Influences of Parasitic Infection and Aging on *Aedes aegypti* Wingbeat Frequency

by

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Abstract

While there are thousands of species of mosquitos, only a few hundred are responsible for transmitting disease-causing pathogens; however, certain of these species, such as *Aedes aegypti* and *Aedes albopictus*, are responsible for the transmission of many urban arboviruses like dengue, chikungunya, and Zika. Due in part to the used tire trade and marine traffic, these mosquitoes can now be found on six continents and in the majority of major urban centers. Mosquitoes have been described as the deadliest animals on earth due to public health concerns; however, many questions remain regarding the ecology and behavior of mosquito as vectors of disease-causing agents. For decades, scientists have noted the acoustic signatures of mosquitoes. These unique wingbeats allow mosquitoes of the same species to locate one another for mating. In recent years scientists have shown that using sensitive acoustic devices like mobile phones, these wingbeat signatures can be used to identify mosquito species without the necessity of complex morphological identifications using taxonomic keys. In this research, I expand upon findings of unique acoustic signatures in mosquito wingbeats and address how additional aspects of mosquito biology may influence wingbeat frequencies. The aim of this work is to understand whether acoustic tools can be used not to identify mosquito species and determine whether mosquitoes are infected with pathogens of veterinary concern. Information on mosquito sex, age and other entomological indicators are essential for public health surveillance of mosquito borne diseases-causing agents. Here we confirm that wingbeat recordings can be used to identify *Ae. aegypti.* We present data on laboratory-reared *Ae. aegypti* infected with the causative agent of canine heartworm, *Dirofilaria immitis,* and show that while there are no clear wingbeat frequencies that can be used to identify mosquitoes infected with *D. immitis* from non-infected

mosquitoes, wingbeat frequency can be used helpful in determining heavy burdens of infective (L3) larvae. We also show that male and female mosquitoes have unique wingbeat signatures. This contribution is important because only female mosquitoes take a bloodmeal and therefore act as vectors of disease-causing agents. We also observed a weak correlation between age and wingbeat frequencies in female mosquitoes, suggesting that wingbeats may be used to identify life history stages such as pre-feeding and post-ovipositing. Results obtained herein provide compelling justification for the integration of acoustic wingbeat recordings in public health entomology. Acoustic recordings may also aid in species identification and as a complementary tool to gather data on infection status, age, and sex.

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Preface

Mosquitoes have contributed to human and veterinary disease for centuries (Rieckmann, 2006; Mullen & Durden, 2019). In 1878, Patrick Mason who was investigating filarial infection in Amoy, revealed mosquitoes as the first hematophagus arthropods that can serve as intermediate hosts of mammalian parasites (Kuno, 2010). Due to their capability of transmitting pathogenic agents, such as malaria parasites and yellow fever, Zika, and dengue viruses, mosquitoes are considered to transmit some of the deadliest agents in the world (Tolle, 2009; Lambrechts et al., 2010; Grard et al., 2014; Mourya et al., 2018; Mullen & Durden, 2019).

Despite the fact that thousands of species of mosquitoes exist, only a few hundred are known to be capable of spreading pathogenic agents (Gubler, 2009; Mullen & Durden, 2019; Gubler, 2009), and are known as mosquito vectors of disease-causing agents. One of the most significant mosquito vector species to human health is *Aedes aegypti,* also known as the yellow fever mosquito, since its global expansion contributed to the emergence and global spread of yellow fever in the 19th century. *Ae. aegypti* is thought to be such a successful medical vector because it utilizes man-made containers as breeding sites, thrives in urban environments, and has a preference for feeding on humans (Mullen & Durden, 2019). *Ae. aegypti* is derived from African ancestral populations, and it consists of two supbspecies, *Aedes aegypti formosus* and *Aedes aegypti aegypti*. *Ae. a. formosus* once was rural, however; it has recently adapted to urban environments (Powell & Tabachinick, 2013; Gloria-Soria et al., 2016; Mullen & Durden, 2019). Recent estimates suggest that by the year 2050, about 50% of the world's population will be threatened by two mosquito vectors*, Aedes albopictus* and *Aedes aegypti* (Kraemer et al., 2019).

Ae. aegypti mosquitoes belong to the family Culicidae and are diurnal mosquitoes that are known to be the vectors of human pathogens like dengue, chikungunya, Zika, and yellow fever

viruses (Tolle, 2009; Mourya et al., 2018; Grard et al., 2014; Lambrechts et al., 2010; Mullen & Durden, 2019). As soon as both female and male mosquitoes emerge, they start to feed on sugar sources such as nectar, honeydew, spoiled or damaged fruits, or even damaged plant leaves and stems (Mullen & Durden, 2019), and they continue to feed upon sugar sources frequently as adults. It is known that some domestic mosquito species, *Ae. aegypti* and *Anopholes gambiae*, rarely feed upon sugar because they live close to their hosts; they use blood for both energy and reproduction (Mullen & Durden, 2019). Unlike male mosquitoes that only feed on sugar sources, female mosquitoes take bloodmeals. Female mosquitoes can take amino acids from honeydew and floral nectar that are important source for initiating egg production and development, however; they don't contain sufficient amounts of amino acids for egg development (Mullen & Durden, 2019). Vitellogenesis, the process of yolk formation begins only after taking a bloodmeal from a host (Briegel, 1985). Water is another important source for mosquito survival and reproduction because mosquitoes require water to oviposit. *Aedes* species generally prefer habitats in two categories: flood water or standing water in containers. Flood waters include floodplains, saltmarshes, and snow pools, whereas container water includes water on leaf axils, in tree holes, or in artificial container (Mullen & Durden, 2019). Because mosquitoes require a water source for egg oviposition, proximity of water is often associated with mosquito prevalence and distribution.

Canine heartworm disease (CHD), one of the most important veterinary vector-borne disease in the United States (US), is caused by a filarial nematode *Dirofilaria immitis. D. immitis* can be transmitted by many species of mosquito belonging to different genera, such as *Aedes, Anopheles, Culex,* and *Ochlerotatus* (Ledesma & Harrington, 2011; Vieira et al., 2014; Mullen & Duren, 2019). The prevalence of heartworm (HW) in the United States (US) has been steadily

increasing along with an increase in temperature (Reiter, 2001; Ledesma & Harrington, 2011; "Climate Changes in the United States," 2014; American Heartworm Society, 2016; American Heartworm Society, 2012-2019). According to the American Heartworm Society (AHS), the average number of HW cases in domestic dogs increased by 21.7 % from 2013 to 2016, although the distribution of HW in the US has not been changed drastically (American Heartworm Society, 2016). Along with increasing HW prevalence, researchers have demonstrated loss of efficacy of macrocyclic lactones (ML) used as preventives for HW infection, and have identified drug-resistant HW strains in the US. This is very concerning to veterinarians who have relied on ML for the last three decades as a method of HW prevention (Bowman, 2012; Campbell, 2012; Pulsaki et al., 2014; Blagburn et al., 2016).

In order to understand *D. immitis* transmission dynamics, it is necessary to study vector ecology and competence. Laboratory models of *D. immitis* have been maintained in *Ae. aegypti* mosquitoes for decades (Serrão et al., 2001; Kershaw et al., 1955). A dog infected with *D. immitis* may have microfilariae (MFF) circulating in the blood (The Centers for Disease Control and Prevention, 2012; American Heartworm Society, 2018). When a female mosquito takes a blood meal from a microfilaremic dog, MFF are ingested. These MFF molt through different developmental stages, and eventually become the infectious L3 stage (Ledesma & Harrington, 2011; The Centers for Disease Control and Prevention, 2012). Mosquito vector ecology and physiology play a critical role in the development of *D. immitis* and influence the transmission and cycling of this pathogen. Even though mosquitoes are studied extensively as vectors of disease-causing agents, there is little understanding of the bidirectional feedback between the parasite and mosquito behavior (Scott et al., 2002; Ferguson et al., 2010). Much emphasis has been placed on mosquito-borne diseases in vertebrate hosts; however, the impacts of infection on

mosquito vectors are less studied. In order to better understand HW epidemiology, it is critical to examine mosquito ecology and physiology in response to pathogen infection.

One of the least understood areas of mosquito biology is wingbeat behavior. The "buzzing" sound which mosquitoes produce and humans hear is actually the sound of mosquitoes moving their wings at a certain high-pitched frequency. This frequency can be heard by other mosquitoes and is a key feature in mate selection. It is thought to allow mosquitoes of the same species to locate one another for mating (Cator et al., 2011). Success in mating leads to effective mosquito reproduction. When male and female mosquitoes mate, each uses wingbeat to locate and recognize the opposite sex (Gibson & Russell, 2006; Cator et al., 2011; Ouyang et al., 2015; Mukundarajan et al., 2017). They recognize and respond to pure tones, by altering wingbeat frequency, so that their flight tone frequencies match for mating (Gibson & Russell, 2006). The matching of flight tone frequencies in male and female pairs is called harmonic convergence (Cator & Harrington, 2011). Each species' wingbeat is like a fingerprint (Mukundarajan et al., 2017). All mosquito species have unique wingbeat frequencies which allow mosquitoes of the same species to find one another (Mukundarajan et al., 2017). A recent study demonstrated that acoustic recordings of female mosquitoes with cell phones can be used as a surveillance method for identifying species (Mukundarajan et al., 2017). Mukundarajan and her team collected and tested both wild and laboratory-raised mosquito species. If a species had different strains, the different strains were also tested for acoustic analysis.

Despite the eradication efforts, mosquito-borne pathogens are responsible for over 4 million deaths per year ("Mosquito-borne diseases," 1970). Aside from their role as vectors of infectious agents, mosquitoes have affected economy development and livestock management (Chuma et al., 2010; Lwande et al., 2013). Because of their ability to transmit pathogenic agents

and to impact to human and animal health, mosquitoes are one of the most studied animals or subjects. While vector control and insecticide resistance are the most important areas of mosquito biology, understanding of mosquito behavior and wingbeat frequency and mating is lacking (Scott et al., 2002; Ferguson et al., 2010). To better understand mosquito ecology and pathogen dynamics, it is important to understand whether infection by a mosquito-borne pathogen influences wingbeat and mating success. *D. immitis* is a prevalent mosquito-borne pathogen and an ideal laboratory model to examine the effect of infection on mosquito wingbeat (Simon et al., 2012). Of four mosquito genera that are capable of transmitting *D. immitis*, *Ae. aegypti.* is particularly considered to be easiest to rear and to manipulate in the laboratory (Ledesma and Harrington, 2011; Vieira et al., 2014; Dickson et al., 2018; Mullen and Duren, 2019). Additionally, *Ae. aegypti* is the most important medical vector of human vector-borne diseases, making it an ideal species to evaluate the impact of infection and aging on wingbeat ecology.

Using a laboratory model of *D. immitis* in *Ae. aegypti* mosquitoes, we aim to evaluate the impact of pathogen infection, sex and age on mosquito wingbeat acoustic signatures.

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Chapter 1:

An Investigation of *Dirofilaria immitis* Infection and its Effects on Mosquito Wingbeat Frequencies

1. Introduction

Heartworm (HW) disease, caused by infection with the filarial nematode *Dirofilaria immitis* and transmitted by female mosquito vectors, is widely recognized as one of the most important veterinary diseases in the United States (Companion Animal Parasite Council, 2016). The primary vertebrate hosts of *D. immitis* are dogs and wild canids; however, it is also known to infect cats, ferrets, otters, other wildlife species, and humans throughout the world (Genchi and Kramer, 2019). Although these infections in less traditional vertebrate hosts typically do not reach patency with circulating microfilariae, HW can mature, even to the adult stage, and disease may manifest in these hosts as well (Bowman and Atkins, 2009). Over 100,000 dogs in the US are diagnosed with canine HW infection each year, with infection rates steadily increasing in many parts of the US (Self et al., 2019). The trend of increasing HW prevalence may be due to a combination of developing resistance to macrocyclic lactone-based preventatives (Bowman, 2012; Wolstenholme et al., 2015; Blagburn et al., 2016), climate change (Genchi et al., 2011; Wang et al., 2014; Bowman et al., 2016) and longterm spatio-temporal changes in mosquito vector populations, particularly *Aedes* spp. mosquitoes (Kraemer et al., 2015; Bowman et al., 2016; Hahn et al., 2017). Understanding the roles that each of these factors play in the transmission of *D. immitis* is critical to developing and targeting prevention efforts (Self et al., 2019).

The ecology of *D. immitis* in vector populations is complicated by the fact that unlike most other mosquito-borne pathogens, which are typically highly vector-specific, *D. immitis* can be transmitted by many mosquito species in several genera, including *Aedes, Anopheles, Culex,* and *Ochlerotatus* (Ledesma and Harrington, 2011; Vieira et al., 2014; Mullen and Durden, 2018). The distributions and densities of mosquitoes are highly geographically variable and routine vector surveillance rarely monitors for pathogens and vectors that are less likely to affect humans and human health (Ledesma and Harrington, 2011; Self et al., 2019). Improved vector surveillance methods, and particularly vector surveillance methods that include data regarding vector infection status, could provide invaluable insight into the eco-epidemiology of *D. immitis* transmission dynamics and help to evaluate infection risk and inform vector control efforts (Self et al., 2019).

Another means to further understand vector species could involve mosquito wingbeat frequencies, which have long been understood to be a goldmine of information regarding mosquito species identification (Kahn et al., 1945; Jones, 1964; Moore et al., 1986), sexual recognition (Roth, 1948; Jones, 1964; Gibson and Russell, 2006; Warren et al., 2009; Gibson et al., 2010), and possibly mate selection and sympatric species divergence (Ritchie and Immonen, 2010; Cator et al., 2009). Recent work by Mukundarajan et al. (2017) also shows that these unique wingbeat frequencies can be captured by commercially available mobile phones, even when used in field settings with background acoustics, offering a potential new paradigm for mosquito surveillance. Abuzz, a new citizen science project developed at Stanford University, aims to bring this tool for vector identification to the masses and to encourage citizen-science and community-based vector surveillance. While this new technology for vector surveillance is

already invaluable, the potential for determining vector infection status could make it even more useful for understanding *D. immitis* transmission dynamics.

Parasite infection has been shown to affect arthropod vectors in a variety of ways, ranging from body size (Lyimo and Koella, 1992; Tseng, 2004) to feeding behavior (Molyneux and Jefferies, 1986; Moore, 1993; Koella et al., 1998; Cator et al., 2013) and fecundity (Javadian and Macdonald, 1974; Christensen, 1981; Suneja et al., 2003; Vézilier et al., 2012). While the exact effects of *D. immitis* infection on their mosquito hosts remain understudied, we hypothesize that the effects of parasitism by *D. immitis* upon its invertebrate hosts may cause a marked variation in the wingbeats of infected mosquitoes. If this variation could be identified and algorithms designed to determine infection status through wingbeat recording using regular commercial phones, it could create an easy, crowd-sourced alternative to the currently utilized, costly parasite surveillance which often requires time-consuming assays and a well-trained work force for identification. For example, identifying microfilariae in mosquitoes may require dissecting field-caught mosquitoes individually, or processing mosquitoes using protein- and nucleic acid-based assays which are often require high-tech laboratory facilities. The goal of this study was to determine the potential of commercial phones and open-source wingbeat analysis programs for *D. immitis* vector surveillance by comparing wingbeats frequencies between *Aedes aegypti* (L.) mosquitoes infected with *D. immitis* and mosquitoes not infected with *D. immitis*.

2. Methods

2.1 Mosquito Rearing and Blood Feeding

The *Ae. aegypti* Liverpool (LVP) strain was used as a laboratory model of *D. immitis* vectors. The strain was originally obtained from University of Georgia and maintained by in vitro feeding and rearing methods at Auburn University College of Veterinary Medicine. Globally, this mosquito strain has been used to maintain *D. immitis* in laboratory settings since 1977 and they have been maintained as laboratory colonies for *D. immitis* continuously since then. Mosquito larvae were raised in plastic pans for one week and then transferred to emergence containers once they had pupated. Emergence containers were then placed into two $30.48 \times 30.48 \times 30.48$ cm^3 mosquito cages (Bioquip Products, Inc., Rancho Dominguez, CA, USA) and labeled I (infected) or N (non-infected). Group I mosquitoes were fed microfilaremic dog blood (600−2,200 mff/mL) and group N mosquitoes were fed blood from a non-infected dog nine days after emergence began. Microfilaremia levels were determined by performing two 20 µL blood counts. A glass bell-shaped membrane feeder (Lillie Glassblowers, Atlanta, GA, USA) covered with a single layer of sausage casing (hog intestine; Syracuse Casing Co., Inc, Syracuse, NY, USA) was placed on top of each cage to feed mosquitoes. Warm water (37 °C) was circulated around the reservoir of blood using a temperature-controlled circulating pump (Cole-Parmer, Vernon Hills, IL, USA). Mosquitoes were provided with 4 mL of blood for 2 h. Approximately 1800 mosquito larvae were counted on D-15, or 15 days prior to blood feeding when larvae were set from hatched eggs (Fig. 1-1), and reared in each cage. Emerging mosquitoes in cages were provided with sugar cubes and cotton pads which were saturated with deionized water. After four days, emergence containers were removed. Mosquitoes were fed a blood meal nine days after emergence began. Blood was collected from non-infected and experimentally infected microfilaremic dogs. A glass bell-shaped membrane feeder (Lillie Glassblowers, Atlanta, GA, USA) covered with a single layer of sausage casing (hog intestine;

Syracuse Casing Co., Inc, Syracuse, NY, USA) was placed on top of each cage to feed mosquitoes. Warm water (37 °C) was circulated around the reservoir of blood using a temperature-controlled circulating pump (Cole-Parmer, Vernon Hills, IL, USA).

2.2 Mosquito Recordings

Mosquitoes were aspirated individually into collection vials and recorded for approximately one minute using the Voice Memo app for iPhone (Fig. 1B). Mosquito wingbeats were recorded before mosquitoes were fed, and at time points targeting each HW developmental stage (total of 4 recordings): day -1 before feeding (BF), day 5 (L1), day 9 (L2) and day 16 (L3). An iPhone 7 plus or 8 with operating systems iOS 10 and 11 was used (Apple, Inc., Cupertino, CA, USA). These phones were updated whenever new software was released. Previous work has shown that signal-to-noise ratios for these phone models are indistinguishable in performance (Mukundarajan et al., 2017). For the BF recording, 10 female mosquitoes were randomly selected from each cage (I & N) and recorded. Mosquitoes were confirmed as female under a compound microscope at 40X magnification using the appearance of the antennae (pilose) and palps (short as compared to males). On days 5, 9 and 16, the same procedures were used for recording mosquitoes. On each sampling day, 10 female mosquitoes from cage N were collected, recorded and confirmed as female. Up to 30 female mosquitoes from cage I were recorded using the same methods on each sampling day, the goal being to record at least 10 infected females. All recordings were collected between 8 and 10 a.m. (CST), and the baseline room noise ranged from -90 dB to-27 dB in the room where the wingbeat recordings were collected.

The infection and recording experiments were repeated 17 times (17 batches). For each time (batch) we used two cages, one cage I of *D. immitis*-infected mosquitoes (fed microfilaremic

blood) and one cage N of non-infected mosquitoes (fed amicrofilaremic blood). No size differences were noted in mosquitoes.

2.3 Infection Confirmation and Parasite Quantification

In addition to confirming sex, the cage I mosquitoes were examined for *D. immitis* larval stages. The larvae were identified to stage using length and appearance, and quantified. The classification scheme for L1, L2, and L3 and descriptions of the morphology used to categorize larvae are shown in Fig. 2. In the mosquito, the L1 and L2 stages of *s* are found in the Malpighian tubules in the abdominal cavity.

2.4 Data Analysis

Recorded mosquito wingbeat files were analyzed using an in-house frequency analysis program written in Python 3.0 (Mukundarajan et al., 2017). Wingbeat frequencies obtained from the program were averaged (average frequency in Hz over time) over recording time and analyzed in R using linear mixed-effects models, which were fit by restricted maximum likelihood (REML) using the R package “nlme” (Pinheiro et al., 2019). To avoid pseudoreplication cage column was added to our data sheets and treated as a random effect. Mosquitoes aspirated from same cage was assigned with same cage number. Other effects, infection and stage, were treated as fixed effects. Interaction between *D. immitis* developmental stages and infection was analyzed. Number of *D. immitis* stages present compared to wingbeat frequencies was also analyzed.

3. Results

A total of 1669 mosquitoes had wingbeats recorded and analyzed from 482 infected and 1175 non-infected female mosquitoes. *D. immitis* infected and non-infected mosquitoes at each developmental stage were analyzed (Fig. 1-3). Overall, we found no significant effect of *D. immitis* infection on wingbeat frequency (P=0.86; β=1.78 Hz ± 19.41 Hz; ±95 % CI). Wingbeats of mosquitoes from cage I had 5.32 Hz (±43.88 ± 95 % C.I.) higher frequencies than wingbeats of mosquitoes from cage N in the BF stage (P=0.81). For the L1 stage, cage I mosquitoes had wingbeat frequencies 10.67 Hz (±14.20 ± 95 % C.I.) lower than cage N mosquitoes (P=0.14). For the L2 stage, mosquitoes from cage I had wingbeat frequencies 13.85 Hz (±18.47 ± 95 % C.I.) lower than mosquitoes in cage N (P=0.14). Mosquitoes from cage I had 4.58 Hz (±10.46 ± 95 % C.I.) lower wingbeat frequencies than mosquitoes from cage N in the L3 stage (P=0.39). Wingbeat frequencies of mosquitoes analyzed in relation to the number of larval parasites at different stages suggests a shift in wingbeat frequency with increased parasite burden (Fig. 1-4). The overall trend was an increase in average wingbeat frequency with increasing number of larvae at both the L1 (β = 3.72 Hz ± 4.16 Hz; ±95 % C.I.; P = 0.08; R^2 = 0.04) and L2 (β = 3.38 Hz ± 4.43 Hz; ±95 % C.I.; P = 0.13; R^2 = 0.04) stage recordings, though neither relationship was statistically significant. However, we did find a significant effect of increasing number of L3 larvae resulting in decreased wingbeat frequency (β=7.32 Hz ± 7.14 Hz; ±95 % C.I.; P=0.04; R^2 = 0.03; Fig.1-4).

4. Discussion

We found no significant difference in wingbeat frequency between *Ae. aegypti* mosquitoes infected with *D. immitis* and those not infected with *D. immitis* (Fig. 1-3). However,

we did find a significant relationship between number of L3 larvae present in infected mosquitoes and wingbeat frequency (Fig. 1-4). This suggests that while it may be these 2 groups of mosquitoes are not distinguishable using average wingbeat frequencies, identifying mosquitoes with high *D. immitis* burdens may be possible. Vectorial capacity and transmission risk of *D. immitis* increase with increased tolerance of the parasite by the vector, so vectors with higher parasite burdens are likely to be more tolerant of infection and thus pose a higher transmission risk (Dharmarajan et al., 2019). Thus, wingbeat frequency may hold the key to identifying vectors with high capacity to transmit HW. More work is needed to compare wingbeat frequency between heavily parasitized individuals and those with low or nonexistent infections.

Despite conducting wingbeat recordings in semi-soundproof containers, it is possible that ambient laboratory and outdoor sounds may have included in the recordings. While the algorithm filters out sounds that fall outside the known mosquito wingbeat ranges (200–700 Hz) by disregarding frequencies outside the expected range (500–1500 Hz) (Mukundarajan et al., 2017), it is possible that external sounds were recorded and not appropriately filtered. However, over 1600 recordings were collected here under the same laboratory conditions and no significant differences in wingbeat frequencies being detected aside from the impact of parasite burden. Additionally, disparities in the sample sizes between infected and non-infected mosquitoes were a result of many females dying before their parasites reached the L3 stage.

While our results are promising for the utility of commercially available phones and software to detect heavy *D. immitis* infection among *Ae. aegypti*, it is possible that this trend is not universal to all vectors of HW. While *Ae. aegypti* is considered a competent vector of *D. immitis* (Vezzani et al., 2011; Brown et al., 2012), some strains of *Ae. aegypti* are also known to

be refractory to *D. immitis* infection; it has been suggested that inter-strain dynamics of both parasite and vector may determine competence (Nayar and Knight, 2002; Tiawsirisup and Nithiuthai, 2006; Ledesma and Harrington, 2011). Furthermore, the anthropophilic nature of *Ae. aegypti* as well as its replacement by *Aedes albopictus* (Skuse) across much of its former North American range reduces its potential role in the actual transmission of *D. immitis* in the US (O'Meara et al., 1995; Ledesma and Harrington, 2011; Kraemer et al., 2015). Examinations of the effects of *D. immitis* infection on wingbeat frequency of more prominent vectors of *D. immitis*, such as *Ae. albopictus*, could provide even more valuable insight into HW transmission dynamics.

Wingbeat frequencies showed no significant differences across *D. immitis* developmental larval stages and no significant difference was found between infected and non-infected mosquitoes, even though the burden of parasites did appear to impact wingbeat frequencies in L1 and L2 stages. While it is possible that we might have seen more significant differences with an increased sample size, the observed lack of variation outside of parasite burden suggests that the algorithm used (Mukundarajan et al., 2017) is robust enough to confirm *Ae. aegypti* species identification even with natural physiological variation. The Abuzz tool (Mukundarajan et al., 2017, http://www.abuzz.stanford.edu) is likely to be effective in identifying mosquitoes (*Ae. aegypti*) in the field with accuracy, although very high parasite burden may affect this. Mosquito wingbeat frequencies may need to remain in the same range for conspecifics to find one another for mating events to occur. Further work investigating the range of wingbeat frequencies and mate attraction may reveal whether infection can impact mating success and fitness.

Other mosquito-borne parasites may also have even more marked effects on vector wingbeat frequencies than does *D. immitis*. Unlike *D. immitis*, which develops in the Malpighian

tubes of its hosts (Angela, 1960), another filarial parasites, *Wuchereria bancrofti*, the causative agent of human lymphatic filariasis, and *Brugia pahangi* and *Brugia malayi* have L2 larvae which develop in the thoracic muscles that also control wing movement (Wharton, 1957; Berry et al., 1986; Somerville et al., 2019). Berry et al. (1986) demonstrated that *B. pahangi* had a significant effect on spontaneous flight activity of *Ae. aegypti*. First 8 days of post-infection (PI), mosquitoes infected with fewer than 20 worms were significantly more active with up to 3 times than uninfected ones and their flight activity decreased PI 8–12 corresponding to the later stages (L2 and L3) in the muscles. After PI 12, flight activity of mosquitoes infected with 1–10 worms equivalent to infected mosquitoes at same day after emergence, indicating L3 larvae migrated out from the muscles (Berry et al., 1986). Future work exploring the impacts of *Brugia* species and *W. bancrofti* development on vector wingbeat frequencies may show unique wingbeat signatures which may be used to distinguish infected mosquitoes from non-infected mosquitoes at a stage before infective (L3) larvae develop.

5. Conclusions

The results of our study showed no significant difference in time and population-average wingbeat frequencies between *D. immitis* infected and non-infected mosquitoes. However, we did find a significant effect of number of L3 larvae on wingbeat frequencies of *D. immitis* infected individuals. These results suggest the while wingbeat frequency may not be useful for determining infection vs. non-infection, it may be able to detect vectors with heavy parasite burdens. Future work should aim to investigate the relationship between parasite burden and wingbeat frequency, as well as investigating these effects for different vector species and different parasites.

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Figures and Tables

Figure 1-1. Mosquito rearing and recording timeline
(A) The timeline was ranged from day -16 (D-16) when eggs were set to develop into larvae to day 0 when adult female mosquitoes were given a *D. immitis* infected or non-infected bloodmeal to day 16 (D+16), the stage when L3 larvae should be present in adult female mosquitoes. (B) Individual mosquito in collection vial was recorded in semi-soundproof container with Voice Memos app.

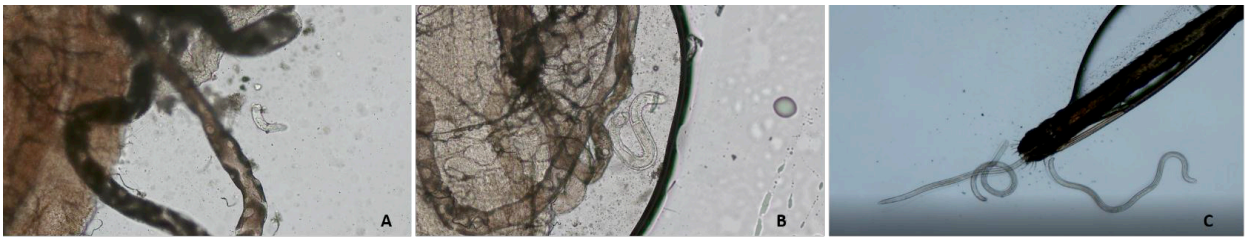

Figure 1-2. Images captured of *D. immitis* L1 (A), L2 (B), and L3 (C) larvae (40X) in laboratory reared *Ae. aegypti*
(A) "Sausage stage" (L1) larvae are short (170–230 µm) and stout and have a thread-like structure at the posterior end. (B) L2 larvae are longer (400–780 µm) and thinner than L1 larvae. (C) The longer infective L3 stage larvae are 900–1,200 µm in length and are typically found the mosquito proboscis. Occasionally, upon examination, some L3 larvae were still detected in the Malpighian tubules.

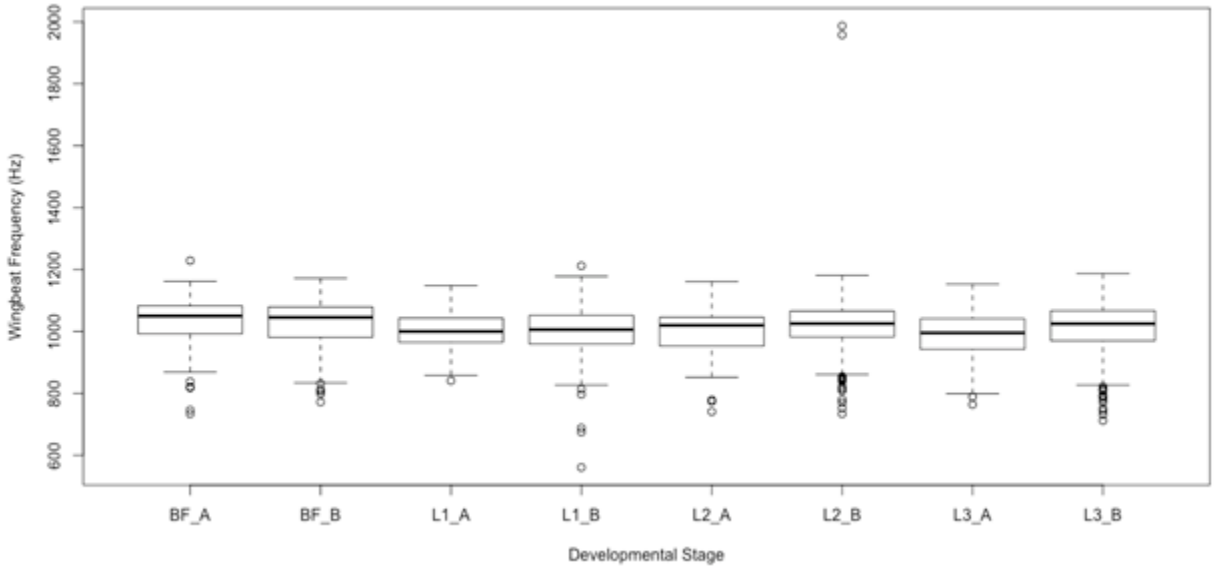

Figure 1-3. Wingbeat frequencies of *D. immitis* infected (cage I) and non-infected (cage N) female mosquitoes
The x- axis represents developmental stages (L1, L2, and L3) of infected (I) and non-infected mosquitoes (N).

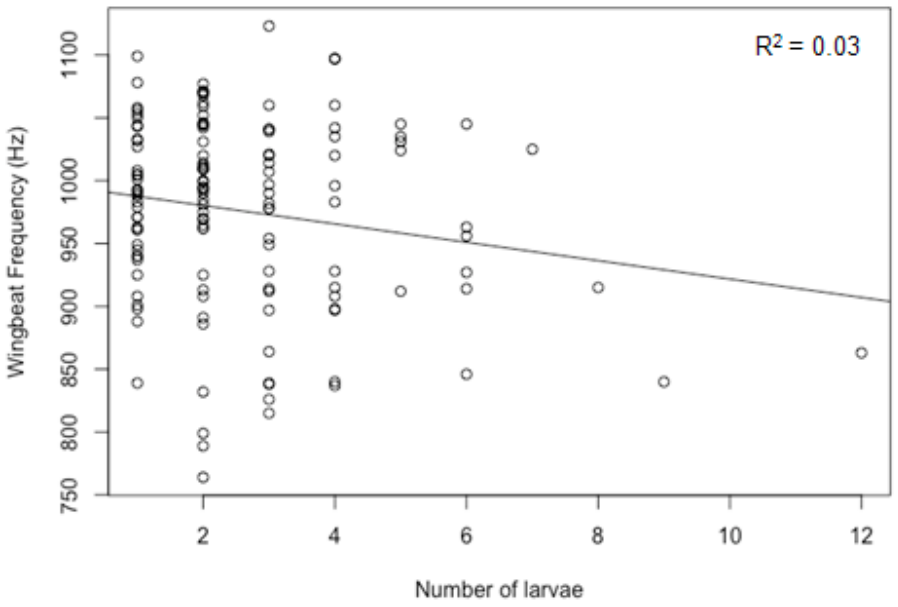

Figure 1-4. Wingbeat frequencies of *D. immitis* in L3 infected female mosquitoes
Ae. aegypti wingbeat frequencies decreased significantly with increasing number of L3 larvae within mosquitoes (P = 0.04).

Chapter 2:

Effect of Age on Wingbeat Frequency of *Aedes aegypti* and Potential Application for Age Determination of Wild Caught Mosquitoes

1. Introduction

Mosquito-borne diseases, such as malaria, yellow fever, chikungunya, dengue, and Zika, have continued to negatively impact human health and economic development (Riekmann, 2006; Mullen and Duren, 2019). According to the World Health Organization, malaria alone was responsible for an estimated 409,000 deaths in 2019 worldwide, and negative effects of the parasite have resulted in huge economic impediments in the endemic regions such as the African region of south of the Sahara (Chuma and Molynexus, 2010; WHO, 2020). To combat these mosquito-borne diseases, a variety of mosquito vector control tools have been developed and implemented, including the use of insecticide-treated bed nets, fumigation, larvicides, indoor residual spraying of insecticides, and biological control tools (Lengeler and Snow, 1996; Clark et al., 2001; Bhattarai et al., 2007; Moore et al., 2007; Benelli et al., 2016; Huang et al., 2017). To determine whether these control interventions are effective, it is essential to examine the age structure of the population to confirm that the interventions are suppressing vector populations.

Assessing age-structure in vector populations has become a critical method for understanding transmission potential in a population of mosquitoes and determining the effectiveness of vector control tools. Lymphatic filariasis (LF), one of the major health problems in tropical and subtropical areas, caused by *Wuchereria brancrofti* or *Brugia* spp., is transmitted by mosquito species in genera of *Anopheles, Aedes,* and *Culex.* In order for these pathogenic parasites to be transmitted by their vector, they must mature into an infective stage, third-stage larva (L3), which takes about 10–13 days of extrinsic incubation. Because blood-feeding by

female mosquitoes does not start until 1–3 days after emergence or until mating for some species (Mullen and Duren, 2019), those that can successfully transmit the agents of LF must survive for 2 weeks following ingestion of the pathogen to allow for L3 development. Therefore, infectious females must be "old" to transmit parasites, thus the age structure of a mosquito population is a critical determinant of transmission potential.

Although there are methods for estimating age of wild mosquito populations, these age grading techniques are laborious and rely on detailed knowledge of mosquito ovarian dissection and morphology. One method to determine age is through collection and dissection of mosquitoes to estimate parity. Parity is an indicator of mosquito age and is determined by examination of ovaries (Lambert et al., 2018; Johnson et al., 2020). Since egg production can be influenced by the number of feeding events and the size of the blood meal (Mamai et al., 2017), age estimation by assessing egg-laying cycles may not directly indicate the precise biological age of mosquitoes. Not only can these methods lead to inaccurate estimates, but they are also laborious and time-consuming tasks. Another well-known method for estimating an average age of wild mosquitoes at the population-level is mark-release-recapture (MRR) (Lambert et al., 2018). This method requires estimation of the average age of trapped mosquitoes in the wild by releasing a known, large number of marked mosquitoes and recording the detection of recaptured mosquitoes over time to develop a population age structure (Guerra et al., 2014; Lambert et al., 2018; Johnson et al., 2020). However, MRR is costly, requires considerable resources, and may negatively impact mosquitoes during the marking processes (Guerra et al., 2014; Lambert et al., 2018; Johnson et al., 2020). Additionally, this method can lead to biased data since recapture rates can be influenced by unintended manipulation such as distances from where traps are placed to releasing sites (Guerra et al., 2014). A few other techniques have been developed as

alternative methods of estimating mosquito age such as evaluating changes of concentration of pteridine, a pigment responsible for eye color in Dipteran species, and transcriptional profiles by assessing age-dependent changes in global genomes (Cook et al., 2006). While these methods' initial applications were promising, they have limitations on estimating the age of wild mosquitoes because certain age-determinant chemical compounds that are measured can fluctuate with the number of blood-feeding events or age of mosquitoes in the limited age range can only be estimated (Cook et al., 2006; Lambert et al., 2018). Recent work from Lambert et al. (2018) demonstrated that near-infrared spectroscopy (NIRS) can estimate the age of mosquitoes at the population-level by measuring the change in light absorbance of organic compounds within the head and thorax of mosquitoes; however, the method requires a costly spectrometer and laboratory capacity to perform this method.

Since the existing methods to predict mosquito age are costly, laborious, and may not be efficient to understand population wide age structure, there is great need for a rapid, scalable, and cost-effective method to estimate mosquito age. Previous studies have utilized wingbeat frequency for surveillance and determination to species of mosquito populations (Mukundrajan et al., 2017) and trapping male mosquitoes by playing the sound of a flying female (Villarreal et al., 2017). Recent works have demonstrated that the body size and ambient air temperature have significant effects on wingbeat frequencies of both *Ae. aegypti* female and male mosquitoes (Villarreal et al., 2017; Staunton et al., 2019), and commercially available cells phones can be used as a method of detecting mosquito wingbeat and identifying to species, which can be used for vector surveillance by researchers or citizen scientists (Mukundrajan et al., 2017).

In this study, we hypothesize that 1) wingbeat frequencies will differ between males and females, 2) wingbeat frequencies increase with chronological age as demonstrated from Staunton

et al. (2019), and 3) wingbeats recorded using commercially available mobile devices and the algorithm from Mukundurajan et al. (2017) can be used as a cost-effective method to estimate sex and age of wild mosquitoes.

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2. Methods

2.1 Mosquito rearing and blood feeding

In this study, the *Ae. aegypti* (LVP) strain was used. It has been maintained by *in vitro* rearing and feeding methods at Auburn University's College of Veterinary Medicine after being originally obtained from the University of Georgia. Mosquito eggs on cotton sheets were placed into a small glass container. After 24 hours, hatched mosquito larvae were transferred into a plastic pan with deionized water, then into emergence containers after they had pupated. About 4 days (D -4) before adult mosquitoes emerge (D 0), emergence containers were then placed into a $30.48 \times 30.48 \times 30.48$ cm^3 mosquito cage (Bioquip Products, Inc., Rancho Dominguez, CA, USA). Sugar was continuously provided for both female and male mosquitoes. On D 10 after emerging, a bell-shaped glass feeder (Lillie Glassblowers, Atlanta, GA, USA) with a layer of hog intestine (Syracuse Casing Co., Inc, Syracuse, NY, USA) was used to feed mosquitoes. They were fed 4 mL of blood from a healthy dog for 2 hours using a temperature-controlled circulating pump (Cole-Parmer, Vernon Hills, IL, USA).

2.2 Mosquito recordings

Each mosquito was aspirated into a collection vial and its wingbeat was recorded in a semi-soundproof container for approximately one minute using the Voice Memo app on an iPhone 8 with the iOS 11 operating system (Apple, Inc., Cupertino, CA, USA). Wingbeat

frequencies of five female and five male mosquitoes were recorded daily for 24 days. All recordings were collected between 8 and 9 a.m. (CST). The obtained wingbeat recordings from female mosquitoes were categorized into five different stages of the mosquito life cycle: 1) Stage 1: Freshly emerged (FE) (D 1–4), 2) Stage 2: Non-fed (NF) (D 5–9), 3) Stage 3: Digestion (DG) (D 10–12), 4) Stage 4: Oviposition (OP) (D 13–17), and 5) Stage 4: Parous (PA) (D 18–24) (Fig 2-1). Male ages were determined different than females; the wingbeat recordings from male mosquitoes were categorized into weeks: Week 1 (D 1–7), Week 2 (D 8–14), Week 3 (D 15–21), and Week 4 (D 22–24). After wingbeat recordings were collected, mosquitoes were put in a freezer (-20 °C) for one minute followed by sex confirmation via light microscopy.

2.3 Statistical analysis

The collected audio files of mosquito wingbeats were analyzing using a frequency analysis program written in Python 3.0. Wingbeat frequencies of each mosquito was averaged and analyzed by linear mixed effects regression (LMER). To avoid pseudoreplication, Cage column was added and treated as a random effect, and mosquitoes aspirated from the same cage were assigned the same cage number.

When wingbeat frequencies of both female and male mosquitoes were analyzed by LMER, the intraclass correlation coefficient (ICC) was extremely low (females: ICC = 0.04, males: ICC = 0.10). Low ICC indicates that the measures in the same cage or batch do not resemble each other, and the random effect of cage is negligible. Therefore, one-way analysis of variance with post-hoc Tukey honest significant difference test was performed to compare the mean wingbeat frequency of each stage (females) or week (male) to that of others.

The overall difference in wingbeat frequency of female and male mosquitoes was analyzed by Welch two sample t-test.

3. Results

3.1 Sex differences in wingbeat frequency between Ae. aegypti *female and male mosquitoes*

A total of 1,520 mosquito recordings of *Ae. aegypti* females (n = 728) and males (n = 792) from 7 replicates were collected and analyzed (Fig 2-2 A). The overall mean of wingbeat frequency was 993.3 Hz (± 65.6 Hz) for female mosquitoes and 964.1 Hz (± 57.3 Hz) for male mosquitoes, and the overall range of wingbeat frequency was 731–1,194 Hz for female mosquitoes and 828–1,180 Hz for male mosquitoes. The overall wingbeat frequency of female mosquitoes across the ages was significantly higher than that of male mosquitoes ($t = 9.19$, $df = 1{,}448.7$, $P < 0.001$).

3.2 Impact of chronological age on wingbeat frequency in male and female mosquitoes

The sample size of each stage of female life cycle was 140 for Stage 1: FE, 163 for Stage 2: NF, 85 for Stage 3: DG, 165 for Stage 4: OP, and 175 for Stage 5: PA. The mean wingbeat frequencies of female mosquitoes were 971.8 Hz (± 63.9 Hz; range 780–1,157 Hz) for Stage 1: FE, 991 Hz (± 74.0 Hz; range 799–1,194 Hz) for Stage 2: NF, 999.7 Hz (± 62.5 Hz; range 700–1,123 Hz) for Stage 3: DG, 1014.1 Hz (± 64.1 Hz; range 835–1,162 Hz) for Stage 4: OP, and 990.1 Hz (± 55.6 Hz; range 731–1,123 Hz) for Stage 5: PA (Fig 2-2 B). The data show a trend of increasing wingbeat frequencies in female mosquitoes from Stage 1: FE to Stage 4: OP, and a decrease of wingbeat frequencies at Stage 5: PA. The mean wingbeat frequency of Stage 2: NF was 19.0 Hz (± 20.3 Hz ± 95 % C.I.) higher than that of Stage 1: FE, however they were

statistically not significant (P = 0.078). The mean wingbeat frequency of Stage 5: PA was 18.3 Hz (± 20.0 Hz ± 95 % C.I.) higher than that of Stage 1: FE, however, there was no significant difference between these stages (P = 0.090), as well. The mean wingbeat frequency of Stage 3: DG was 27.9 Hz (± 24.2 Hz ± 95 % C.I.) significantly higher than that of Stage 1: FE (P = 0.014), and the mean wingbeat frequency of Stage 4: OP was 42.3 Hz (± 20.2 Hz ± 95 % C.I.), significantly higher than that of Stage 1: FE (P < 0.001). The mean wingbeat frequency of Stage 3: DG was 8.9 Hz (± 23.5 Hz ± 95 % C.I.) higher than that of Stage 2: NF, however, they were not significantly different from each other (P = 0.84). The wingbeat frequency of Stage 4: OP was 23.3 Hz (± 19.4 Hz ± 95 % C.I.) significantly higher than that of Stage 2: NF (P = 0.0096) but was 14.4 Hz (± 23.5 Hz ± 95 % C.I.) higher than that of Stage 3: DG, which was not significant (P = 0.79). While the wingbeat frequency of Stage 5: PA was not significantly lower than that of Stage 2: NF (0.71 Hz (± 19.2 Hz ± 95 % C.I.), P = 1.0) and Stage 3: DG (23.3 Hz (± 9.6 Hz ± 95 % C.I.), P = 0.79), it was 24.0 Hz (± 24.0 Hz ± 95 % C.I.) significantly higher than that of Stage 4: OP (P = 0.0056).

The overall range of wingbeat frequencies of male mosquitoes was 828-1,001 Hz. The mean wingbeat frequency for each week was 949.7 Hz (± 66.1 Hz; range 832–1,137 Hz) for Week 1, 964.2 Hz (± 53.5 Hz; range 842–1,180 Hz) for Week 2, 976.9 Hz (± 52.5 Hz; range 828–1,143 Hz) for Week 3, and 967.1 Hz (± 46.5 Hz; 828–1,044 Hz) for Week 4 (Fig 2-2 D). The data showed wingbeat frequency of male *Ae. aegypti* mosquitoes increased until Week 3 and decreased on Week 4. The wingbeat frequency of Week 2 was 13.4 Hz (± 13.6 Hz ± 95 % C.I.) higher than that of Week 1, however, these stages were not significantly different (P = 0.055). On the other hand, the wingbeat frequency of Week 3 was 26.4 Hz (± 13.5 Hz ± 95 % C.I.) significantly higher than that of Week 1 (P < 0.001). While the wingbeat frequency of Week 4

was 16.6 Hz (± 17.1 Hz ± 95 % C.I.) higher that of Week 1, they were not significantly different from each other (P = 0.062). The wingbeat frequency of Week 3 and 4 were higher (Week 3: 13.0 Hz (± 13.7 Hz ± 95 % C.I.); Week 4: 3.2 Hz (± 17.3 Hz ± 95 % C.I.)), there was no significant difference between two groups. From Week 3 to 4, the wingbeat frequency showed a decreasing trend; the wingbeat frequency of Week 4 was 9.8 Hz (± 17.3 Hz ± 95 % C.I) higher than that of Week 3. However, they were not significantly different.

4. Discussions

We hypothesized that wingbeat frequencies of laboratory-reared *Ae. aegypti* female and male mosquitoes will show an increasing trend as they age, and the algorithm used in Mukundarajan et al. (2017) can capture the effect of aging on mosquito wingbeat frequency and be used as a method of estimating the age of wild caught live mosquitoes. Unlike recent work from Staunton et al. (2019) that demonstrated aging significantly increases the wingbeat frequencies of laboratory-reared *Ae. aegypti* female mosquitoes, our female wingbeat frequency data showed a pattern of weak correlation between wingbeat frequency pre- and post-oviposition (Stage 4: OP) (Fig 2-2 B) with significant differences found between some stages of the female mosquito life cycle. The wingbeat frequency increased significantly from Stage 1: FE to 3: DG (P = 0.014) and 4: DG (P < 0.001) and decreased significantly from Stage 4: OP to Stage 5: PA (P = 0.0056) (Fig 2-2 B-C). While Staunton et al. (2019) collected wingbeat samples for 3 weeks and categorized age by week (Week 1–Week 3), our wingbeat frequency recordings of female mosquitoes were categorized by different stages of the life cycle. When reevaluating our data when categorized solely by week, our results showed similar results that were shown in Staunton et al. (2019). Wingbeat frequencies of female mosquitoes significantly increased from

Week 1 to Week 2 (27.2 Hz ± 15.5 Hz; ±95 % C.I.; $P < 0.001$) and Week 1 to Week 3 (33.3 Hz ± 16.1 Hz; ±95 % C.I.; $P < 0.001$) and significantly decreased from Week 3 to 4 (-26.6 Hz ± 22.5 Hz; ±95 % C.I.; $P = 0.013$) (Fig 2-2 C). Our analysis of wingbeat frequencies cannot accurately determine the specific age in days of lab reared female *Ae. aegypti*; however, wingbeats provide an indication of pre- and post-oviposition stage (Stage 4: OP), including stages where visual examination of a gravid abdomen would be inconclusive to determine prior feeding according to traditional aging methods.

The increasing trend of wingbeat frequency from Stage 1: FE to Stage 3: DG suggests that the frequency recorded may be explained by nutritional states of mosquitoes. While mosquito females take a blood meal for reproduction, sugar is the primary energy source for flight (Nayar and Handel, 1971). When sugar is consumed, it takes a few days to be metabolized and converted for flight fuel energy for mosquitoes to reach full flight capacity. Work by Kaufmann et al. (2013) demonstrated that flight performance of sugar-fed *Ae. albopictus* (Skuse) with a continuous sugar supply was significantly improved at three days after emergence, and that blood- and sugar-fed female mosquitoes were flying significantly faster than only sugar-fed or non-fed mosquitoes. Maximal continuous flight of sugar- and blood-fed *Ae. albopictus* was longer than sugar-fed and non-fed mosquitoes.

Once a female mosquito takes a bloodmeal, it puts a burden on her flight ability. However, our data suggest that wingbeat frequency did not decrease until mosquitoes became old or reached Stage 5: PA (Fig 2-2 B). The improved flight performance of blood-fed mosquitoes can also be a result of oviposition site preference seeking-behaviors. After female mosquitoes take a bloodmeal, they locate species-specific preferred resting sites by using chemical and visual cues, thus allowing for blood meal digestion and egg production to take

place. However, blood-fed females of many species are known to leave their resting sites to feed on sugar and obtain supplementary blood meals during the gonotrophic cycle (Mullen and Durden, 2019). Additionally, gravid females need to relocate to their appropriate oviposition site that can provide a good environment for successful larval development. For example, *Anopheles* and *Culex* tend to lay eggs on edges of lakes and ponds, whereas *Coquillettidia* and *Mansonia* prefer water with floating or aquatic plants to which the submerged immatures can attach (Mullen and Durden, 2019).

Along with the nutritional states of mosquitoes, the body size may have affected the increasing and decreasing trend of wingbeat frequency. Previous studies have shown that *Ae. aegypti* mosquitoes with larger body sizes had higher wingbeat frequencies than those with smaller body sizes (Villarreal et al., 2017; Staunton et al., 2019), and similar results have been shown in *Anopheles gambiae* (Giles) (Cator et al., 2010). One of the factors that can influence the body size of adult mosquitoes is food availability during the larval stage, where a positive relationship was found between food availability during the larval stage and the size of adult mosquitoes (Staunton et al., 2019). The change in body size of mosquitoes during the different stages of the life cycle can also influence wingbeat frequencies. After a female mosquito suddenly increases her body mass by about 2-times her original weight after taking a blood meal (Roitberg et al., 2003), she needs to generate higher push-off forces to escape from a host undetected for her survival (Muijres et al., 2017). Work from Muijres et al. (2017) demonstrated that *Anopheles coulzzii* yield the highest forces by beating their wings with additional push-off forces from the legs to reach maximum body accelerations during the push-off from a surface from which they are escaping. After their legs are free from a surface and they are in the aerial phase, constant wing-derived aerodynamic forces are required for flying (Muijres et al., 2017).

Muijres et al. (2017) might support our increasing trend of wingbeat frequency during digestion. Change in body mass also may explain the decreasing trend of wingbeat frequency after oviposition, thus a decrease in body mass and less need to generate push-off forces for flight (Fig 2-2 B).

Unlike the work from Staunton et al. (2019) that demonstrated that wingbeat frequencies of male mosquitoes increased as they aged in time intervals of Week 1 to Week 3, our data showed that wingbeat frequencies increased from Week 1 to Week 3 and decreased as males aged to Week 4 (Fig 2-2 D). This result could indicate that the wingbeat frequencies of male mosquitoes do not fluctuate as much as female mosquitoes because, unlike female mosquitoes, males do not undergo dramatic life stages involving blood-feeding and oviposition which can influence wingbeat frequencies. Even though male mosquitoes are not responsible for pathogen transmission, understanding male wingbeat frequencies is critical for a better understanding for sex recognition, mate selection, and male-focused traps, which utilize wingbeat frequencies of flying female mosquitoes to lure male mosquitoes.

Unlike other age grading techniques, our method of using commercially available mobile phones for obtaining wingbeat frequency recordings of mosquitoes and analyzing them by using the Python algorithm from Mukundarajan et al., (2017) is rapid and cost-effective. Our wingbeat frequency data of female and male *Ae. aegypti* demonstrated that the algorithm can capture and compute the effect of aging and sex difference of wingbeat frequencies; however, the wingbeat frequencies of *Ae. aegypti* female and male mosquitoes (female: 731–1,194 Hz; males: 828–1,001 Hz) captured with the mobile phone are higher ranges when compared to the reported wingbeat frequencies (female: 350–664 Hz; male: 571–832) of previous studies (Mukundarajan et al., 2017; Staunton et al., 2019). The differences in results could have resulted from the use of

mobile phones and the Python algorithm in this experiment; alternatively, certain environmental factors of the location where these mosquito recordings were collected may have diminished the accuracy of wingbeat frequency estimation.

5. Conclusions

We concluded that there was no precise threshold indicator of specific age of both *Ae aegypti* female and male mosquitoes. However, our data demonstrated the trends that could be used to determine pre- and post-oviposition stage. The results could suggest that the change in wingbeat frequency is influenced by effect of their nutritional state and body mass on flight capacity. While estimating the age of individual mosquitoes is important, the overall mean and age distribution across mosquito populations is more valuable for assessing efficacy of the interventions and understanding the disease transmission dynamics. While estimating an individual mosquito is important, the overall mean and age distribution across the mosquito populations is more valuable for public health surveillance of mosquitoes that vector disease-causing pathogens.

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Figures and Tables

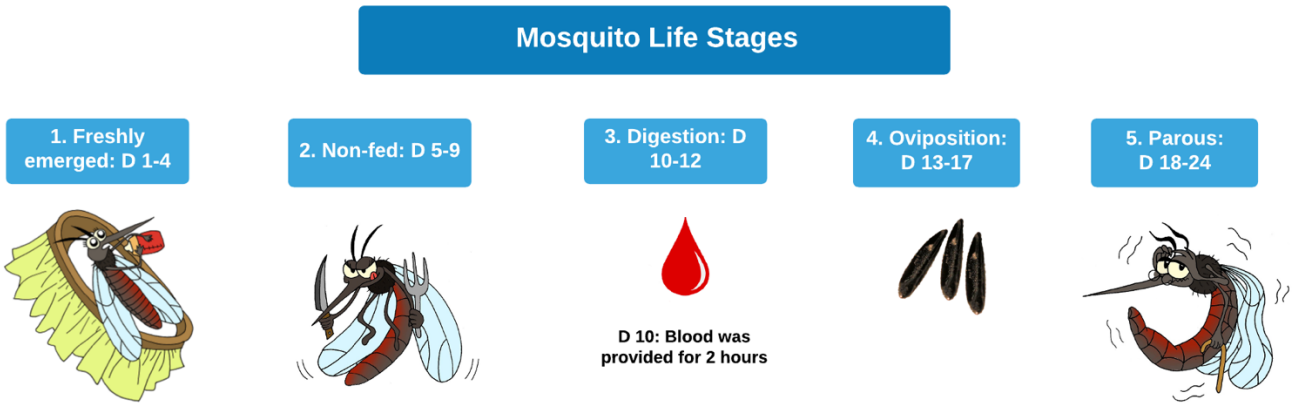
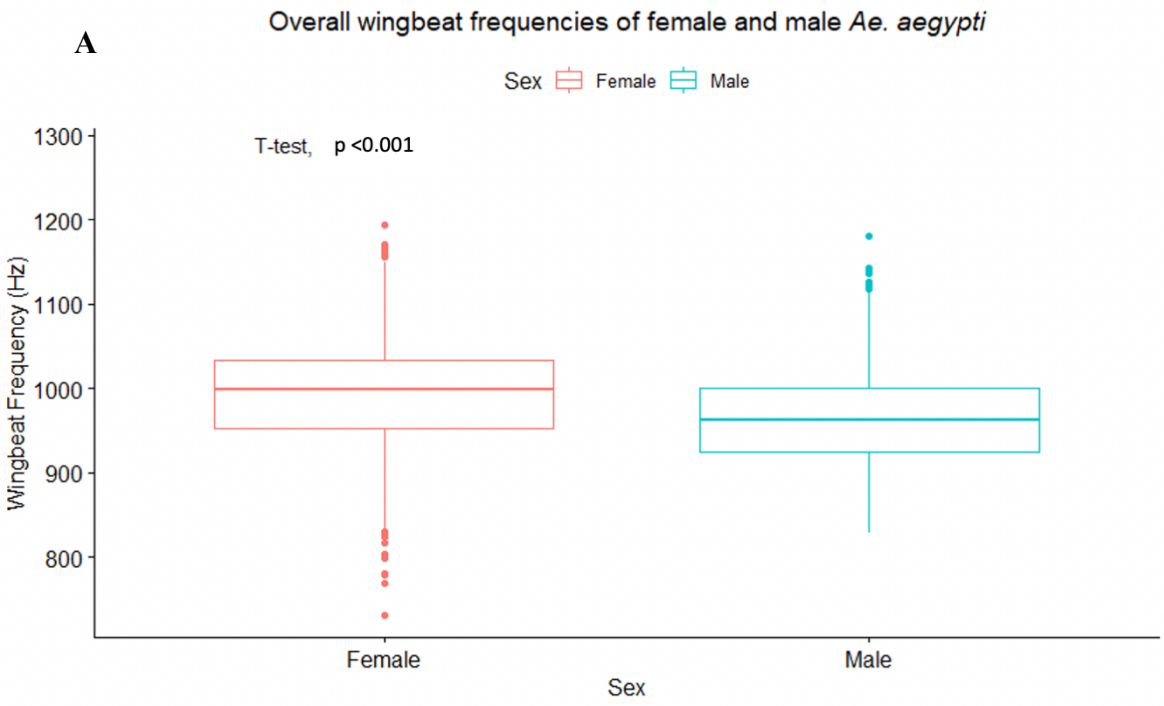
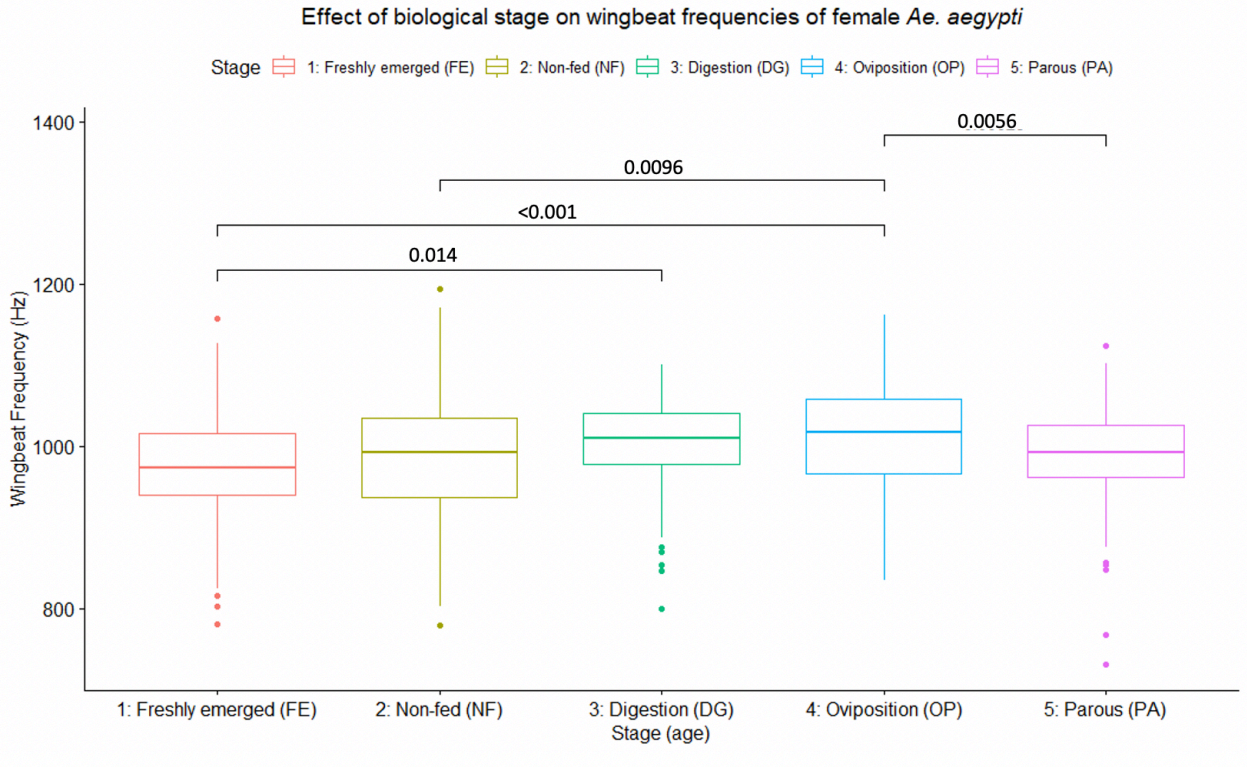


Figure 2-1. Mosquito life stages
Female mosquito recordings were categorized into five stages: 1) Stage 1: Freshly emerged (FE), 2) Stage 2: Non-fed (NF), 3) Stage 3: Digestion (DG), 4) Stage 4: Oviposition (OP), 5) Stage 5: Parous (PA).

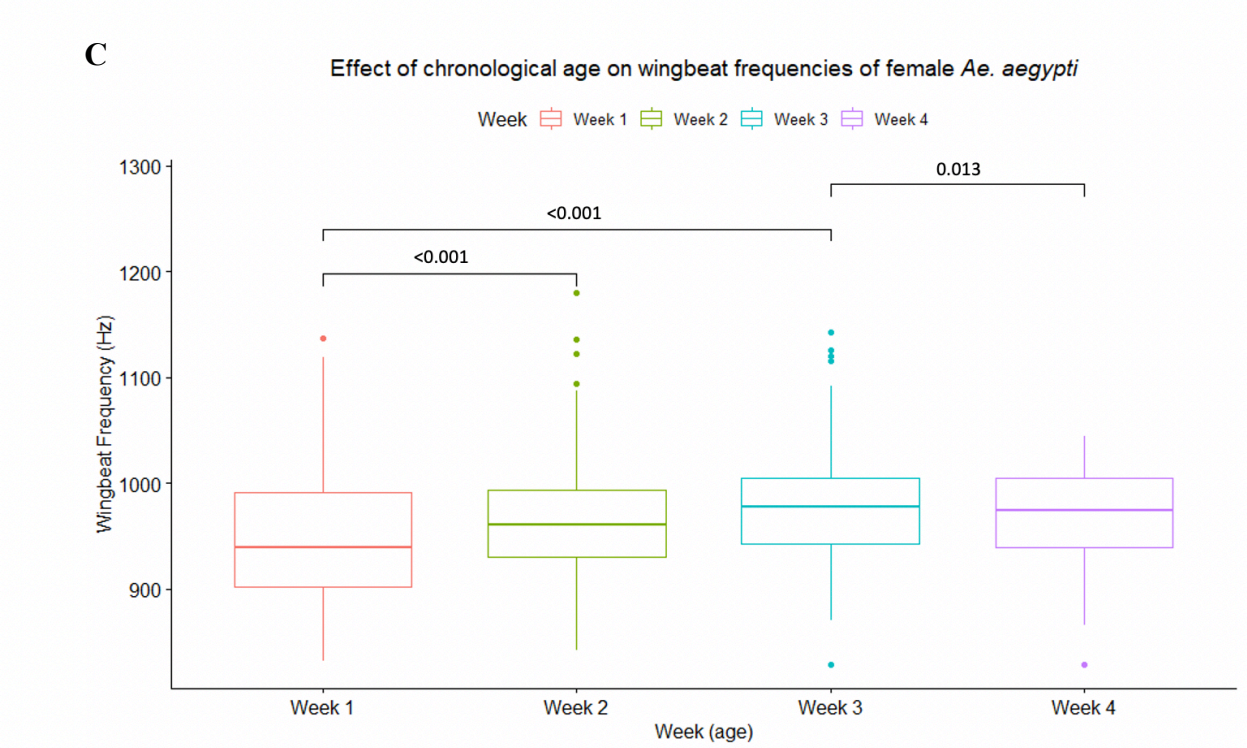
A


Overall wingbeat frequencies of female and male Ae. aegypti
Sex Female Male
T-test, p <0.001
1300
1200
1100
1000
900
800
Wingbeat Frequency (Hz)
Female
Male
Sex

B


Effect of biological stage on wingbeat frequencies of female Ae. aegypti
Stage 1: Freshly emerged (FE) 2: Non-fed (NF) 3: Digestion (DG) 4: Oviposition (OP) 5: Parous (PA)
0.0056
0.0096
<0.001
0.014
1400
1200
1000
800
Wingbeat Frequency (Hz)
1: Freshly emerged (FE)
2: Non-fed (NF)
3: Digestion (DG)
4: Oviposition (OP)
5: Parous (PA)
Stage (age)

C


Effect of chronological age on wingbeat frequencies of female Ae. aegypti
Week
Week 1
Week 2
Week 3
Week 4
0.013
<0.001
<0.001
Wingbeat Frequency (Hz)
1300
1200
1100
1000
900
Week 1
Week 2
Week 3
Week 4
Week (age)

D

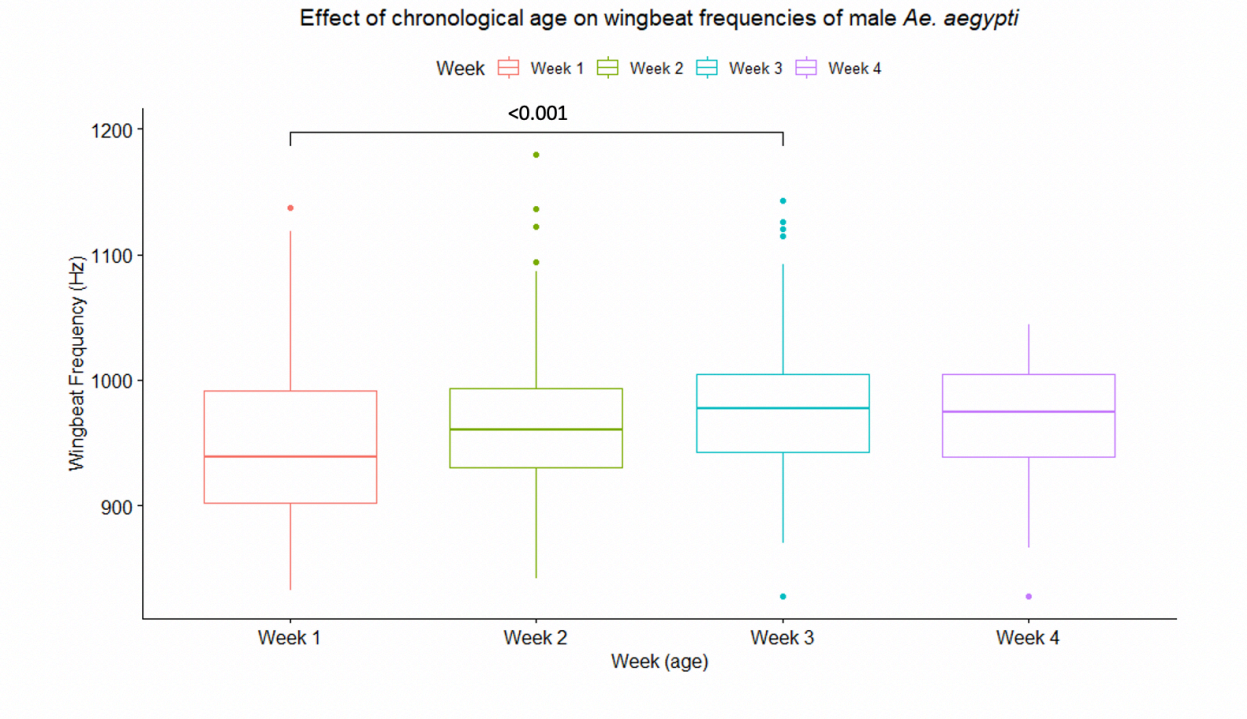

Effect of chronological age on wingbeat frequencies of male Ae. aegypti
Week
Week 1
Week 2
Week 3
Week 4
<0.001
Wingbeat Frequency (Hz)
1200
1100
1000
900
Week 1
Week 2
Week 3
Week 4
Week (age)

Figure 2-2. Results of effect of aging on wingbeat frequency of *Ae. aegypti* female and male mosquitoes

A. The overall wingbeat frequencies of *Ae. aegypti* female and male mosquitoes.

B. Each biological stage of female mosquitoes was compared with others. Stage 1: FM was significantly different compared with 3: DG ($P = 0.014$) and 4: OP ($P < 0.001$), but 2: NF ($P = 0.052$) and 5: PA ($P = 0.090$). Stage 2: NF was significantly different compared with 4: OP ($P = 0.0096$), but 3: DG ($P = 0.84$) and 5: PA ($P = 1.0$). Stage 3: DG was only scientifically different from 1: FE ($P = 0.014$) but other stages. Stage 4: OP was significantly different from 5: PA ($P = 0.0056$).

C. Each week of female mosquitoes was compared with others. Wingbeat frequencies of female mosquitoes significantly increased from Week 1 to Week 2 ($P < 0.001$) and Week 1 to Week 3 ($P < 0.001$) and significantly decreased from Week 3 to 4 ($P = 0/013$).

D. Each week of male mosquitoes was compared with others. Week 1 and Week 3 were the only groups showing a significant difference ($P < 0.001$).