used in every experiment, only groups of two, three and four mosquitoes flying simultaneously were analysed. For each experiment, recordings (2.5 h in length) were analysed in 10 min segments. In some instances, multiple recordings were made from the same group of mosquitoes. All recordings were performed at 26±2°C between 16:00 and 18:30, corresponding to the 2 h prior sunset in order to guarantee enough light for visual recordings.

4.2.2.3 Audio signal analysis. Signals from each of the four microphones were used to extract frequency components of the flight-tones. By obtaining the cross-spectrum among the microphones (FFT length of 4096, window length 0.08 s, 50% overlapping) (Swami et al. 2001), we generated a high order spectrogram which was used to analyse the signals continuously, regardless of the distance between mosquitoes and microphones. Depending on the interaction analysed, we extracted different frequency features from the spectrograms: 1) for male and female individual flight, the WBF distribution and range was determined. 2) For male-female interactions, we visually selected relevant steps of mating and analysed the WBF ratio (♀WBF/♂WBF) prior to mating and changes in frequency displayed during the interactions. 3) For male groups, we measured the average difference between the highest and the lowest WBFs, referred hereafter to as frequency difference.

4.2.2.4 Video analysis. Audio recording from the microphone integrated to the camera was used to synchronize audio and video. We used a 1 kHz pure-tone to synchronize the camera and electret microphones. Once audio and video were synchronized, we extracted 5 s video fragments from recordings in which interaction was observed. Each fragment was segmented into individual frames and each frame converted to grayscale and adjusted through the equalization of its histogram to improve the contrast of the image. Finally, for each frame, X and Y coordinates of each mosquito were obtained manually to reconstruct flight trajectories using Matlab® (2016a). Trajectories were represented by ellipses following Gibson (1985) and characterized to differentiate behaviours quantitatively. The area of the ellipses was calculated to represent the area covered by the movement of each mosquito. The average distance among the centroids of each ellipse to the others was calculated to assess the extent of the aggregation of the group. Finally, the loop period of each trajectory was calculated to assess the periodicity of the movement, as in (Gibson 1985).

4.2.3 Statistical analysis

Male-female interactions during free-flight trials were assessed using the non-parametric Mann-Whitney U test. The frequency difference broadcast by male groups was evaluated using multifactor analysis. As factors, we evaluated the number of mosquitos flying and whether males were displaying swarm-like flight patterns. Normal distributions were compared by using t-tests. Residuals of t-tests and factorial models were tested for normality, homogeneity of variance and independence using Shapiro-Wilks, Bartlett and Durbin-Watson tests, respectively. To determine differences between the characteristics of flight trajectories, the non-parametric Mann-Whitney U test was performed. Results are presented as mean ± SEM and the sample size is indicated by letter "n" in each one of the experiments. Reports from statistical analyses include test statistic, degrees of freedom and p value (e.g. Multifactor ANOVA, F_{df_effect,df_error}, p). In the case of nonparametric tests, we indicate the sample size of each one of the distributions.

[redacted]Results

4.3.1 Harmonic convergence between tethered mosquitoes

We observed harmonic convergence in 41 of 52 male-female couples (78.8%). By analysing the ratio of the female WBF with respect to the males', we observed two distinct groups where convergence was observed: in 21 couples (40.4%), the females third harmonic converged with the males second (Figure 4-1, left panel), while in 20 cases (38.5%), the female's fourth harmonic converged with the males third (Figure 4-1, right panel). The average time of convergence was 2.00±0.81 s and the response latency ranged from 4 to 13 s. The remaining 11 couples did not match harmonics. We did not detect a

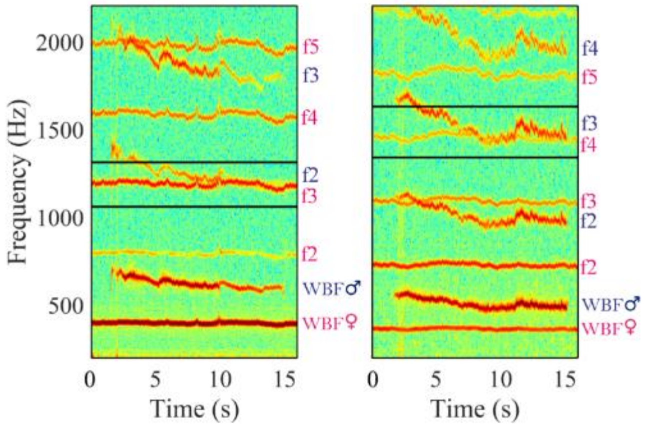

Figure 4-1. Harmonic convergence between tethered male-female couples. Spectrograms of males and females are shown, displaying the WBF and harmonics. Pink labels indicate female harmonics, blue labels indicate male harmonics. Black rectangles highlight harmonic convergence between female f3 and male f2 (left) and female f4 and male f3 (right).

relationship between the ratio of convergence and ratio of couple size (♂size/♀size)—couples where the female's third harmonic converged with the males second had a size ratio of 0.954±0.015; couples where the female's fourth harmonic converged with the males third had a size ratio of 0.942±0.007 (t-test, $t_{1,40}$=0.68, p=0.49).

4.3.2 Male-female precopulatory acoustic behaviours during free-flight

To characterize mosquito flight-tones prior to mating, we examined male-female acoustic behaviours during mating attempts. Male mating attempts were determined by visual and audio analysis—a male "chasing" a female was classified as the onset of a mating attempt. From 33 different females, 134 mating attempts were analysed. Mating attempts resulted in either copulation (i.e. the female was inseminated) or mating rejection; 12 of 33 females mated. The duration of a mating interaction was brief (1.38 ± 0.32 s; n=33). Ten of the mated females rejected a male at least once, while two mated after the first mating attempt. All mated females copulated within 1 to 5 minutes after introduction to the arena. Females that did not mate in our assays rejected males 1 to 9 times, with females that rejected once (n=3) not flying the remainder of the assay. To compare mating attempts that ended in a successful copulation or a rejection, we examined audio recordings of the 12 interactions that resulted in a successful copulation and randomly selected one mating attempt from each of the 21 females that did not mate in order to identify male- or female-specific acoustic behaviours that occur immediately prior to *An. albimanus* copulation or mate rejection.

We consistently observed that all mating attempts, regardless of the outcome, were characterized by an increase in male WBF followed by an increase in female WBF (Figure 4-2a; Supp. Video 4-V1). The increase in the WBF of the male coincided with the onset of the female chase, while that of the female coincided with her flying away from the approaching male. After increasing their WBFs, males and females rapidly modulated their frequency until couples departed or stopped flying. Although these oscillations appear to be similar to those reported in other species, it was not always possible to observe this behaviour in every mating attempt as 25% of the couples that copulated landed upon making contact. The time interval of rapid frequency oscillation after reaching maximum WBF ranged from 0.34 - 1.38 s and 0.18-1.56 s for males and females, respectively.

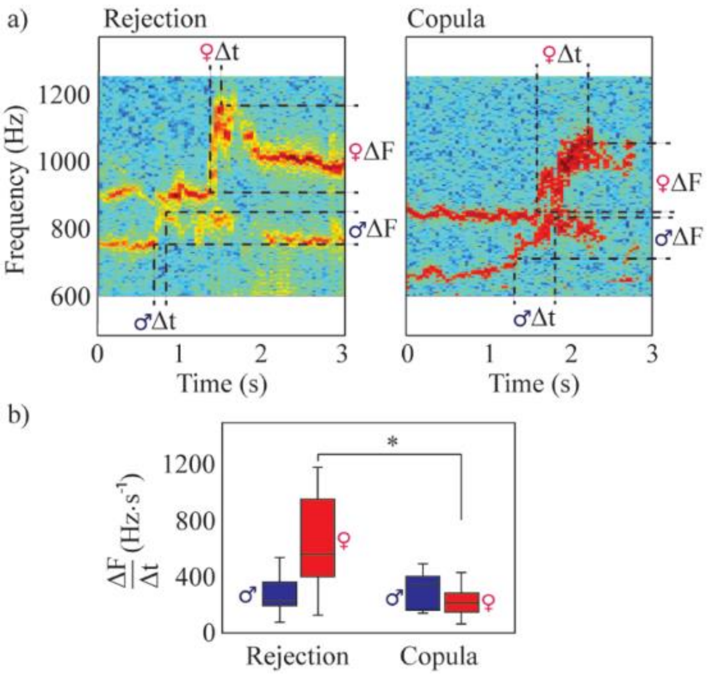

Figure 4-2. Acoustic analysis of free-flying mosquito during courtship. (a) Representative spectrograms of a mating attempt that resulted in mating rejection (left) or copulation (right). For visual simplicity, the spectrograms show the males fundamental frequency and the females second harmonic (analysis was done using the WBFs of each sex). The change in WBF (ΔF) and the time interval between the initial WBF increase and the maximum frequency reached (Δt) is shown for each sex. (b) Comparison of the rate of frequency increase (ΔF/Δt) for male-female interactions that ended in a rejection (n=21) or copulation (n=12). * signifies a significant difference for a Mann-Whitney U Test (p<0.01).

Using the criteria for tethered (Aldersley et al. 2016), we did not detect harmonic convergence in free-flying pairs. The brevity of the mating interaction, the WBF range of individuals in free flight, and that several couples ceased flying upon contact suggest that our methods were not sufficient to detect this phenomenon. However, we analysed the latency of the response of the female, the female/male WBF ratio prior the interaction and the WBF increase during the mating attempt to determine if these characteristics influenced the outcome of a mating attempt. The latency of the females' WBF increase in response to that of the male did not significantly differ between interactions that resulted in copulation or a rejection (copulation: 0.375 ± 0.074 s, rejection: 0.542 ± 0.047 s; Mann Whitney U Test: $U_{12,21}$=78, Z=-1.79, p=0.07). The female/male frequency ratio average (♀WBF/♂WBF) 1-1.5 s prior to a mating, before any observable male-female interaction occurred, was similar for mating attempts that resulted in copulation (♀WBF/♂WBF: 0.595±0.024) or rejection (♀WBF/♂WBF: 0.596±0.024; Mann-Whitney U Test: $U_{12,21}$=125, Z=-0.03, p=0.97) and did not influence mating outcome. Finally, to analyse the WBF increase during the mating attempt, we measured the change in WBF (ΔF) and the time interval between the initial WBF increase and the maximum frequency reached (Δt), allowing us to calculate the WBF rate of increase (ΔF/Δt) of both sexes during this interaction (Table 4-1, Figure 4-2a). In males, we found that the rate of WBF increase was similar for mating attempts that resulted in copulation or rejection (Mann-Whitney U Test: $U_{(12,21)}$=109, Z= 0.63, p=0.52) (Table 1, Figure 4-2b). While females also increased their WBFs regardless of mating outcome, the rate of the frequency increase was significantly greater prior to a mating rejection compared to a copulation (Table 1, Figure 4-2b) (Mann-Whitney U Test: $U_{(12,21)}$=24, Z= -3.81, p<0.01), owing to differences in the extent of the frequency increase (Mann-Whitney U Test: $U_{(12,21)}$=58, Z= -2.54, p=0.01) and the time interval of the increase (Mann-Whitney U Test: $U_{(12,21)}$=40.5, Z= 3.19, p<0.01) (Table 4-1).

Table 4-1. *Values of frequency interval (ΔF), time interval (Δt) and the rate of frequency increase (ΔF/Δt) during mating attempts.*

	Copulations (n=12)	Rejections (n=21)
ΔF (Hz)	♂: 91.2 ± 6.3 ♀: 81.1 ± 10.3	♂: 90.9 ± 4.9 ♀: 111.6 ± 5.3
Δt (s)	♂: 0.325 ± 0.034 ♀: 0.393 ± 0.048	♂: 0.364 ± 0.025 ♀: 0.220 ± 0.029
ΔF/Δt ($Hz\ s^{-1}$)	♂: 314.68 ± 36.60 ♀: 218.32 ± 29.59	♂: 282.69 ± 27.35 ♀: 655.52 ± 67.71

As we detected differences in the female rate of WBF increase between mate rejection or copulation subsequent to a mating attempt, we further analysed this behaviour. In females that rejected a male more than once (n=18), we performed a paired comparison between two different mating attempts of the same female and found no differences in the rate of WBF increase (Wilcoxon Matched Pairs Test: Z=0.97, p=0.32) (Figure 4-3a). Thus, females consistently

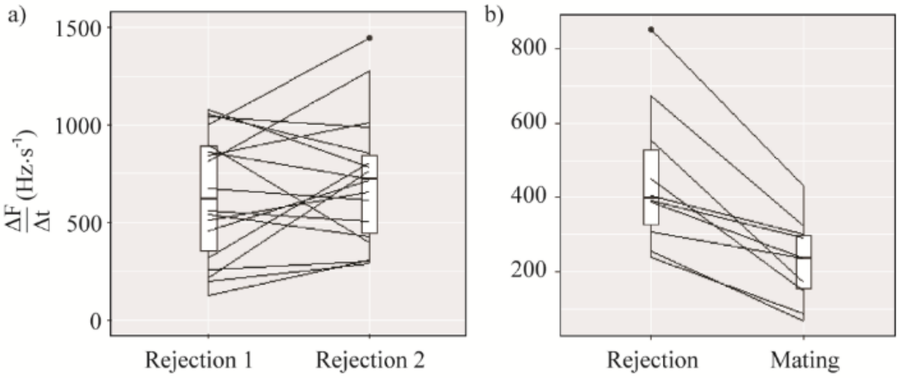

Figure 4-3. Paired comparison between (a) two rejections and (b) a rejection and successful copulation of the same female. While there is no significant difference between rejections (Wilcoxon matched-pairs test: Z = 0.97, p = 0.32), there is a significant decrease of the WBF rate of increase (Wilcoxon matched-pairs test: Z = 2.84, p < 0.01) between rejections and successful copulations.

increased their WBF at similar rates during rejections. However, in females that rejected and then copulated with a male (n=10), we performed a paired comparison between the copulation and a rejection of the same female and found significant differences in the rate of WBF increase between outcomes (Wilcoxon Matched Pairs Test: Z=2.84, $p<0.01$) (Figure 4-3b)—the rate of increase was lower for mating attempts that resulted in copulation compared with a rejection. Thus, in our assays, the increasing rate of the female WBF predicted copulation or rejection.

4.3.3 Male-male acoustic behaviours during free-flight

We next analysed acoustic and flying behaviours among groups of males. We introduced eight males into a soundproof chamber and made an audio record of their flight-tones and a visual record of their flight characteristics. As only a portion of the introduced males flew at any given time, we have differing numbers of recordings of two, three and four males flying simultaneously. For our analysis, we selected 5 s intervals in which flying behaviour was clear to analyse male flight trajectories using an ellipse, determining the distance of each individual in relation to the rest of the group and following the X and Y coordinates of each individual over this time frame. To assess flight acoustics of male groups, we analysed the average frequency difference of each group—the difference between the highest and lowest WBFs observed in the spectrogram, representing the frequency bandwidth of the interaction among males, during the 5 s segments analysed.

Two distinct flying behaviours were apparent among groups of males flying simultaneously: random flight and a swarm-like, patterned flight (Figure 4-4a and 4-4b; Supp. Video 4-V2). During random flight, male WBFs diverged or were distinct (Figure 4-4c and 4-4d, bottom panels), but converged once patterned flight was initiated, reducing the frequency differences of the male flight-tones (two males: Figure 4-4c, four males: Figure 4-4d; Supp. Video S2). By examining flight behaviour in 5 s intervals, we observed a significant change in the frequency difference between males in random flight and those in patterned flight. This change was consistent among groups of two (Mann-Whitney U Test: $U_{8,8}=0$, $Z=-3.36$, $P<0.01$), three (Mann-Whitney U Test: $U_{5,5}=0$, $Z=-2.61$, $P<0.01$) and four (Mann-Whitney U Test: $U_{5,5}=0$, $Z=-2.61$, $P<0.01$). The difference in WBF of groups in patterned flight was always smaller than groups in random flight (Figure 4-4e). The mean frequency difference of groups of two, three and four males in patterned flight was 38.20±3.33 Hz, 52.60±4.01 Hz and 68.21±7.11 Hz, respectively. On the other hand, the mean frequency difference of groups of two, three and four males in random flight was 110.88±19.91 Hz, 177.05±31.16 Hz and 167.65±19.88 Hz, respectively.

During patterned flight, we observed that *An. albimanus* fly in an ellipsoid pattern around a central position, similar to what has been reported in *Cx. quinquefasciatus*. Therefore, we quantified visual differences between random and patterned flight (where we observed acoustic interactions) by examining the trajectory of individual mosquitoes and analyzing the ellipsoid shape formed while flying. We found significant differences in the characteristics of male trajectories between the two types of flight observed—the ellipse area representing random flight is significantly larger than the area representing patterned flight (random flight: $(1.55 \pm 0.29) \times 10^5$ px^2, patterned flight: $(0.49 \pm 0.06) \times 10^5$ px^2; Mann-Whitney U Test: $U_{(18,18)}=58$, $Z=3.24$, $P<0.01$). We further measured the distance (in pixels) of each mosquito in relation to the rest of the group by calculating the average distance among the centroids of each ellipse in comparison with the others. We found that mosquitoes flying randomly are significantly less aggregated than males in patterned flight (random flight: 244.66±21.03 px, patterned flight: 95.79±11.15 px; Mann-Whitney U Test: $U_{18,18}=69$, $Z= 2.89$, $P<0.01$). Finally, by separately analysing the X and Y trajectories of males in flight, we observed characteristic loops (i.e. periodic movement) like those defined in previous studies. Mosquitoes in patterned flight display loops with significantly shorter periods (random flight: 2.63 ± 0.39 s, patterned flight: 0.90 ± 0.08 s; Mann-Whitney U Test: $U_{(18,18)}=33.5$, $Z= 4.03$, $P<0.01$) (Figure 4-4f). Taken together, these data show that patterned flight is associated with distinctive acoustic behaviours.

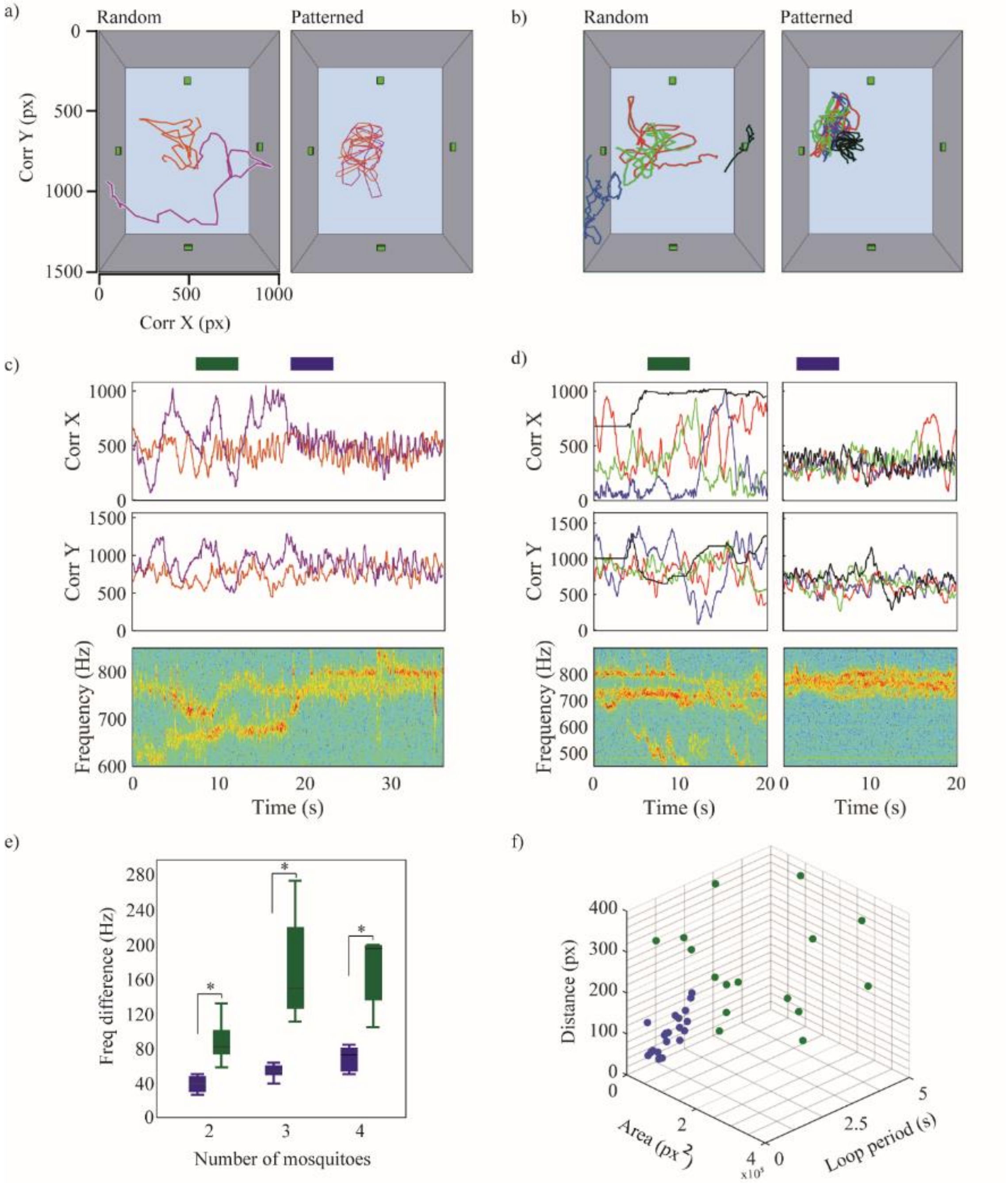

Figure 4-4. Acoustic and visual analysis of free-flying males. (a) Two and (b) four males in random flight (left panels) and patterned flight (right panels). Grey boxes represent the spatial distribution of the experimental arena. Green squares within the boxes indicate microphone location. Coloured lines show the 2D trajectory for each male during 5s recordings. c. and d. The X (top) and Y (middle) coordinates of the flight trajectories and the high order spectrogram (bottom) of groups of two (c) and four (d) mosquitoes during random and patterned flight. Coloured rectangles indicate the 5 s interval analysed to associate flying pattern with acoustic behaviour (green: random flight; blue: patterned flight). e. Box and whiskers plots of the frequency difference of males flying randomly (green) and in patterns (blue). The box ranges from the first quartile to the third quartile of the distribution. A horizontal line across the box indicates the median. The whiskers extend from the quartiles to the extreme data points. Males flying randomly exhibit a higher frequency difference than males in patterned flight. f. Differences in the characteristics of the flight trajectories of mosquitoes in random (green) or patterned flight (blue).

[redacted] Discussion of results and contribution to the research field

In this chapter, we describe several aspects of the acoustic behaviours of the New World malaria vector *An. albimanus*. While tethered mosquitoes display harmonic convergence, we describe a new female-specific acoustic behaviour related to mating success. Additionally, by examining *An. albimanus* males in free flight, we characterized male-male acoustic interactions, uncovering distinct audio and visual characteristics when males display patterned, swarm-like flying behaviour. To our knowledge, this is the first study to describe male-male acoustic interactions in free flight linked to their flight trajectories and a female-specific acoustic behaviour that predicts acceptance or rejection of courting males in *An. albimanus*.

Harmonic convergence, or the synchronization of frequencies between two specific harmonics during male-female interactions, has been described in *Ae. aegypti* (Cator and Harrington 2011), *Cx. quinquefasciatus* (Warren et al. 2009) and *An. gambiae* (Cator and Zanti 2016) using tethered individuals. We also observed this behaviour in tethered *An. albimanus*. The time of convergence and the latency of the response were similar to *An. gambiae*. In our assays, a common convergence event occurred between the females' third harmonic and males' second. This relation between harmonics during convergence is associated with mating success in other species (Cator and Harrington 2011). Another common convergence occurred between the females' fourth harmonic and the males' third, which has been reported in *Ae. aegypti*.

Unlike when tethered, we were unable to observe harmonic convergence in free-flying mosquitoes. Harmonic convergence presumably happens rapidly during courtship or is an epiphenomenon linked to the male auditory strategy used to track females (Simões et al. 2018). However, we observed a common precopulatory behaviour: a rapid increase in male WBF immediately followed by a rapid increase in female WBF. A similar behaviour has been observed in *Culex quinquefasciatus*(Simões et al. 2016), *An. coluzzii* and *An. gambiae*(Simões et al. 2017), where males increase their WBF in response to female flight-tone. The rate of WBF increase in males was similar regardless of the mating outcome, while a more rapid increase in female frequency resulted in mate rejection. It is possible that the frequency increase results from controlled flight to reach or escape a potential mate. Results from the *Culex* (Simões et al. 2016) and *Anopheles*(Simões et al.

2017) auditory system suggest that to locate females, males use the difference between their own frequency and that of the female rather than the female WBF itself (Simões et al. 2018). The rapid change in female WBF during mate rejection immediately modifies the male-female WBF ratio. When a female reaches her highest WBF, the ♀WBF/♂WBF ratio is minimized, and the odds of locating a female decline. Remaining within the optimal auditory sensitivity range may be a critical characteristic to reach and copulate with a female.

Male swarming behaviour appears to be an obligatory feature of mating for some *Anopheles* species (Yuval 2006, Howell and Knols 2009). However, swarming behaviours have not been described for Latin American species. Further, male-male interactions during swarming and female mate selection within a swarm are not well understood. Acoustic signalling might be an important factor during these interactions, although studies of male-male and male-female interactions in free flight are few (Simões et al. 2016, Simões et al. 2017, Aldersley and Cator 2019). In our assays, *An. albimanus* males performed two identifiable types of flying behaviour regardless of the number of males assessed: random and patterned flight. During random-flight, males flew in large trajectories within the entire experimental arena. Once the patterned flight was initiated, the large trajectories immediately gave way to flight patterns composed of small loops in a specific region of the arena. Males in patterned flight acoustically interacted within a narrower band of frequency, linking flight pattern and acoustic behaviour. This result is likely related to the clustering of male flight-tones of closely located, tethered *Ae. aegypti* males (Aldersley et al. 2017). During swarming, males must coordinate their flight patterns and recognize females that enter the swarm. It has been proposed that flight-tones are used to coordinate movement during group flight (Aldersley et al. 2017), dividing into frequency clusters to reduce acoustic interference (Lapshin and Vorontsov 2013).

Linking acoustic and flying behaviours under more natural, unrestricted conditions, as shown in this thesis, provides important information regarding behaviours during courtship and ultimately, female mate selection. Control programs that rely on the mass release of lab-reared mosquitoes (Carvalho et al. 2015, Flores and O'Neill 2018, O'Neill 2018) need to consider acoustic behaviour, as the results presented here, as well as those of previous reports, demonstrate the importance of precopulatory acoustic behaviour in mating success (Cator and Harrington 2011). Our characterization of *An. albimanus* acoustic flight behaviours, an important Latin American malaria vector, is a step towards understanding male-male and male-female interactions prior to copulation and will aid in the improvement of vector surveillance and control programs in areas affected by this species.

In this chapter, we examined acoustic behaviours of tethered and free-flying mosquitoes. From the next chapter, we present the applied component of the research. By using the information obtained far in this thesis, we go on to evaluate novel approaches to improve the acoustic-based surveillance of mosquitoes. In the next chapter we develop an acoustic trap for *Ae aegypti*, using recordings of their own flight-tones to synthetize acoustic attraction cues.

Chapter 5: A new approach to improve acoustic trapping effectiveness for male and female *Ae. aegypti* (Diptera: Culicidae)

[redacted] Introduction

As current adult surveillance tools need improvement and tend to have high operational cost, it is crucial to find new mosquito attractants that improve capture rates of traditional traps or develop new trapping strategies (Sivagnaname and Gunasekaran 2012). Given that these trapping methods rely on reproductive behaviours, 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 behaviour prior to mating (Gibson and Russell 2006, Cator et al. 2009) given that they are used for mate selection (Cator and Harrington 2011). 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 swarms like other mosquito species, the presence of a host or the onset of the sun elicits small aggregations of males with specific flight patterns (Cabrera and Jaffe 2007, Fawaz et al. 2014). Within swarms, *Ae. aegypti* use stereotypical acoustic signals for courtship (Cator et al. 2011a). As males track females by the sound of 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 wing-beat (Johnson and Ritchie 2015). These devices, however, have not been employed yet as a surveillance tool, probably due to their low effectiveness at collecting female mosquitoes. The low effectiveness at attracting females mosquitoes by acoustic signals is surprising given their high hearing sensitivity, which is comparable to that of males (Göpfert and Robert 2001, Cator and Harrington 2011, Lapshin 2013). In this chapter, we present a description of a new prototype of an acoustic trap and the results of investigations to determine the effectiveness of signals synthesized from mosquito wingbeat recordings. Signals aimed at minimizing discomfort to humans inhabiting areas where the traps would be used were also tested. 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 thus establish the effectiveness of both the design of the prototype trap and a variety of acoustic attraction signals, including some previously evaluated in other studies (e.g. pure-tones).

[redacted]Methods

Mosquitoes flying individually or in male-female couples were recorded following 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 synthesized by using the fundamental frequency of recordings of tethered males or females flying at 25 ± 1 °C. Based on the fundamental frequency of 80 males (range of wingbeat frequency: 650 - 750 Hz) and 80 females (range of wingbeat frequency: 650 - 750 Hz), we synthesized 10 s pure. These 80 pure-tones were broadcast alternating from two speakers (Figure 5-1a), at a continuous intensity of ~10 dB SPL. In one experiment, however, the sound intensity of the stimuli was changed to test the role of this acoustic feature. The specific values used for each one of the stimuli are indicated in the description of this particular trial (Under "Acoustic traps performance under semi-field conditions" below). In contrast, complex signals were synthesized by using over 200 clips (10 to 30 s length) extracted from 240 recordings of male and female mosquitoes flying alone or in couples. 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 mosquitos or a male and female interaction (Figure 5-1b). Complex signals were broadcast for one minute at a high sound intensity (~10 dB SPL) followed by nine minutes of low intensity (~2 dB SPL). Complex signals were synthesized by using a sinusoidal representation of mosquito's flight-tones. From the short-time Fourier transform (Fast Fourier Transform (FFT) length of 4096 points, a 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 mosquitos without generating distortion or increasing the noise.

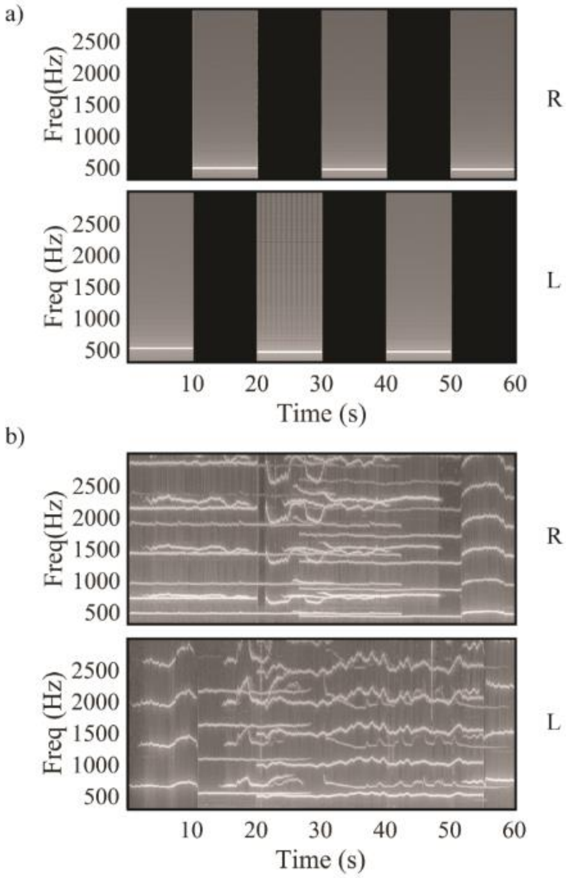

Figure 5-1. Representative spectrograms (one-minute segments) of attraction signals used in the experiment. Spectrograms of the left (L) and right (R) channels displaying (a) pure-tones and (b) complex signals.

5.2.1 Acoustic trap prototype

Our prototype trap is designed to be used under indoor conditions. It is composed of an acrylic structure that holds an acoustic stereo system to broadcast the acoustic lure and a vacuum fan that captures mosquitoes into a collection bag (Figure 5-2a). The base of the trap is a 15 cm height isosceles trapezoid with a short and long base of 20 cm and 12 cm, respectively. The frontal face is 31 cm in length, and it has a 11×14.5 cm^2 black and white area to increase the orientation of mosquitoes towards 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 work with a rechargeable 3.7 V power supply.

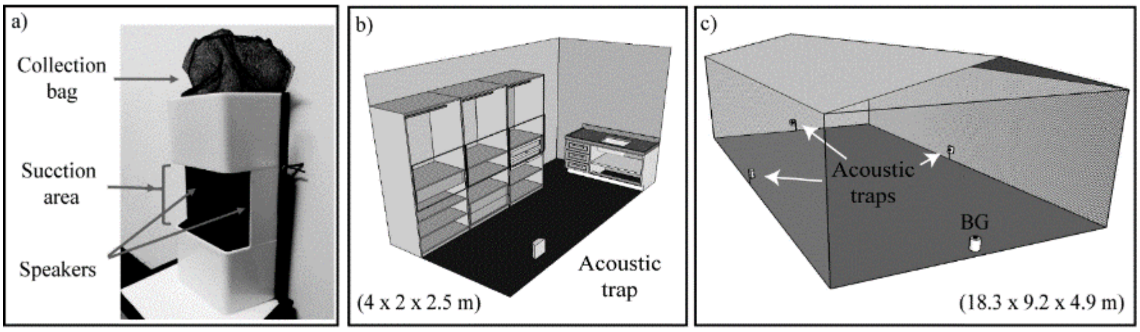

Figure 5-2. (a) Prototype of the acoustic trap developed in this study and the (b) set-up used to test it under indoor and (c) semi-field conditions. For semi-field trials, the location of traps was rotated to the right at every replica. Each trap was thus placed at a given location at least one time during the experiment.

5.2.2 Acoustic trap performance under indoor laboratory conditions

5.2.2.1 Experimental Site. This experiment was conducted at the University of Antioquia in Medellín, Colombia. In particular, the trials were performed in medium-sized rooms (~4 × 2 × 2.5 m) that simulated standard household conditions in which we controlled temperature (25 ± 1 °C), humidity (60 %RH) and light intensity (artificial light from standard fluorescent bulbs Philips F32T8/Super 84, 32 W). We also kept the distribution of furniture constant during and between trials and prevented the use of the room by human visitors (Figure 5-2b). 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 SPL).

5.2.2.2 Mosquitoes. The colony and the method to obtain virgin mosquitoes are described in Chapter 2 (Section 2.4.1.). Virgin 4-7 days old mosquitoes were used for the experiments.

5.2.2.3 Experimental design. Recapture rate of 40 mosquitoes originally released in the room at the beginning of each trial, was assessed every 30 min for a period of two hours. We compared the males' capture rate of traps broadcasting no sound, pure-tones and complex signals and females' capture rate of traps broadcasting no sound and complex signals. All trials were performed between 1600-1800 hrs. We replicated each treatment seven times.

5.2.2.4 Data analysis. A one-way ANOVA followed by a Fisher's LSD post hoc test was 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. The analysis was performed using R© V 3.3.1. Results are presented as mean ± SEM. Reports from statistical analyses include test statistic, degrees of freedom and p value (e.g. Multifactor ANOVA, F_{df_effect,df_error}, p).

5.2.3 Acoustic trap performance under semi-field conditions

5.2.3.1 Experimental Site. Semi-field trials were conducted in large enclosures (18.3 x 9.2 x 4.9 m) at the United States Department of Agriculture, Mosquito and Fly Research Unit at Gainesville, FL. 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 at 700 m from the enclosures). Background noise intensity was measured from the centre of the enclosure pointing to the corners and ranged between 63 and 70 dB SPL.

5.2.3.2 Mosquitoes. Two to seven-day-old mosquitoes from the colony at the USDA Mosquito Unit at Gainesville Florida (Orlando 1952 strain) were used for this experiment**.** Males and females were separated one hour before every trial to test them separately. Six hundred males or females were used for every replica of the experiment. A total of 4800 males and 2400 females were used for the semi-field experiments.

5.2.3.3 Experimental design. To assess the performance of the acoustic trap under semi-field conditions, we determined the trapping effectiveness for males and females separately. Between 9:00-10:00 hrs, 600 individuals (either all males or females) were released into the enclosure. Experiments followed a 4 × 4 Latin square design with three acoustic traps broadcasting different acoustic stimuli and, for comparison, a commercial trap BG-Sentinel (BioGents, Germany) using a BioGents skin odour lure (Figure 5-1c). We established the recapture percentage for every trap after a period of six hours. Three experiments were performed following the same methodology but varying the stimuli used for the three traps additional 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 broadcasting pure-tones at a high intensity (90 dB SPL), another trap broadcasting the same pure-tones with a 10 min fading of intensity (90-65 dB SPL), and a third (control) trap from which no sound was played. Experiment two 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 broadcast pure-tones, a second one used the same pure-tones plus a commercially available odorant cue (Biogents®, skin odour lure), and the third trap played complex signals with no additional cues. A final third experiment was performed to investigate the effectiveness of acoustic lures at attracting females. In this case, one trap broadcast pure-tones, the second trap used the same stimulus as the previous trap paired with an odorant cue (skin odour lure, BioGents, Germany) and, as in the experiment with males, the third trap used complex signals. In all treatments, in the second and third experiment, the acoustic stimuli were played at an intensity of 10 dB SPL above the noise floor.

5.2.3.4 Data analysis. We used a Generalized Linear Model (GLM) to evaluate differences between treatments. We also include the position of the trap and the environmental variables of the day of the experiments as random variables. 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$^{\copyright}$ V 3.3. Results are presented as mean ± SEM. Reports from statistical analyses include test statistic, degrees of freedom and p value (e.g. Multifactor ANOVA, F_{df_effect,df_error}, p).

[redacted]Results

5.3.1 Indoor acoustic tests

During two hours in which the traps were used, the acoustic signals broadcast in this study varied significantly in their ability to capture mosquitoes. When male mosquitoes were released into the room (Multifactor ANOVA, $F_{2,19}=30.44$, $p<0.001$), the traps broadcasting pure-tones (69.2 ± 2.1 %) and complex signals (77.7 ± 2.9 %) recaptured a higher number of individuals than the trap broadcasting no sound (52.1 ± 3.1 %). There was, however, no significant difference between the performance of the traps playing pure-tones or complex acoustic signals (P=0.064). Most male mosquitoes (>60%) were recaptured after one hour (Figure 5-3a). After this time, the recapture rate decreased considerably. Similarly, the capture rate for females increased significantly by using complex signals (Multifactor ANOVA, $F_{1,13}=23.02$, $p<0.001$). Female’s capture rate, however, was considerably lower than the male’s capture rate for traps broadcasting both swarm signals (32.9 ± 1.64 %) and no sound (20.8 ± 1.11 %) (Figure 5-3b).

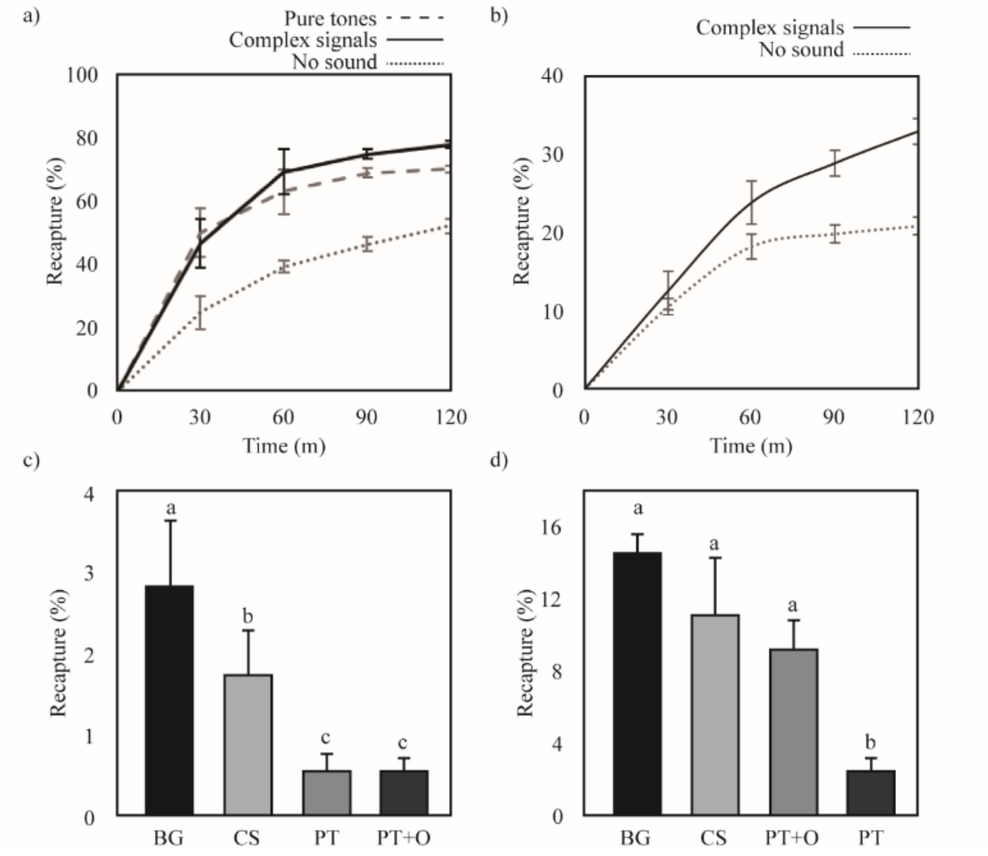

Figure 5-3. Capture effectiveness of the acoustic traps at attracting (a) male and (b) female mosquitoes under indoor conditions. The traps used indoors broadcast no sound (dotted light grey), pure-tones (dashed grey) or complex signals (continuous black). Mean and SEM is shown for the average number of mosquito recapture at 30, 60, 90 and 120 min. Captures effectiveness of (c) males and (d) females under semi-field conditions of BG sentinel trap (BG) and acoustic traps broadcasting complex signals (CS), pure-tones (PT) and pure-tones plus odour cue (PT+O). Mean and SEM is shown for each treatment. Letters upon the bars indicate statistically different groups ($p<0.05$ [Fisher's LSD]).

5.3.2 Semi-field acoustic tests

During the experiment designed to test the effect of sound intensity on the males' capture rate, there were significant differences between the performance of the BG sentinel and the acoustic traps (GLM, $F_{3,11}=3.94$, $p=0.02$). 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 in the capture rate among acoustic traps (LSD test $p>0.1$ for every comparison). While the UV index of the day of trapping affected the capture rate of this experiment (GLM, $F_{1,11}=4.02$, $p=0.04$), no effect of any other environmental variable measured was detected.

When we evaluated the effectiveness of the acoustic stimuli and odour as lures for trapping males, the capture performance differed between treatments (GLM, $F_{3,11}$=8.25, p=0.03). Even though capture rates were low; BG-Sentinel trap (2.85 ± 0.69 %) captured the highest number of males (Figure 5-3c). 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 odour lure (0.57± 0.28 %). No effect of any environmental variable measured was detected during this experiment.

When females were tested, the BG-Sentinel trap did not outperform the acoustic traps (Figure 5-3d). The traps using complex signals and the combination of pure-tones and the odour lure trapped as many individuals as the BG-Sentinel trap. The trap broadcasting only pure-tones, however, captured a significantly lower number of females. While the UV index of the day of trapping affected the capture rate of this experiment (GLM, $F_{1,11}$=6.37, p=0.04), there were consistent differences between treatments (GLM, $F_{3,11}$=6.16, p=0.02).

Analysis of the recapture rates of the reference trap (BG) revealed that the capture rate was considerably higher for females (14.6 ± 2.9 %) than males (3.4 ± 1.2 %). In addition, there was a strong effect of UV index of the day of the trial on the capture rate of males (Linear regression, r=0.73, R^2=0.53, p=0.02). While the sample size for females is limited to evaluate the effect of UV light on their capture rates on different days, standardization of the recapture rates for both sexes using normal distributions suggest that females were also affected by the UV index to a similar degree (Figure 5-4). No effect of any other environmental variable measured was detected.

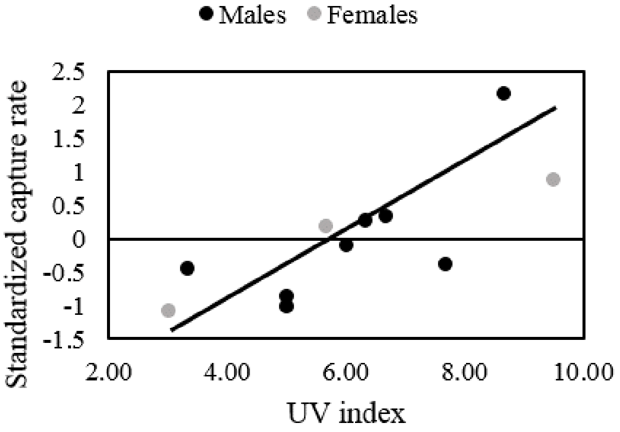

Figure 5-4. Relationship between ultraviolet (UV) index and standardized capture rate of mosquitoes under semi-field conditions. Linear regression for both males and females.

[redacted] Discussion of results and contribution to the research field

Our results show that signals synthesized from recordings of individuals flying independently and male-female interactions are effective at attracting both sexes under indoor and semi-field conditions. This work contributes to the body of recent literature that is actively exploring venues to increase the trapping efficiency of *Ae. aegypti* (Jakhete et al. 2017, Johnson et al. 2018). Our findings also reveal sexual differences in attraction to lures and represent, to the best of our knowledge, the first report suggesting successful acoustic attraction for female mosquitoes.

Under indoor laboratory 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. Even though the capture rate of females was considerably lower, using complex signals also increased the number of females trapped. Thus, in accordance with a previous study showing that signals with multiple harmonics and frequency sweeps are more attractive for male *Ae. aegypti* (Jakhete et al. 2017), our results also show that the increased complexity of the signals does not deleteriously affect the capture rate. Indeed, our results suggest that it is possible to include male and female harmonics and frequency modulations to increase the capture rate of both sexes. Broadcasting just one minute of high-intensity complex signals every ten minutes was sufficient to match the males' capture rate of traps using pure-tones at a constant intensity. This finding is critical due to the necessity of using traps for *Ae. aegypti* within houses, where the presence of humans constrains the use of prolonged high-intensity pure-tones. Development of acoustic stimuli that minimize discomfort for people, such as the use of interrupted signals as the one we evaluated, is critical to implement the use of acoustic traps in households. 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. Finally, although females were attracted in lower numbers than males by swarm signals, there was a significant increase in the capture rate of traps broadcasting these signals. Our indoor trials thus indicate complex signals are effective lures for both males and females even in the presence of abundant potential visual 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. While acoustic traps have proved to be effective when positioned in dark areas, sheltered from the wind, sunlight and rain, 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 corroborate that the specific location of where the trap is set is critical to reaching 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.

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

Under semi-field conditions, capture rates of males were significantly lower than females. This discrepancy in capture rates between sexes may be explained by the fact that females move more, covering larger areas as they search for potential hosts or oviposition sites while males focus on finding a harborage site (Ball and Ritchie 2010). Therefore, the probability of trapping females may be higher especially in large enclosures with several potential harborage sites. Higher mobility of females and the use of the BG-Sentinel trap lure, which is designed specifically to attract females could explain the difference between capture rate of males and females in our semi-field trials. This effect, however, was not evident under indoor trials in which the recapture rate of males was considerably higher than that of females. We attribute higher male trapping effectiveness under indoor conditions to the fact that males perform stereotypical flight patterns near the suction area of the trap. This behaviour increases significantly the capture probability as males fly near the suction area until they are captured. It was possible to clearly observe this behaviour by turning off the suction fan (e.g., Supp. Video 5-V1). This behaviour, however, was not observed under semi-field trials. We propose that differences among indoor and outdoor conditions like the presence of wind and sunlight in semi-field trials, prevented male mosquitoes from flying close to the suction area for long enough to be captured, which resulted in lower capture rates.

It is well recognized that environmental factors can strongly influence mosquito capture rates (Lebl et al. 2013, Chuang et al. 2018). Our data revealed a strong effect of UV light on the capture rate of BG-Sentinel traps. 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 likely increases the conspicuousness of the funnel of the traps, which results in an increase in the 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, this study suggests acoustic signals can be used to design new surveillance tools for monitoring *Ae. aegypti* males and females or improve traditional strategies. There are, however, some details that deserve further attention to enhance the performance of the traps. The sound pressure level of the signals, for instance, should be investigated in further studies to find values that maximize attraction while minimizing discomfort by residents. Combinations of long and short distance attractants will likely enhance attraction to the traps and result in more effective trapping strategies.

Traps playing complex signals with no odorant lure captured comparable numbers of mosquitoes to the BG-Sentinel. As females move more than males within the space they are located (Ball and Ritchie 2010), it was more likely for them to reach the surroundings of the acoustic trap and been attracted by the signals. In addition, capture rates of females obtained by the trap using a combination of pure-tones and the odour lure was comparable to the capture rate of the BG-Sentinel trap. These results suggest our capture system is effective enough to capture similar numbers of females compared to the BG-Sentinel trap. From the application perspective, it is important to highlight that our results show acoustic and chemical lures can be effectively used together.

In this chapter, we present a novel approach to improve trapping effectiveness. Yet, the operational cost of traps constrains their use, especially in poor countries. In the next chapter, we explore a different approach of acoustic surveillance based on the differentiation of species by their flight-tone. This approach aims to generate high-throughput and low-cost surveillance methods by generating an automatic technique to recognize mosquito species.

Chapter 6: Recognizing mosquitoes by their flight-tones. Opportunities from the entomological surveillance perspective

[redacted]Introduction

Monitoring populations of mosquitoes gain importance when the observed species are responsible for transmitting diseases to humans (WHO 2014). By using different surveillance technics like human landing catches or trapping, questions about mosquito ecology and behaviour are normally addressed (Ferguson et al. 2010). These questions are used in many instances to plan or evaluate control programs designed to mitigate mosquito-borne diseases (Schaffner et al. 2013, Osório et al. 2014, Petrić et al. 2014). The traditional surveillance strategies, however, demand high operational costs or are not effective enough (Utzinger et al. 2001, Sivagnaname and Gunasekaran 2012, Pepin et al. 2013). In addition, due to the absence of high throughput and low-cost surveillance methods, there is a scarcity of data about the abundance, temporal variation, and spatial distribution of mosquito species (Hay et al. 2013, Sedda et al. 2019). The impact of this phenomenon is more evident in developing countries, where the cost of trapping and identifying specific species reduce the success of such surveillance strategies.

Novel acoustic-based strategies are now accessible to enable the detection and surveillance of mosquitoes. By using the specific characteristics of mosquitoes' flight-tones –the sound produced by the wingbeat– it is possible to effectively recognize the presence of mosquitoes or even differentiate among species (Mukundarajan et al. 2017). Using common cell-phone microphones is a plausible option to enable extensive acoustic mosquito surveillance. The automated mosquito detection from microphone recordings, however, presents a signal processing challenge since flight-tones are week signals compared to the environmental noise (Kiskin et al. 2018). Different methods have been developed to solve this problem. An array of photoreceptors and infrared emitters is capable of recording optoacoustic signals generated by the wingbeat of mosquitoes (Chen et al. 2014, Potamitis and Rigakis 2016). Even though this approach overcomes the technological limitations of recording flight-tones with microphones, it uses is restricted by the cost and the global scalability of the equipment required.

Regardless of the sensor used to record wingbeats, recognizing species is problematic when their flight-tones share similar frequency characteristics (Chen et al. 2014, Mukundarajan et al. 2017). In this scenario, data about the location and the moment of the capture can be used to increase the probability of an accurate classification. There are some scenarios, however, where two species simultaneously inhabit the same location, share similar WBF distributions and exhibit similar behaviours during the day (Kraemer et al. 2015). In this context, differentiating one species from the other is challenging but necessary. Finding new approaches to increase the classification effectiveness is critical to move towards the development of high throughput and low-cost acoustic-based surveillance tools.

In this chapter, we review two approaches used to perform an acoustic recognition of species and discuss their advantages and limitations. Finally, by analysing a database containing optoacoustic recordings, we briefly evaluate the feasibility of differentiating species sharing similar WBF.

[redacted] Flight-tones recording approaches

6.2.1 Optoacoustic

The foundation of the optoacoustic sensors to record mosquitoes is the gradual interruption of a path of light between an emitter and a receiver (Chen et al. 2014). The interruption caused by the wing movement generates an electrical signal with frequency characteristics like those of flight-tones recorded with microphones. The main advantage of optoacoustic sensors is the low noise levels compared to microphones (Potamitis and Rigakis 2016). Due to its optical nature, optoacoustic sensors are not affected by common environmental noise. Consequently, the high signal to noise ratio provides accurate data for classification purposes. The possibility of recording mosquitoes one by one is an additional advantage. In the presence of numerous mosquitoes, using microphones impedes the analysis of individual flight-tones. In addition, the signature of a mosquito flying across the path of light is unique and therefore can be rapidly detected. In the context of an automated surveillance strategy, obtaining accurate signatures is essential to avoid false positives and consequently, to increment the efficiency of the classification algorithms. In spite of the advantages, the cost and global scalability of the hardware limit this approach. The hardware necessary on this kind of applications is out of the reach for poor communities where there is a need for mosquito surveillance.

6.2.2 Microphone-based

Although less accurate than optoacoustic sensors, microphones are widespread around the world due to the existing mobile phone global network. Using any kind of microphone to record mosquitoes is emerging as a new paradigm for mosquito surveillance. Recent studies have demonstrated that recordings from different microphones provide highly similar acoustic characteristics from the same population of mosquitoes (Mukundarajan et al. 2017). This fact would enable a worldwide acoustic-based platform for mosquito identification. Unfortunately, good quality microphone recordings are hard to obtain. The noise of recordings reduces the classification accuracy and consequently, it is necessary to develop more elaborated classification algorithms in order to improve this strategy.

Both microphones and optoacoustic sensors generate new opportunities for mosquito surveillance. There are some challenges, however, that need to be addressed to effectively use flight-tone recordings to differentiate species. One of the main challenges is to differentiate flight-tones of species with similar frequency distributions (Mukundarajan et al. 2017).

[redacted] Differentiation of species with similar frequency characteristics

As the characteristics used for classification are estimated mainly from the power spectrum of the signals, when two species share similar frequency distributions the classification accuracy is reduced considerably (Chen et al. 2014, Mukundarajan et al. 2017). Using additional information as the hour and location of the recording reduces the chance of comparing two species with similar distributions and thus, increase the accuracy (Chen et al. 2014). In specific cases, however, it is necessary to differentiate species inhabiting the same location, exhibiting the same behaviour during the day and with similar wingbeat frequency distributions (Kraemer et al. 2015). In this scenario, exploring new classification features and technics are required to gradually increase the effectiveness of the differentiation.

Here we explore the classification of two species with similar frequency characteristics. By using optoacoustic recordings from an available database containing 10 classes of mosquitoes, we identify relevant species for public health and evaluate the possibility of accurately classifying two species with similar frequency characteristics. Two classification approaches are evaluated, Artificial Neural Networks (ANN) and Support Vector Machines (SVM).

6.3.1 Database

The database used in the present analysis is available online and it comprises 50000 optoacoustic recordings from ten flying insect classes – 5000 recordings for each class (Chen et al. 2014). Each sample consists of a frame of 100 ms or 1600 points at a sample rate of 16000 Hz. The available classes are *Ae.aegypti* (male), *Ae. aegypti* (female), *Culex quinquefasciatus* (male), *Cx. quinquefasciatus* (female), *Cx. tarsalis* (male), *Cx. tarsalils* (female), *Cx stigmatosoma* (male), *Cx. stigmatosoma* (female), *Musca domestica* and *Drosophila simulans*. The recordings of *Mu. domestica* and *Dro. simulans* are not discriminated by sex because males and females share the same frequency distribution. The first eight classes consist of mosquito species in which males always exhibit a higher fundamental frequency than females.

6.3.2 Signal processing and feature extraction

Recordings used in this experiment were obtained under controlled conditions and already pre-processed to eliminate background noise (Chen et al. 2014). The high signal-to-noise ratio achieved by optoacoustic sensors (Potamitis and Rigakis 2016) and the characteristics of the database, set an ideal scenario to reduce noise that might affect the classification features. Here, we used the power spectrum to estimate the classification features. Aiming to improve the frequency resolution, we used a 4096 fast Fourier transform to analyse each one of the 1600-point segments. From the power spectrum, two different set of classification features were generated.

Truncated spectrum: Previous works demonstrated that truncating the spectrum to use the information of the first harmonic increase the classification accuracy (Chen et al. 2014). Here, we truncated the spectrum between 100 and 2000 Hz; this frequency range includes the first and second harmonic of all the species analysed. By using this method, each recording was represented by an array of 487-points containing the information of the power spectrum between 100 and 2000 Hz. The 487 points where hence used as inputs for classification purposes.

Mel Frequency Cepstral Coefficients (MFCCs): Mel frequency analysis is based on human perception experiments (Rabbani et al. 2004). Concentrating the analysis on low-frequency components, we used 12 triangular filters in the Mel scale to represent the spectral information of each one of the recordings. This filter bank transforms the spectrum into the Mel-spectrum. A cepstral analysis was performed on the Mel-Spectrum to represent each flight-tone frame as a sequence of 12 cepstral coefficients.

6.3.3 Classification approach

Artificial Neural Networks (ANNs): We designed a two-layer feed-forward network with a sigmoid transfer function in the hidden layer and a softmax transfer function in the output layer. A scaled conjugate gradient backpropagation approach was used during the training. The number of neurons in the hidden layer was modified to evaluate the effect of the size of the network on the accuracy.

Support Vector Machines (SVMs): A Radial Basis Function (RBF) kernel was used to evaluate the classification rate. An analysis of the parameters of the kernels was performed to improve the classification accuracy. The regularization parameter C and the polynomial degree γ were optimized following previous works (Hsu et al. 2003). We performed a “grid search”, increasing exponentially the sequence of each one of the parameters.

We used 80 % of the data to construct a training set of 4000 samples for each one specie. Folds of 1000 samples were used to perform a 4-fold cross-validation. The 20 % (1000 samples for each class) left was used in order to evaluate the classification accuracy. Similarly, for two-class classification problems, training and testing sets comprise 8000 samples (4000 for each specie) and 2000 samples (1000 for each specie), respectively. Results are reported as mean ± standard-deviation. From the entire database, we performed a prelaminar classification among the 10 classes by using ANNs. We used the truncated spectrum and MFCCs as classification features to evaluate the effect of increasing the number of neurons in the classification accuracy. From the analysis, we identified species with similar frequency characteristics by inspecting the confusion matrix. Finally, we selected two classes of mosquito species that share similar frequency characteristics and compared the performance of ANNs and SVMs.

6.3.4 Results

In order to use the truncated spectrum to differentiate among 10 classes, input and output layers comprised 487 and 10 neurons, respectively. We used two hidden layers as suggested in previous studies (Chen et al. 2014). A total classification accuracy of 71.53±0.29% was obtained by using an ANN with 100 neurons. Increasing the number of neurons above 100 did not improve the classification among 10 classes. On the other hand, in order to use MFCCs as classification features, input and output layers comprised 12 and 10 neurons, respectively. The classification performance in this case was comparable to the one obtained by using the truncated spectrum (Table 6-1).

Table 6-1. Effect of the increment of the number of neurons on the classification accuracy and comparison between the truncated spectrum and MFCCs

Class	**Accuracy per number of neurons in the hidden layer**				**MFCCs**
	Truncated spectrum				
	10	**20**	**100**	**200**	**100**
Ae. aegypti (♂)$^{+}$	89.06	90.00	90.73	91.60	91.13
Ae. aegypti (♀)*	58.28	56.91	59.83	58.07	63.10
Cx. stigmatosoma (♂)^{x}	82.82	83.90	85.07	83.87	83.22
Cx. stigmatosoma (♀)^{*,o}	64.14	64.83	65.07	63.77	65.63
Cx. tarsalis (♂)^{x}	55.36	54.07	53.63	55.50	57.46
Cx. tarsalis (♀)^{o}	77.80	77.57	79.27	80.07	78.16
Cx. quinquefasciatus(♂)$^{+,x}$	57.58	61.60	61.93	60.53	65.12
Cx. quinquefasciatus (♀)*	53.84	61.13	62.40	63.87	57.8
D. simulans♠	71.42	75.30	75.80	76.73	73.45
M. domestica♠	77.08	80.07	81.57	81.43	76.26
Total Classification Accuracy ±Confidence interval (%)	**67.78±1.14**	**70.57±0.19**	**71.53±0.29**	**71.52±0.36**	**71.08±0.25**

From the analysed classes corresponding to females, *Ae. aegypti* and *Cx. quinquefasciatus* share a special interest due to their competence as pathogen transmitters. Both classes were misclassified during the classification process (Figure 6-1a). A comparison between their WBF distributions reveals there is a 97. 22 % of overlapping data (Figure 6-1b).

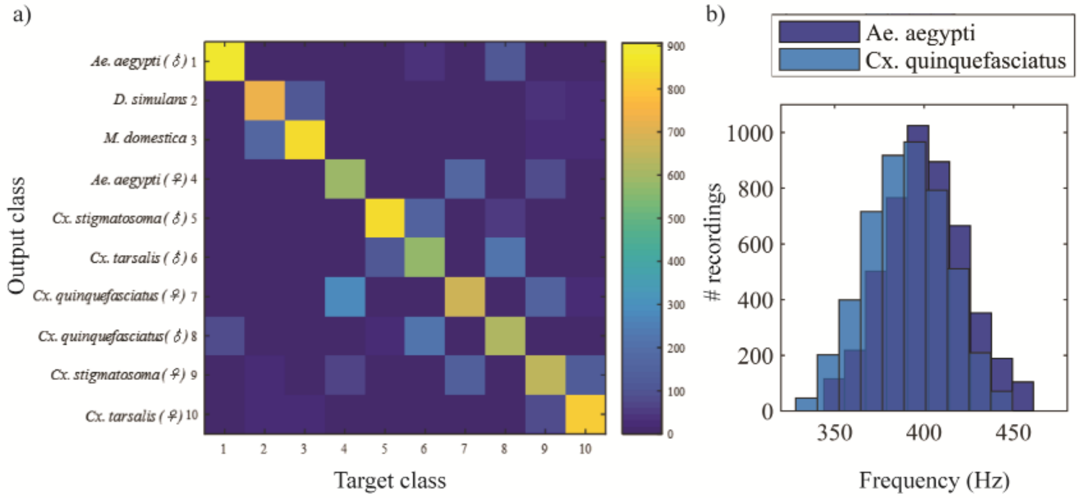

Figure 6-1. Analysis of the classification among ten classes of dipterans. (a) Confusion matrix indicating the classification results. Cell colour indicates the number of samples classified within each class. (b) Comparison of the wingbeat frequency distributions of females *Ae. aegypti* and *Cx. quinquefasciatus*.

By reducing the classification problem to a differentiation between *Ae. aegypti* and *Cx. quinquefasciatus* females, we further explored the possibility of differentiating species with similar frequency characteristics. A 100-neuron ANN reached a maxim accuracy of 69.50% by using MFCCs as classification features (Table 6-2). It was possible, however, to increase the classification accuracy by implementing SVMs. We optimized the parameters of the SVM over the training data set selecting the maximum classification rate and the lowest number of support vectors (Table 6-3). The maximum classification rate obtained was 72.26±3.98 %. Despite the increment of the accuracy was not higher than 10%, the overall performance of the SVMs was superior (Table 6-4).

***Table 6-2**. Confusion matrix*

Output	*Ae. aegypti*(♀)	671	272
	Cx. quinquefasciatus(♀)	338	719
Accuracy = 69.50% **Number of samples classified correctly = (1390/2000)**		*Ae. aegypti*(♀)	*Cx. quinquefasciatus*(♀)
		Target	

Table 6-3. Grid search for the optimization of the regularization parameter C and the polynomial degree γ. Top values indicate acuracy bottom values indicate the number of support vectors

		γ			
		2^{-6}	2^{-4}	2^{-2}	2^{4}
C	2^{-4}	62.31±5.27	66.72±3.40	66.82±5.69	50.00±8.86
		5609±53.5	4875±38.7	5597±76.8	6000±86.6
	2	66.24±4.52	71.27±3.58	70.17±5.24	59.57±6.47
		4786±67.9	4150±57.8	4744±41.7	6000±44.7
	2^{4}	67.38±6.78	72.10±6.45	66.30±4.83	
		4696±30.4	4064±38.7	4532±42.3	
	2^{-8}	68.62±3.41	72.26±3.98	65.35±6.87	
		4373±79.8	3730±55.1	4369±59.6	
	2^{-10}	70.00±4.78	70.87±4.08		
		4215±43.7	3490±31.6		
	2^{-14}	71.14±5.02			
		3984±61.5			

Table 6-4. Performance comparison between ANNs and SVMs on the detection of *Ae. aegypti*.

	Accuracy (%)	**Sensibility**	**Specificity**
ANN	69.28±1.22	0.665	0.725
SVM	77.09±3.19	0.804	0.721

[redacted] Discussion of results and contribution to the research field

Despite the recent advances in acoustic-based surveillance of mosquitoes and dipterans, classifying two species with similar WBF distributions is a prevalent problem (Chen et al. 2014, Mukundarajan et al. 2017). Here, we use different approaches to classify species that share similar frequency distributions by evaluating two different classification features. Our results show it is possible to use a short dimension array of MCCFs to represent the spectral information of mosquito's wingbeat without negatively affecting the accuracy of the classification.

By using ANNs it was possible to reach classification rates comparable to previous works (Chen et al. 2014, Potamitis 2014). The truncated spectrum and MFCCs produced similar outcomes. The truncated spectrum, however, only contains the spectral information about the first and second harmonic while the MFCCs represented the whole spectrum through 12 coefficients. Hence, using MFCCs stands as an efficient option since it facilitates the use of classification algorithms that computationally are more demanding.

The maximum accuracy was obtained by using SVMs was ~77%. Although from the mosquito surveillance perspective this accuracy is not enough to develop an automatic identification method, we consider that a further exploration of the classification features and new classification methods will lead to improving the accuracy. We attribute this limitation to the overlapping of WBF frequency distributions among the species analysed in this study. The low classification accuracy between females *Ae. aegypti* and *Cx. quinquefasciatus* appears to be explained by this fact. Because the truncated spectrum only provides information of frequency components between 100 and 2000 Hz and due to the characteristics of the database, it is not likely that noise influenced the classification. Including the rest of the spectral information represented by MFCCs increased the accuracy of the classification between females *Ae. aegypti* and *Cx. quinquefasciatus*. This result suggests that the information contained in the rest of the spectrum increased the accuracy. It is necessary to evaluate what frequency components are influencing the classification.

Using SVMs instead of ANNs increased the accuracy of the classification as well. The RBF kernel was used similarly than in previous works and slightly outperformed the ANNs (Bisgin et al. 2018). Although the exact reason for this improvement is difficult to explain and is out of the reach of the present work, this result reveals the advantages of exploring different classification approaches. Previous studies have tested machine learning and deep learning approaches to classify optoacoustic recordings of mosquitoes. A Bayesian classifier based on k-Nearest-Neighbours reached an accuracy of ~ 79% in the classification of the same 10 classes used in our experiment. This approach required to include, besides spectral information, additional classification features based on the circadian rhythm of each one of the species (Chen et al 2014). Although the authors mention the problem of classifying two species with similar WBF distributions, it is unclear the impact of this issue on the classification. On the other hand, deep learning approaches have reach higher classification rates. Classifiers based on Convolutional Neural Networks (CNNs) use spectrogram images for training and classification (Kiskin et al. 2018). The main advantage of this approach is that, by using CNNs, not only spectral traits but the time-varying characteristics of flight-tones influence the classification process. By using this approach, an accuracy of 96% was reported in the classification of six species that presumably share similar WBF distributions. Regretfully, the distributions were not reported and it is not possible to determine if this approach overcome the challenge of differentiating species with similar WBF distributions.

In this chapter, we reviewed acoustic approaches to improve surveillance strategies. In addition, we briefly investigated the feasibility of using flight-tones to differentiate species with similar WBF distributions. Nevertheless, the synergy between engineering and biology generates novel thoughts. From the engineering perspective, a deeper exploration of the characteristics of flight-tones should provide information to improve the performance of the classification algorithms. From the biological perspective, exploring the nature of the features used to improve the classification might lead us to highlight relevant acoustic characteristics associated with mosquito communication.

Chapter 7: Conclusions and future work

[redacted]Conclusions

We have identified three interconnected targets to improve mosquito control and surveillance strategies: 1) understanding mosquito bioacoustics associated with reproduction, 2) developing acoustic traps and 3) designing algorithms to recognize species by their flight-tones. The information obtained during the exploration of each one of these targets is associated with specific aspects of mosquito control. First, by studying mosquito bioacoustics, we provide valuable information about the mechanisms that modulate swarming and mate selection. This information is critical to improving the implementation of control strategies that rely on reproduction. Second, the development of a custom acoustic trap for indoor conditions constitutes a step forward in the development of an effective acoustic-based trapping technic in Colombia. Finally, classifying mosquitoes by their flight-tones may constitute the basis to develop cost-efficient surveillance tools. Our results contribute to the core of the recent research that is actively improving the accuracy of sound-based mosquito classification algorithms.

The first objective of this thesis was to characterize the auditory response of mosquitoes. We conclude that mosquito sound reception is determined by the non-linear characteristics of the mosquito auditory system. Even though the frequency range of the response does not overpass thousands of Hz in any of the studied species, when we compared the response of *Ae. aegypti* and *An. albimanus,* species-specific characteristics appear to modify the reception of acoustic signals. In males, changes in the autonomous vibrations suggest there are physiological differences between species. In females, we found critical differences in the response between species. It is necessary, however, to study in detail the response of *An. albimanus* in order to understand the particularities of its auditory response. In general, our exploration of mosquito audition revealed the complexity of its mechanisms. We are convinced, however, that the characterization presented in this thesis will lay the groundwork to address future questions, especially on *An. albimanus*. To the best of our knowledge, this is the first study on *An. albimanus* audition.

The second objective of this thesis was to characterize the mosquito acoustic behaviour from the emitter perspective. By using audio and video recordings we have established a relationship between flight-tones and flight-trajectories. These results highlight the relevance of using an adequate recording protocol to obtain meaningful results. Although the election of the recording protocol depends entirely on the research question, it is necessary to consider that flight-tones are communication signals linked to mosquito motion. Hence, combining audio and video offers an ideal opportunity to study their behaviour but the low intensity of flight-tones constrains the implementation of such methods. We conclude there are sex-specific acoustic behaviours associated with *An. albimanus* swarming and mating. Males are actively communicating at a local scale when they perform swarm-like flight patterns and females perform stereotypic behaviours associated with the outcome of a mating attempt. These findings contribute to the literature that, since the last decade, is constantly dissecting acoustic behaviours associated with mosquito reproduction. Understanding mosquito acoustic behaviours underlaying mating is critical to improve current control strategies based on reproduction.

The third objective aimed to develop an acoustic trap for *Ae. aegypti* surveillance. Capturing mosquitoes is a challenging task. In the market, successful traps use CO_2 as principal attractant — e.g. Mosquito Magnet ® (American Biophysics Corporation, PA, USA) and BG-Sentinel (BioGents, Germany). These traps were designed to capture mosquitoes in open spaces rather than houses. Using CO2, therefore, does not constitute a cost-efficient alternative, for control and surveillance programs in Latin American. In this thesis, we present a novel method to improve acoustic trapping under indoor conditions. We conclude that acoustic attractants synthesized from flight-tones effectively increase the capture rate of males. We also present promising results regarding female attraction. Even though our results are promising, there are details that need to be addressed before accomplishing the development of a commercial trap. It is necessary, for instance, to minimize the intensity of the acoustic cues in order to avoid discomfort to humans. We hope the results presented in this thesis are the first step towards the development of an acoustic trap for males and females, designed for indoor conditions.

The fourth objective aimed to explore flight-tones to recognize species in the context of surveillance. In this objective we addressed the challenge of recognizing species when they share similar WBF distributions. Our results show that by using optoacoustic recordings it is possible to differentiate species with similar WBF distributions with a minimum accuracy of ~70%. This result highlights the opportunity of increasing the effectiveness of classification algorithms by exploring new features and evaluating different classification approaches. Our results generate expectations regarding mosquito surveillance. It is necessary, however, to further explore strategies to obtain a correct classification between closely related species. Microphone and optoacoustic sensors are generating a new paradigm for mosquito species identification. Each one of them offers advantages that can be used in different scenarios. Optoacoustic sensors provide cleaner information compared to microphones and consequently increase the chance of improving the classification accuracy. Mobile microphones, on the other hand, are widespread around the world and can be used to empower the community to participate in surveillance activities. Currently, both approaches are being implemented in real scenarios and therefore, exploring new classification strategies is necessary to generate more efficient and effective tools. Differentiating species with similar WBFs, however, remains the main challenge.

Overall, the results presented in this thesis reveal the potential of combining fundamental and applied research to move forward in the fight against mosquito-borne diseases. By exploring mosquito bioacoustics, it was possible to identify targets to improve control and surveillance strategies. We are convinced this work has expanded the horizon of possibilities regarding the study of mosquito bioacoustics, from fundamental and applied standpoints.

[redacted] Future work

Studying mosquito bioacoustics presents several engineering challenges. One of the achievements during the execution of the present thesis was to set up a laboratory with the technology necessary to address questions from different standpoints. In addition, we standardized methodologies to examine mosquito audition and developed algorithms to analyse, simultaneously, mosquito motion and flight-tones. We hope these tools will be used in the future, to accomplish pioneer research projects on mosquito bioacoustics. Here, we list some of the possible orientations of future research.

-Exploring mechanisms of mosquito audition. To increase our understanding of mosquito acoustic behaviour, it is necessary to answer questions related to the mechanisms underlaying audition. Acquiring and processing the electrophysiological signals of the JO is essential to address further questions. The methods standardized here generate new opportunities of research, oriented to uncover the particularities of antennal ears.

-Expanding the reach of the experiments to study mosquito behaviour in more realistic scenarios. Using semi-field enclosures to address questions about mosquito behaviour will provide information to form a wider picture of the role of acoustic cues in mosquito biology.

-Developing acoustic traps for males and females by combining different attraction modalities. In this thesis, we have shown that it is possible to use different attractants simultaneously without deleteriously affecting the capture rate. We propose to use a combination of hormones and acoustic attractants as the next step to generate novel approaches to improve capture rates for both males and females.

-Developing automatic surveillance devices is one of the primary needs of control and surveillance programs. Increasing the accuracy on the classification of two species with similar frequency characteristics remains the main challenge. However, there are many applications from a surveillance perspective that can be already developed. Monitoring general mosquito population, for instance, does not require high accuracy of classification. We hope results presented here will promote further research in this field.

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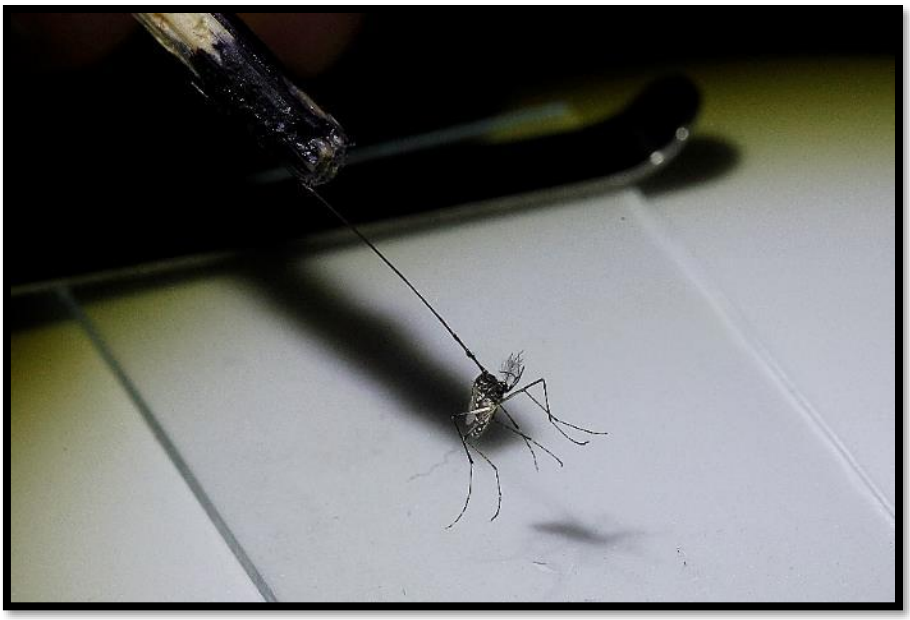
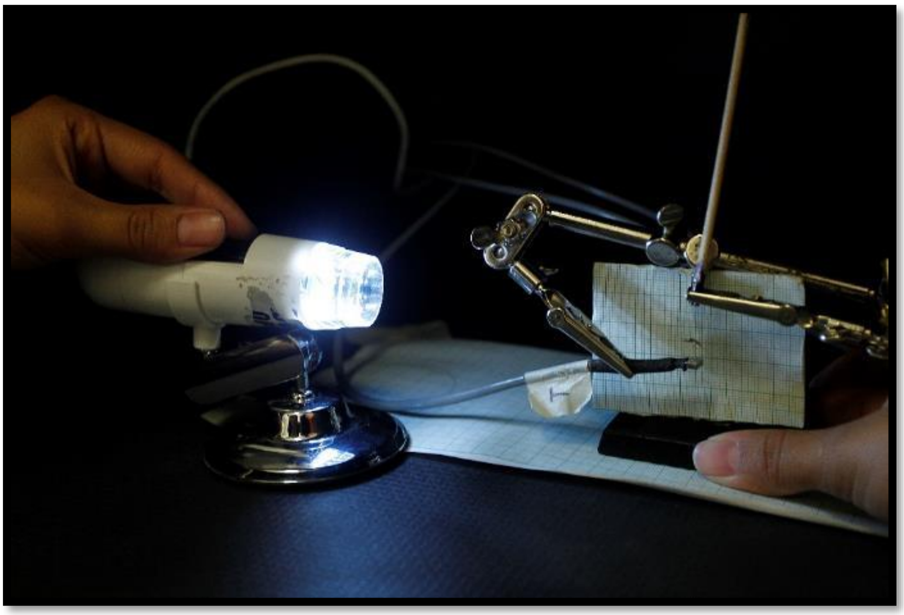
Appendix: Pictures

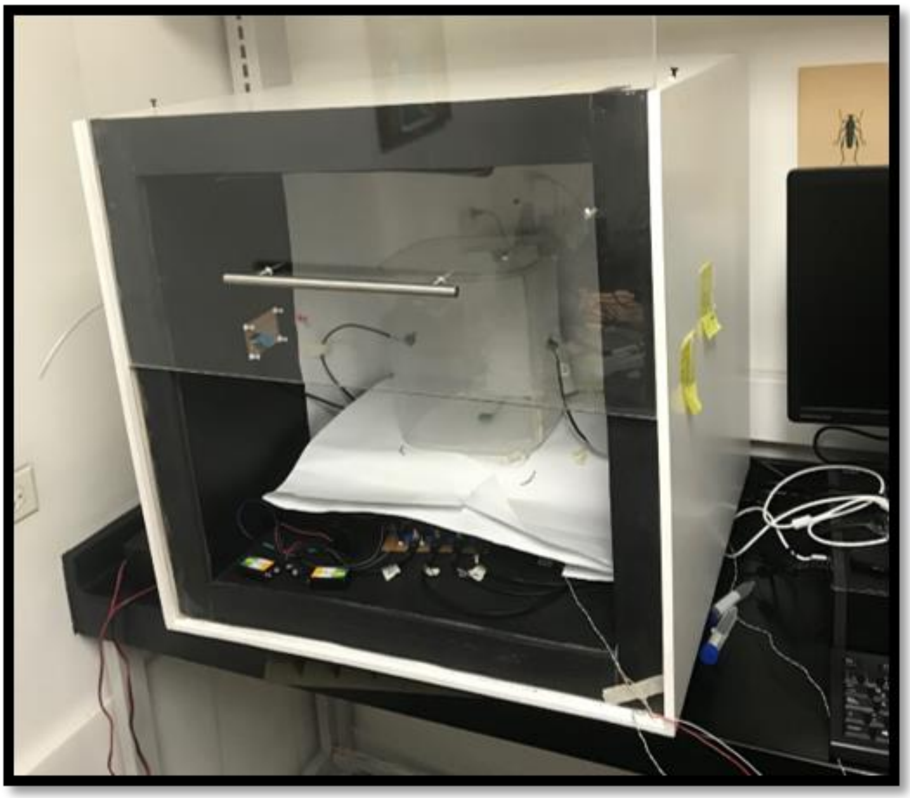
Tethered mosquito



Visual and acoustic recording of a tethered mosquito



Sound proof chamber and the experimental arena used to record mosquitoes flying free



Acoustic trap, indoor experiment set up

Acoustic trap semi-field enclosure for experimentation