THE ROLE OF BIOACOUSTICS IN THE MATING BEHAVIOR OF MEDICALLY IMPORTANT MOSQUITOES

A Dissertation
Presented to the Faculty of the Graduate School
of Cornell University
In Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy

by
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May 2011

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Cornell University 2011

Understanding the basic behavioral ecology of mosquitoes is important for the development of new disease control strategies and the improvement of classical control. Mating behavior is severely understudied and we lack even the most basic information about mosquito mating systems. This stage of the mosquito life cycle may hold important targets for disease control.

I investigated the role of bioacoutics in mosquito mating behavior. I found that male and female mosquito engage in a dynamic acoustic interaction when they meet in flight. I went on to investigate the role of this behavior, termed harmonic convergence, in the mating behavior of both *Aedes* and *Anopheles* mosquitoes. I found that flight tone is correlated with body size and that large individuals produce higher flight tone frequencies. In *Anopheles gambiae,* I conducted playback experiments and was able to determine that males and females are able to discriminate between the signals produced by large and small potential mates. In *Aedes aegypti*, I found that successful convergence in a mating attempt predicted the formation of a copula and that female rejection behaviors were less likely when convergence preceded a mating attempt. The male offspring of converging pairs had higher mating success when compared with sons of non-converging pairs and where more likely to converge themselves.

These results indicate the mate assessment is a key factor in mosquito mating systems. Further characterization of mate assessment and its mechanism has obvious applications to trangsgenic mosquito release programs and may provide the opportunity for new control strategies.

BIOGRAPHICAL SKETCH

Lauren Jennie Cator was born into a loving family in Fairfax, Virginia on August 8, 1984. She became interested in Medical Entomology during a semester abroad in Tanzania in 2004, where she studied the behavior of tsetse flies. After graduating with a B.A. in biology from The Colorado College in 2006, she applied to work with Dr. Laura Harrington at Cornell University. There, under the guidance of Dr. Harrington and committee members Dr. Ron Hoy and Dr. Jennifer Thaler, Lauren completed the studies described here on the role of bioacoustics in the mating behavior of mosquitoes.

"Self-confidence is the first requisite to great undertakings."

-Samuel Johnson

To Mom, Dad and Aaron, for everything, with love.

ACKNOWLEDGMENTS

This dissertation would not have been completed without the valuable advice and unwavering support of my doctoral advisor, Laura C. Harrington. I have learned everything I know about working as a scientist from Dr. Harrington and I consider it a great privilege to have been able to work with her. In addition my committee member, Ron Hoy, has been extremely important in teaching me how to be a successful and happy academic. For this and many other things (summer barbeques) I am extremely grateful. Dr. Jennifer Thaler has offered excellent advice as a member of my committee and has kept the "mosquito express" on track. My labmates Mari Kimura, Melissa Hardstone, Jon Dabro, Alongkot Ponlawat, Prasit Deetateawong, and Michelle Helinski have been inspirational and supportive. Kija Ng'habi severed as my host and facilitated my studies at the Ifakara Health Institute in Tanzania. I am honored to have him as a friend and a colleague. Dr. Ponlawat and his team at AFRIMS made the work on Thai field populations of mosquito possible.

I would have been unable to complete my dissertation without the statistical and moral support of Sarah Short and Sarah Jandricic. Official statistical consulting from Fraincoise was also appreciated. Punita Juneja, Madeline Galac, and Susan Villarreal provided stimulating conversation, occasionally related to this research. Matthew Milner should be recognized for ensuring my general well being and happiness throughout the 5 years which it has taken me to complete this dissertation. Dear, I'm sure I could have done it without you, but it would not have been nearly as fun. Last and perhaps most importantly, I would like to thank my parents, Tom and Becky Cator. You have supported me in every adventure. What a blessing it is to be your daughter.

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CHAPTER 1

INVESTIGATING SEXUAL SELECTION IN MOSQUITOES: BACKGROUND AND APPLICATIONS

Introduction

Mosquito-transmitted diseases are a major and growing public health threat. Malaria is thought to infect 500 million people annually (Hill et al. 2005). In addition to 1-3 million deaths each year, these infections result in a 2% decrease in the economic growth rate of disease endemic nations (Sachs & Malaney 2002). The yellow fever mosquito, *Aedes aegypti,* transmits several viruses which lead to illnesses in humans and cause thousands of deaths each year. Dengue virus alone is responsible for 50,000 deaths and 50 million cases each year (Jelinek 2010) and the numbers are increasing annually. The World Health Organization reported a 30-fold increase in dengue cases in the last 50 years (Guzmán & Kouri 2002). Unfortunately, malaria and dengue infections are difficult to prevent and cure. Currently, vaccination and treatment are not practical solutions for control of malaria, and are not available for the control of dengue. Consequently, vector control is an essential component of public health campaigns and disease prevention programs for both infections. Despite their medical importance, the basic biology of the mosquito vectors of dengue and malaria has remained relatively unstudied. Insights into mosquito behavior are critical for designing and implementing mosquito control programs (Pates & Curtis 2005).

Several evolutionary, ecological, and cultural factors have lead to the emergence of and reemergence of these diseases in spite of conventional control efforts. Earlier this century, many public health programs specializing in vector surveillance and control were cut. This has resulted in poor study of vector populations and has lead to a decrease in the number of vector biologists prepared to deal with disease outbreaks (Beaty 2000). The evolution of resistance both in mosquito populations and parasites and continues to render many once effective chemical control measures impotent (Gratz 1999; Beaty 2000; Read et al. 2009). Finally, unchecked human population growth compounded by unplanned urbanization, deforestation, and globalization, have created the ideal situation for already existing vector-borne diseases spread. These conditions have also resulted in interactions between previously isolated vector, parasite, and host populations (Beaty 2000).

Several innovative control techniques have been proposed that focus on using transgenic or sterile mosquitoes to replace or eliminate wild vector populations. These approaches rely primarily on male releases and successful implementation of these strategies will require an intimate knowledge of mosquito mating behavior. Researchers have identified several chemical and genetic methods that may be used to create such transgenic organisms (Phuc et al. 2007), but to date, these programs have had limited success due to low mating competence of released males (Benedict & Robinson 2003). In one such instance, an attempt to release sterilized *Culex tarsalis* in the United States failed despite laboratory experiments that indicated that sterilized males were able to compete with wild type males for mates. In the field they were not successful. Researchers later discovered that laboratory colonization had selected for males with different swarming behaviors than the wild-type (Reisen 2003).

The low success of these programs highlights the need for increased study of mosquito mating systems both in the laboratory and the field. Such knowledge would increase efficiency of current control practices, allow for better assessment tools of transgenic strains, and may lead to novel control measures. With these applications in mind, I explored the role of sexual selection in the mating systems of several medically important mosquitoes. By dissecting the characteristics and functions of intersexual signaling, I gained novel results and a new perspective on the habits of these insects. I have focused my studies on *Aedes aegypti*, an important vector of arboviruses, including dengue, and *Anopheles gambiae* s.l. a key vector of malaria in Sub-Saharan Africa.

Study Species

Ae. aegypti is an important vector of many viruses important to human health including those that cause dengue, Chikungnya, and yellow fever. Although it is biologically competent to transmit all of these pathogens, it is the behavior of *Ae. aegypti* that makes it important public health. This species is anthropophagic and females feeding on human hosts have significantly greater fitness (Harrington et al. 2001). Additionally, *Ae. aegypti* prefers to rest indoors and to (Schoof 1967) oviposit in man-made containers (Harrington et al. 2008). It is this close proximity to humans and greater human contact that make this species a particularly efficient vector.

The *An. gambiae* complex is composed of seven morphologically indistinguishable *Anopheles* species (Besansky et al. 1992) with an eighth potential species recently described (Riehle et al. 2011) . This dissertation describes experiments on two of these complex members, *An. gambiae* and *An. arabiensis*. Both of these species are recognized as being important in the transmission of human malaria in Sub-Saharan Africa. Several subspecies and groups of *An. gambiae* rests and feeds indoors and is most frequently implicated in human malaria outbreaks (Tuno et al. 2010), although the GOUNDRY subgroup has been shown to feed and rest outdoors (Riehle et al. 2011). More recent work has emphasized the importance of *An. arabiensis* in malaria transmission.

Sexual Selection and its Predictions for Mosquito Mating Systems

In his theory of natural selection, (Darwin 1859) explained bizarre and seemingly maladaptive male traits as being the product of "sexual selection". He defined this type of selection as a category of natural selection, in which males sacrificed one component of fitness, such as longevity, in order to gain more matings and thus increase their *overall* fitness. Males were concerned with maximizing the number of matings because it was this parameter, and not gamete number, that determined male fitness. Females, on the other hand, possessed a limited number of gametes and thus, Darwin (1859) hypothesized, should be more concerned with quality of their mates instead of the number. Males, therefore, were predicted to be in competition for the limited resource of female gametes. In 1948, (Bateman 1948) provided quantitative support for Darwin's theory using fruit flies. In his classic experiment with *Drosophila melanogaster,* Bateman found that female reproductive success did not vary significantly with number of matings while male success varied greatly (Bateman 1948).

It is the evolutionary pressure of selection that dictates the development of mating systems. These systems are diverse, but they can be classified simply by using three generalized terms. Monogamy describes a system in which both males and females mate with a single partner each mating season. Polygyny refers to systems in which males copulate with more than one female and polyandry describes the other extreme, in which the female mates multiple times (Thornhill & Alcock 1983). The degrees to which these types of relationships occur within a mating system allow us to make predictions about mate choice and other reproductive parameters.

There were conflicting observations about female mosquito promiscuity in the laboratory up through the 1950s. In *Ae. aegypti,* (Gwadz et al. 1971) pointed out that this confusion likely resulted from an imprecise use of the terms mating, copulation, and insemination. Subsequent studies found that females only mated as virgins and that after this mating they became refractory to male insemination attempts (but see Harrington Lab, unpublished data). Some authors believed that males chemically induced this state through a substance called Matrone which was found in the ejaculate (Gwadz & Craig Jr 1968). Other authors presented evidence of female *Ae. aegypti* physically preventing genital contact with males (Gwadz & Craig Jr 1968). Sirot et al. (2008), have provided evidence of male accessory gland proteins that are transferred to females and may modulate female behaviors in this species. Helinski et al. (In Submission) found up to 14% multiple mating using a semi-field cage experiment. In the laboratory Orteza et al. (unpublished) found that multiple mating resulting in successful insemination occurred up to 4.8%.

In the *Anopheles* species studied here, it appears that males and females, for the most part, only mate once per gonotrophic cycle with low levels of multiple inseminations detected in field populations (Yuval & Fitz 1994; Tripet et al. 2001; Charlwood et al. 2001; Charlwood et al. 2002; Tripet et al. 2003; Charlwood et al. 2003).

The structure of this system leads to two predictions about mating behaviors in mosquitoes; (1) males should compete for access to female gametes and (2) females should be choosey about which males they allow to fertilize their limited gametes, especially since they will likely only have one mate.

The mating systems of mosquitoes are diverse (Clements 1999). In all of the species I studied for my dissertation, mating has been observed to occur in swarms. The timing and location of swarms is usually species specific (Clements, 1999). The act of mating in flight is not unique to mosquitoes, or even to the Dipterans. Evidence of aerial mating aggregations has been reported across insect taxa. In all of these diverse groups, it is thought that swarming allows dispersed populations to encounter the opposite sex for mating (Downes 1969). Swarming individuals are thought to respond to marker cues and orient individually to them. It is important to note that swarming is a description of a set of behaviors and that these behaviors are not contingent upon a congregation of any kind. A single insect, therefore, can be said to be “swarming” (Downes 1969).

Ae. aegypti males are capable of detecting host cues and use these cues to form diurnal mating aggregations at the host (Hartberg 1971). *An. gambiae* and *An. arabiensis* form swarms over visual markers in response to light cues (Charlwood, Pinto, Sousa, Madsen et al. 2002). Visual markers typically posses some sort of contrast and by using dark or light cloth, males can be made to change swarm location (Charlwood et al., 2002). Swarms have been recorded to occur at these marker locations at both dawn and dusk (Charlwood & Jones, 1967; Marchand 1983; Charlwood et al. 2003).

At first, only males gather at swarm sites and gradually single females enter. Males recognize conspecific females by wingbeat frequency (more on this later) and this stimulus initiates male pursuit. Mating in these species is relatively brief lasting from between 5- 30 secs. Currently, it is believed that there is no courtship behavior in these mosquitoes (reviewed in (Yuval 2006).

It should be mentioned that some researchers believe that mosquitoes may mate earlier in life and that swarming behavior is not related to mating behavior (Nielsen et al. 1960). While there is evidence for alternative mating strategies (Dao et al. 2008), mating can still be observed in swarms, and they remain the only dependable place to observe mating behavior in nature. Also, participation in swarms requires a high cost, both energetically (Yuval et al. 1994) and in predation risk (Yuval & Bouskila 1993), so it is difficult to imagine how this could be a vestigial behavior with no benefits.

One important component of the dipteran swarming behavior is a highly specialized directional response that leads the male to pursue the female. In the Culicidae, Ceratopogonidae and Chironomidae this response is triggered by acoustic stimulus (Downes 1969). Males of these orders possess highly sophisticated hearing organs that are mechanically and neurologically tuned to the sounds of conspecific females. It has long been accepted that male mosquitoes use acoustic cues from females for orientation (Roth 1948). Later, I will discuss how studying these signals may lead to important insights into mosquito behavior.

The Evidence for Male-Male Competition

Roth (1984) stated that male *Ae. aegypti* are likely to copulate at "any time of day, until death" and the male drive to obtain as many mates as possible seems to have resulted in competition between males. In an observational study, Jones & Pilitt (1973) observed that male flight and pursuit behavior increased with the number of females present. They also recorded that copulations were often interrupted by rival males. Despite these observations, few studies have attempted to find correlations between male traits and mating success. Ponlawat and Harrington (2007) determined that larger male *Ae. aegypti* produce and are able to transfer more sperm to females. If intrasexual competition is occurring, I would expect traits that enhance the male ability to locate mates, to attract them, and finally capture them. The production of attractive courtship signals to improve mating success would be expected to evolve under competition pressures.

There is some evidence from the field that suggests that male *Anopheles* with higher caloric reserves are able to participate in swarms longer, and thus, obtain more matings (Yuval et al. 1994). Variation in reserves may be dictated by larval nutrition or by genetic predispositions that allow for ideal nectar foraging in males (Yuval et al. 1994). There are conflicting reports on the effect of male body size and mating success in *Anopheles* species. Some researchers have found that larger males obtain more matings and others have found either no correlation or that in fact smaller males are more successful (Yuval & Bouskila 1993; Charlwood, Pinto, Sousa, Ferreira et al. 2002; Ng'habi et al. 2008). Similarly, (Sibley et al. 2001) determined that small male midges had greater success in swarms due to "acrobatic ability". However, they also found that these males have decreased acoustic sensitivity and thus are more likely to mistake male conspecifics for females, an error which certainly does not lead to increased fitness.

The Evidence for Female Choice

Female choice may take two forms; active and passive. Passive female choice describes female behaviors that increase the intensity of male competition and the subsequently female's chance of mating with the most fit male (Thornhill & Alcock 1983). There is some observational evidence for such behaviors in these mosquito species. Jones & Pilitt (1973) observed that female *Ae. aegypti* were able to escape chasing males for extended periods by out maneuvering them or simply avoiding flight. Charlwood et al. (2002) suggested that female *An. gambiae* would not approach swarming sites until males had reached a critical mass. This would allow the female to only expose herself to potential mates during a period of maximum intrasexual selection. Similar types of observations in the love bug, *Plecia nearctica* have been used to suggest that females adjust behaviors to incite male competition or even to postpone mating until the female is at her maximum fecundity (Thornhill 1980).

Active female choice is characterized by the rejection of unsuitable males and selection for males with either genetic or material benefits. Hybrid offspring are often sterile and thus females should prefer males of the same species. Within their species females should select males with adequate sperm and who are in the possession of superior benefits (Thornhill & Alcock 1983). At the species level, most mosquito studies have found low levels of hybridization in the field (Tripet et al. 2003). As would be predicted in a mating system with persistent males, female mosquitoes have been observed to actively reject both males from other species and conspecifics by either kicking them away or bending their abdomens to prevent copulation (Gwadz & Craig Jr 1968). It is currently not known how females of the *Ae. aegypti* or *An. gambiae* s.l. identify conspecifics in nature or if they discriminate between males of their own species. In the mosquito, *Sabethes* males and females actively court using elaborate movements and visual signals (Zsemlye et al. 2005; South et al. 2009). Such obvious intrasexual communication is absent from the swarming mosquitoes.

Table 1: Previous studies on male-male competition, female choice, and sexual selection in mosquitoes.

Author	**Study Species**	**Component Measured**	**Notes**
(Gwadz et al. 1971))	*Ae. aegypti*	female choice	Observed and described active female rejection of males
(Zsemlye et al. 2005)	*Sa. chloropterus*	female choice	Documented female rejection behaviors
(South 2009)	*Sa. cyaneus*	female choice	Measured cost to male of courtship to males
(Okanda et al. 2002)	*An. gambiae*	male choice	Large females were more frequently mated in cage trials in Tanzania with Ifakara strain
(Hancock et al. 1990)	*Sa. cyaneus*	male choice	When female leg paddles were removed males did not approach them to court
(Yuval et al. 1994)	*An. freeborni*	male-male competition	Larger males with higher reserves were found to swarm longer
(Ponlawat & Harrington 2007)	*Ae. aegypti*	male-male competition	Determined that larger males have increased sperm production and transfer
(Ponlawat & Harrington 2009)	*Ae. aegypti*	male-male competition	Found that older males collected as pupae from the field transferred more sperm than younger lab reared males
(Yuval et al. 1994)	*An. freeborni*	male-male competition	Reported that larger males have higher mating success and males found in the swarm are larger than those found resting

Author	**Study Species**	**Component Measured**	**Notes**
(Yuval & Bouskila 1993)	*An. freeborni*	male-male competition	Found that time when large males were at highest abundance in the swarm that mating frequency was high and dragonfly predation frequency was low.
(Charlwood, Pinto, Sousa, Ferreira et al. 2002)	*An. funestus*	male-male competition	Observed no effect of body size (male or female) on copula formation in the field
(Ng'habi et al. 2005)	*An. gambiae*	male-male competition	Compared mating success of males reared under different crowing conditions and found that males reared under low crowding were most likely to acquire the first mating
(Voordouw & Koella 2007)	*An. gambiae*	male-male competition	Measured genetic variation in male fitness (proportion of females inseminated) in G3 colony population.
(Benjamin & Bradshaw 1994)	*W. smithii*	male-male competition	Reported that flight activity and male size effect reproductive success.
(Jones & Pilitt 1973)	*Ae. aegypti*	both	Found that male flight activity increases when females are present. Described that females avoid or outmaneuver male in flight
(Chambers & Klowden 2001)	*An. gambiae*	both	Female held with 2-day old males laid more eggs than females held with 6 day old males.
(Ng'habi et al. 2008)	*An. gambiae*	both	Medium males achieved the most matings (with medium females)
(Charlwood, Pinto, Sousa, Madsen et al. 2002)	*An. gambiae*	both	Females do not enter the swarm until it reaches a threshold size. Males caught in copula did not significantly differ in size with average swarming males.

Sexual Communication and Mating Systems

There is limited, but mounting, evidence for male competition and female choice in mosquitoes (see Table 1). The traits that dictate mate choice and success have thus far remained elusive. Determining these factors will be critical in the development of rapid and accurate assessment of genetically modified mosquitoes (Knols 2006). I propose that the study of intersexual signaling systems may reveal these correlates. If female choice occurs in mosquitoes, then male mosquitoes may be able to communicate information about material or genetic benefits to females. Such signals would likely be costly for males to produce and for females to attend. Therefore, signals will only be maintained if they in fact result in greater fitness. If there is a correlation between signal cues and male fitness, females will benefit from selecting males with these cues (Searcy & Nowicki 2005).

This type of signaling system is found in many organisms. Playback studies in frogs, have found that females preferential move to speakers generating lower frequency calls that are stereotypical of larger males (Ryan et al. 1990). (Gwynne 1982) found, that in one katydid species, the females use the acoustic signals of males to locate larger males that supply females with larger and more nutritious spermatophores (Gwynne 1982). In the South African bulldog fish, larger males have a greater electric organ discharge pulse duration and dipoles emitting signals at these pulse durations were approached more frequently by females (Machnik & Kramer 2008).

There is evidence from various insects, including crickets (Wedell & Tregenza 1999) and sandflies (Jones et al. 1998), that males are able to confer indirect benefits to their offspring. Further in many of these insects it has been determined that components of male signals are mechanism by which females select males for these types of benefits. For example, Tregenza et al. (2006) found evidence that female field crickets are able to discriminate between males with higher innate immunity by their mating calls. Hoikkala et al. (1998) elegantly demonstrated that the frequency of male courtship song was correlated with offspring survival in *Drosophila monatana.* Additionally, the authors found that females were able to differentiate these males and that they were preferred as mates.

The discovery of an intersexual communication mechanism in mosquitoes, with which males provide females information about fitness, would allow for us to tease apart the relative roles of male-male competition and female choice in mosquito mating systems. In order to function in this capacity the signal would need to be (1) perceived by females, (2) correlate with male fitness characteristics, and (3) attention to this signal should increase female fitness.

The Flight Tone of Mosquitoes: A Potential Signaling System

In flight, mosquito wingbeats produce a non-sinusoidal tone. Most organisms produce such types of sounds; pure sinusoidal waves are very difficult to produce. Fourier analysis dictates that such signals are composed of several sinusoidal waves of differing frequencies (Fourier & Freeman 1878). In mosquitoes (as well as many other animals), this combination of waves produces a fundamental frequency and several harmonic frequencies. The fundamental (F0) is the loudest dominant component of the signal, with harmonic frequencies occurring at twice the fundamental (F1), three times the fundamental (F2) and so on. As these signals increase in frequency they decrease exponentially in amplitude (Clements 1999).

The fundamental frequency of male and female mosquitoes differs. This difference can be as small as that between male and female *Toxorhynchites brevipalpis* (female, 400 Hz, male, 500 Hz) or more dramatic, as seen in *Ae. aegypti* (female, 500 Hz, male, 750) (Clements 1999). Male mosquitoes produce much louder stimulus than females. Female frequencies are lower and wavelengths longer, thus energy produced when the wing oscillates in one direction can cancel the energy produced then the wing moves the other way (Clements 1999). The relatively small amplitude of the female signal has acted a driver to produce elaborate sound detection organs in males.

As early as 1855, (Johnston 1855) speculated about the auditory function of mosquito antennae. In fact, it was in a Culex mosquito that Johnston first described the Johnston's organ (JO), an acoustic apparatus that has been found in 99% of insect species (Göpfert & Robert 2001). A.Mayer (1874) studied the stereoscopic response of mosquito antennae to various frequencies produced by tuning forks. Up through the 1990s it was agreed that male mosquitoes used the fundamental frequencies of females to locate conspecifics and it was only these fundamental frequencies that presented any biological significance (Tischner 1954; Roth 1948; Wishart & Riordan 1959; Downes 1969; Belton 1994; Clements 1999).

Recent discoveries from the fields of insect physiology and mosquito behavior have suggested that mosquito acoustics may play a larger role in mosquito mate selection behavior. First, it has been demonstrated that males are able to hear with an extremely high degree of sensitivity and selectivity (Göpfert et al. 1999). Mosquito antennae function as particle receptors. This means that instead of detecting changes in pressure, the antennae are stimulated by the actual movement of air particles in the sound field (Göpfert et al. 1999). The antennae of a male mosquito can sense a displacement of these receptors as small as 7nm (Göpfert & Robert 2000). They are able to accomplish this because of precise mechanical tuning and 7,500 sensory neurons contained in each male JO. This is almost as many as are found in the human cochlea (Göpfert et al. 1999).

Göpfert and Robert (2001) demonstrated that male antennae are able to use self-sustained motion to amplify select frequencies while simultaneously responding to them. Using this complex mechanism, males are able to selectively amplify the flight tone of conspecific females in order to pursue them in crowded chaotic swarm conditions. This type of "active" hearing was previously thought to be confined to vertebrates. Jackson and Robert (2006) went one step farther and characterized the response of the male antennae to a moving female. They found that the antennae were able to use the same mechanism to extend their range of detection. As females approached active hearing mechanisms amplified their flight tone and as the female signal began to overwhelm male receptors the antennae attenuated the signal. This type of dynamic hearing process is very advanced and represents a high degree of adaptation in mosquito sensory biology.

As studies revealed the complexity of audition in male mosquitoes, researchers pondered the apparent absence of auditory function in females. (Göpfert & Robert 2001) elegantly capture this sentiment.

> "It may be that an entire aspect of female mosquito sensory biology has been overlooked. Considering the relevance of male audition in mosquito reproduction, unraveling the enigma of the significance of female audition is expected to be an important step towards future understanding of the sensory and behavioural ecology of mosquitoes."

There are several differences between the physiology of the male and female hearing organs. The female antennae do not possess the plumose hairs found on most male antennae and thus, have reduced receptor surface and mechanical sensitivity (Göpfert & Robert 2001). The female JO contains half the number of neurons found in males. This still leaves females with 7,500 neurons, with no apparent function. Boo and Richards (1975) found several similarities between the morphological organization of the male and female JO. There are, however, differences. Females have three types of scolopedia (A, B and C) and males have four (A, B, C, and D) and these types of cells are found in different concentrations (Boo and Richards, 1975).

Gibson & Russell (2006) demonstrated that males and females of the large, non-blood feeding mosquito species, *Toxorhynchites brevipalpis*, match fundamental frequencies when flown in tandem with the opposite sex. This study presented the already established mechanical frequency range of females, but did not present physiological evidence that females can hear the flight tone frequencies that they are matching. In other words, these authors showed that the female antennae are moved by the displacement of particles, but offered no evidence that this displacement is perceived by the nervous system. Despite this oversight, the study was the first to infer an active acoustic role for the female. The authors proposed that this synchronization may be used by males and females to act as a species isolation mechanism. I argue that flight tone has also evolved as part of signaling system between male and female mosquitoes and that this signaling system allows for the assessment of the quality of potential mates.

To address this hypothesis I tested three predictions. First, I described a novel precopulalory mosquito behavior, termed harmonic convergence in *Ae. aegypti* and *An. gambiae* (Chapter 2). I went on to explore the utility of this behavior as a mate assessment signal. I determined the relationship between harmonic convergence and body size and that the variation in the body size of potential mates was detectable by acoustic stimulus (Chapter 3). Next, I measured the benefits associated with variation in harmonic convergence behavior prior to copula formation (Chapter 4). Finally, I determined whether harmonic convergence behavior was heritable (Chapter 5).

CHAPTER 2

CHARACTERIZATION OF HARMONIC CONVERGENCE IN *AEDES AEGYPTI* AND *ANOPHELES GAMBIAE*

Introduction

It has long been accepted that sound plays an important role in mosquito mating behavior. Informal reports of male mosquitoes being attracted to sounds are documented as early as 1878 (Howard 1901). Several experiments have demonstrated that male mosquitoes are attracted to the fundamental component of the female flight tone and that this stimulus elicits mating behaviors (Roth 1948; Kahn & Offenhauser 1949; Wishart & Riordan 1959; Belton 1994).

The exact role of female flight tone in mating interactions has not been resolved. Some work has suggested that there are species specific components of female flight tone and that males used these differences in tone as a precopulatory isolation mechanism (Tischner 1954). When Wishhart and Riordan (1959) played recordings of the flight sounds of four unnamed species of *Aedes,* a *Culex* species, and an *Ae. aegypti* female at the same frequency they found that there was no difference in the attraction of male *Ae. aegypti*. Durkopf and Hartberg (1992) reported that when male *Ae. aegypti* were presented with recordings of female *Ae. albopictus* they did not respond, but the same study found that *Ae. albopictus* was not responsive to either stimulus. Later studies found differences between the flight tone of different species, but none of these studies tested attraction or orientation to these sounds (Brogdon 1994). Yet other studies found no difference between reproductively isolated groups of the *Anopheles gambiae* complex (Tripet et al. 2004).

All of these experiments operated under the presumption that only males were receiving acoustic information and that only the female fundamental frequency was biologically significant. Males were only thought to be acoustically sensitive between 300-800 Hz. This assumption was based on the behavioral work of Roth (1948) in which females did not respond to sound and Wishart and Riordan (1959) in which males did not orient to harmonic frequencies played alone.

Although several studies were published on both the vibrational and neurophysiological properties of male mosquito hearing (Tischner 1954; Apasov et al. 1986; Belton & Costello 1979) it was not until Göpfert et al. (1999) that the properties of the female Johnston organ and antennae were examined as sound receptors. Boo and Richards (1975) described anatomical differences between male and female Johnston's organs and hypothesized that females were unable to hear because their Johnston's organs lacked type D scolopedia.

As newer technology has been applied to insect physiology the ability of females to hear came under debate. Using microscanning laser vibrometry, Göpfert et al. (1999) measured mechanical tuning in both male and female *Ae. aegypti*. They followed up this study and measured neural responses in the Johnston's organ to sound in both males and females (Göpfert & Robert 2000). In this study they found that both male and female mosquito Johnston's organs responded to particle displacement, although the female was far less sensitive than the male.

There are sporadic reports citing evidence for female audition. Borkent & Belton (2006) published a study in which female mosquitoes were collected in acoustically baited CDC traps. Gibson and Russell (2006) found that when pairs of

Toxorhynchites brevipalpis were recorded in flight that they altered their wingbeats to match at fundamental components of their flight tone (400-500 Hz). However, these authors failed to determine how and if females were actually hearing the flight tone of conspecific males using any physiological measurements of the Johnston's organ.

I was interested in whether similar convergence behaviors occurred in medically important mosquitoes. I recorded paired tethered flight in both *Ae. aegypti* and *An. gambiae* and found that, like *Tx. brevipalpis*, these mosquitoes matched at components of their flight tone signals, but instead of matching at fundamental frequencies, they matched at harmonics above 1 kHz. This was well above the accepted 800 Hz acoustic ceiling for males and provided further behavioral evidence that females were able to hear. I then conducted a series of playback experiments with both male and female *Ae. aegypti* to further determine the ranges of male and female audition. These experiments were combined with electrophysiology data collected by Dr. Ben Arthur and resulted in a publication Cator et al. (2009). The results from this study drove the remainder of the experiments I conducted for my dissertation.

Methods

Mosquito Rearing

Ae. aegypti (Mexico strain) were reared from eggs collected from Rio Florido, Mexico (N 14.85483, W 092.34100) in August 2006. The colony was supplemented with field material yearly. Adults were maintained in an environmental chamber. Under the following conditions were as follows: a fluctuating temperature range of 24 ± 4˚C, 80% RH and a photoperiod of 14 h L: 10 h D with a 2 h period of dusk/dawn conditions.

I hatched eggs (F5 progeny) taken from a mixture of females under a vacuum for 30 min. After a 30 min period, I added 0.2 mg of *Aedes* food (1:1 ratio of lactalbumin: brewer's yeast) to the flask. Larvae were held for 24 hrs at 27˚C, sorted into 3.9 L trays filled with 1 L of distilled water and fed 0, 38, 75, 113, and 150 mg of *Aedes* food on days 1, 2, 3, 4 and 5 respectively.

To obtain virgin mosquitoes, I individually distributed pupae into vials (15 ml) containing 3 mL of distilled water and plugged with cotton wool. Emerged adults were grouped by sex and age, maintained in the same environmental chamber conditions as described above, and fed on a 20% sucrose solution. Mosquitoes were held under these conditions for 3 days and on the 4th day were used in experimental trials. Unless otherwise specified, all *Ae aegypti* used in experiments were large 3-day old virgins reared in the manner described above.

An. gambiae eggs were taken from over 5,000 females in the Ifakara Health Institute (IHI) colony; established from 1996 collection from Njage Village, Tanzania. Consistent with field observations, matings in colony cages began at dusk and continued for several hours into the evening indicating that the colony had not significantly diverged from temporal behavior of field populations. After hatching, first instar larvae were sorted into two groups of 1,000. Larvae were held in large 15 L basins containing 4 L of water and were fed a diet of ground fish food (Tetramin, Melle, Germany). For paired flight experiments larval basins received 400 mg of diet per day. Pupae were removed from larval trays and transferred to bowls in small (30 cm x 30 cm x 30 cm) mesh cages for emergence. We separated newly emerged males and females into 1.4 L cylindrical cartons within 12 hours of eclosion (prior to male maturity i.e. rotation of the male genitalia) daily (Clements 1999). Adults were held in cages for three-days and supplied a 10% glucose solution. All mosquitoes used in experiments were 3-5 days old and virgin. Mosquitoes were held in the IHI center insectary at approximately 25-29°C with 60% humidity and 12:12 hr dark: light photo regime from fluorescent bulbs.

Tethering and Recording Procedures

Males and females were immobilized with wet ice for 15 min at which time individuals were tethered to size 02 insect pins (Bohemia ®, Czech Republic) using super glue (Ross Products, Inc., Columbus, OH). I tethered each mosquito to a blunted sharp end of the pin and positioned subjects in an upright orientation conducive to flight with the tether fastened to the anterior end of the thorax. The tether was positioned so as not to interfere with either the thoracic flight muscles or antennae.

Paired Flight

I clipped tethered pairs of male and female *Ae. aegypti* to micromanipulators. Jackson and Robert (2006) established that male antennal response was fully active when females were within 2 cm. Therefore, I placed the microphone (NR-21358, Knowles, Itasca, IL) 1.5 cm from each mosquito (Figure 1). I initiated flight using tarsal stimulation. In many insects the loss of tarsal contact with a substratum triggers the initiation of flight (Chapman 1998). Solitary male flight was recorded for 20 s. I then initiated female flight and moved females into the male auditory field for 10 s of paired flight recording. I removed the female for 5 secs before beginning another 10 sec “fly-by”. I repeated this cycle for 90 secs or until one of the subjects stopped flying.

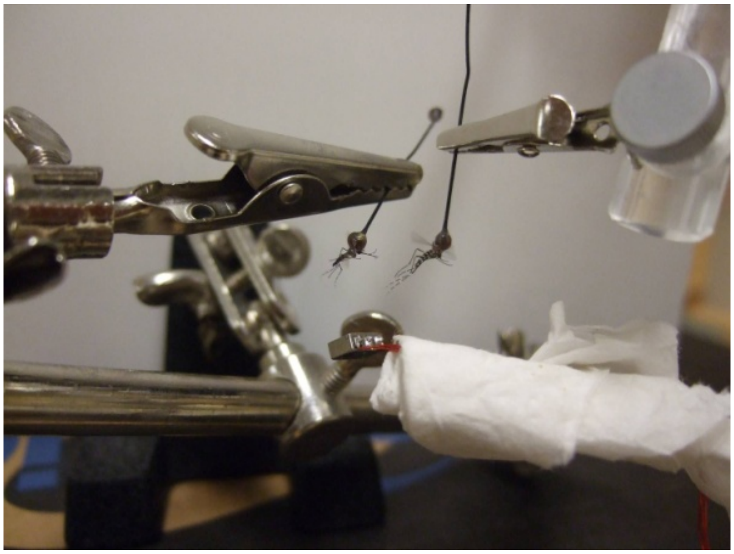


Figure 1 Paired flight in experimental rig.

For *An. gambiae,* I used a modified tether designed to allow for free orientation. A 1 cm long section of a human hair strand was attached to the end of an insect pin (size 02) on one end and to dorsal thorax of anesthetized adults on the other. I positioned males and females approximately 1.5 cm apart and placed a particle velocity microphone (NR-21358; Knowles, Itasca, IL) 2 cm below the pair and equidistant between them. Recordings began when one or both individuals initiated flight and continued until both individuals stopped flying.

Recordings were taken using gPRIME software (Lott 2007) and processed using Raven 1.0 (Cornell University Lab of Ornithology, Ithaca, NY). I defined synchronization by two criteria. First, male and female flight tone had to match at any convergence of harmonic frequencies. Sec, this match had to be achieved by one or both parties adjusting flight tone frequency. After recording, I removed and measured the right wing from each individual.

Playback Experiments

Preliminary Experiments

A sub-sample of 10 *Ae. aegypti* (Mexico) females were removed from the Cornell lab colony. Individual recordings of these females were taken. The carrier frequencies and power spectra of these females were averaged to produce a standard harmonic series. This averaged harmonic series was recreated using Koé software (Hoy Lab 2008). Ten large 3-day virgin males were then tethered and individually exposed to playbacks using Koé files played through a bud earphone (MDR-E828LP,Sony Corp. Tokyo Japan). Each recording proceeded as follows: male flight alone (5 sec), male exposed to all frequencies (10 sec), male alone (5 sec), male exposed to harmonics only (10 sec), male alone (5 sec), male exposed to carrier only (10 sec).

A series of playbacks were conducted to identify the carrier frequency which elicited the most consistent response from males and females. The averaged frequency from the colony sub-sample was used as an estimator. I measured the responses of large male *Ae. aegypti* to 400, 415, 430, 445, and 460 Hz. I used the male optimal playback frequency as the estimator for the optimal female frequencies. A behavioral tuning curve was conducted using 600, 650, 700, and 750 was the test fundamentals. Five females were exposed to playbacks of the first thru fourth harmonic based on these fundamental frequencies. A “response” in these experiments was characterized by a convergence of the test individual to any of the tones in the playback. Again, Koé was used to create playbacks which had the experimental carrier frequency and the power spectra taken from the original sub sample average. Five individuals were recorded for each increment for a total of 20 individual. Each recording was analyzed in Raven to determine if convergence occurred.

I constructed an intensity tuning curve for males. Four groups of 20, 3-day old, virgin, male *Ae. aegypti* were exposed to a 1200 Hz stimulus at four different intensity levels. The percent of males responding to 1200 Hz stimulus played at 0.0122, 0.0224, 0.0511, 0.3086 and 0.800 mm/s was recorded. The results from this study were compared with electrophysiological measurements from the Johnston's organ (Arthur, 2008). The right wing of each male was removed, measured, and recorded.

Male Playback

After these preliminary experiments, two different playback experiments were conducted. First, a group of 20 males was exposed to the 400 Hz harmonic stack alone without the carrier frequency. Sec, an additional group of 20 males were exposed to only the 1200 Hz simulated female 3rd harmonic. In both cases the male was permitted a 5 s acclimation before a stimulus was introduced as well as a 5 s acclimation period in between each playback in the recording series.

Female Playback

Based on preliminary experiments, twenty females were exposed to a synthesized male frequency with the fundamental at 700 Hz and a harmonic stack of 1400, 2100, and 2800 Hz. Females were exposed to stimulus in 10 sec bursts with 5 sec breaks. Next, a group of twenty females were exposed to only the male 2nd harmonic (1400 Hz). Finally, 10 two-day old virgin females were transferred into cartons containing 3-4 day old virgin males. The females were held with males overnight and provided a 20% sucrose solution. Within 24 hours these females were exposed to a simulated male flight tone containing the first four harmonics. Stimulus was introduced in the same pattern as described previously. After recordings were completed, I dissected the female reproductive tracts to confirm insemination. This experiment was replicated three times.

Controls

Controls were conducted to confirm that (1) mosquitoes were fact responding to the stimulus, (2) that the mosquitoes perceived the stimulus with their antennae, (3) that the frequencies observed were not a product of tether resonance, and (4) that the phenomena observed were not an artifact of laboratory culture.

Ten 3-day old, virgin, male *Ae. aegypti* were tethered using the methods described above and exposed to two treatments. In the control treatment, males were allowed to fly for 60 sec without exposure to stimuli. In the subsequent experimental treatment, they were exposed to 10 sec periods of a 1200 Hz tone, which simulated the female 3rd harmonic or male 2nd male harmonic. The distance which males moved toward the 1200 Hz stimulus was recorded for each individual under experimental and control flight conditions.

Both males and females were anesthetized with cold narcosis. I deafened males by removing the antennae using a fine razor blade and fine point forceps. I deafened females by immobilizing the antennae with super glue so that there was no movement at the intersection of the antennae and the JO. Test subjects were given a 1 hr recovery time after which I assessed their condition. If post-procedure individuals were able to fly, they were tethered. Males were exposed to a female 3rd harmonic stimulus and females to a male 2nd harmonic (described under “playback experiments”).

In order to confirm that the frequencies recorded were not being distorted by the resonance of the tether; I recorded 5 male mosquitoes with full (4 cm) and half (2 cm) length tethers. If the frequencies being picked up by the microphone were in fact a product of the tether resonance then dividing the tether in half should double the original frequency. The frequencies of half tethered flight were compared to double those of full tethered flight.

In order to ensure that synching behavior was not an artifact of laboratory culture, I examined the paired flight dynamics of field caught mosquitoes. Pupae were removed from breeding sites in several barrios of Tapachula, Mexico and the neighboring community of Rio Florido (N 14.85483, W 092.34100) from January 17-24th, 2008. The eggs used to begin the laboratory colony utilized in these studies was established aith eggs from the same field sites. Pupae were individually placed in 3ml tubes with 1.5ml of filtered water and plugged with cotton wool. Upon emergence, I separated males and females into small cartons and held them at approximately 28°C with 20% sucrose solution for 3 days. Recordings were conducted outdoors with natural lighting and temperature (average 26.5 °C) conditions. The identical equipment was used in these recordings with the exception of a slightly different converter to connect the lab top to the amplifier.

I collected 4th instar *Anopheles* larvae of unknown species from Sagamaganga village, in the Kilombero Valley of Tanzania (S 08.11437, E 036.550). Larvae were held in a 15 L basin contain 4 L of water under the IHI conditions detailed above until pupation and placed into individual vials for emergence. Adults were held in the IHI insectary and provided 10% sucrose solution. I repeated paired flight recording with 3-

day old emerged adults as described above. After recording, each individual was stored on desiccant for species confirmation (Scott, 1993).

Free Flight Assay

Virgin 3-4 day old male and female *Ae. aegypti* were used in free flight experiments. Experiments were conducted between 0700 and 1800 hrs. I anesthetized females on wet ice for 5 mins. I then tethered the female using Elmer's glue to a 3 cm strand of hair, which at the opposite end was attached to an insect pin (the "semi"-tether). Females were positioned so that they are within 4 cm of the sensitive face of the microphone at all times. Host stimuli were provided by a human arm. I stimulated flight using tarsal inhibition and gentle puffs of air.

Males were released into a 12 x 6 x 8 cm test plastic arena containing the female and the host stimuli. Five of the six arena faces were opaque (covered with white paper) and the front face was clear plastic to allow the researcher to observe interactions. Recordings were started when males were released in the mating arena and terminated upon successful copula formation. The right wing of successful males was at this time removed and measured as a proxy for body size.

Comparison of Flight Tone of An. gambiae found in copula

I reared large and small cohorts as previously described. As detailed in Ng'Habi et al. (2005) eggs for the small cohort were hatched 4 days prior to the large one to facilitate synchronized emergence. I segregated newly emerged adults by age and sex prior to sexual maturation of males (rotation of the male genitalia) and held them for 3 days with a 10% glucose solution.

On the evening that experimental mosquitoes were 3-5 days of age, 20 large and 20 small males were transferred to a 15 x 15 x 10 cm mesh cage. Swarms of males were established as described in Ng'habi et al.(2008). Briefly, I exposed them to natural light beginning at 17:00 hrs and approximately 10 mins before sunset began checking the cages for swarming activity. Females were transferred in groups of ten consisting of a 1:1 ratio of large: small body sizes (5 small females, 5 large females). Copulating pairs were removed with a mouth aspirator as they formed and placed into an individual vial for the remainder of the swarming period. I replaced males and females removed with fresh individuals of the same size to maintain the same ratio of body sizes and overall number of test individuals throughout the swarming period. At the conclusion of swarming collection and on the same evening, I recorded the flight tone of males and females found in copula. After recordings were completed, I removed the right wing from each individual.

Recording Analysis

Recordings were analyzed using Raven 1.0. If convergence was determined, then I additionally recorded the time of convergence in relation to paired interaction, the movement of male and female fundamental frequencies leading to convergence, the rate of convergence, and the duration of synch.

Data Analyses

I used Fisher's Exact Test to determine if the frequency data were significantly different between control and test treatments (Fisher 1922). Frequencies recorded using half tethers were compared to double the full tether frequency using a student t-test. Binary logistic regression was used to determine the effect of stimulus intensity and frequency on response. For free flight assays, a generalized linear model was used to determine the effect of convergence on mating.

Results

Paired Flight

Ae. aegypti. Paired flight recordings resulted in 21 quality recordings of paired sustained flight. Of these 21 pairs, 14 pairs (67%) met the criteria for matching behavior. Matching tones occurred between the male 2nd and female 3rd harmonic and averaged 1354.5 ± 31.5 Hz (Figure 2B). Synchronization between pairs lasted 9.71 ± 1.05 secs. Males and females both adjusted frequencies. Individuals flown in pairs modulated frequency an average of 42.25 ± 5.78 Hz. Females altered their frequency significantly more than males (Student's t test, t=-3.01, d.f.=41, P=0.004).

An. gambiae. Twenty pairs of *An. gambiae* were recorded in paired flight. Of these 30% showed evidence of synchronization at harmonic frequencies. Most commonly, synchronization occurred between the male 2nd and female 3rd harmonic (Figure 2A). Only two pairs synchronized at other harmonics (1 pair at the male 1st and female 2nd, and one at the male 3rd and female 4th). The average frequency of synchronization was 1368.0 ± 165 Hz and synchronizations lasted an average of 3.83 ± 0.872 secs. Rate of synchronization was calculated as the change in frequency (Hz) divided by the time (secs) the subject was exposed to the stimulus. The average rate of synchronization (change in frequency Hz/ time with conspecific in flight secs) for males was 8.20 ± 3.38 Hz/sec. Female synchronized at a rate of 6.59 ± 2.51 Hz/sec. This difference was not significant (Student's t test, t- = 0.38, d.f.= 9, P= 0.711

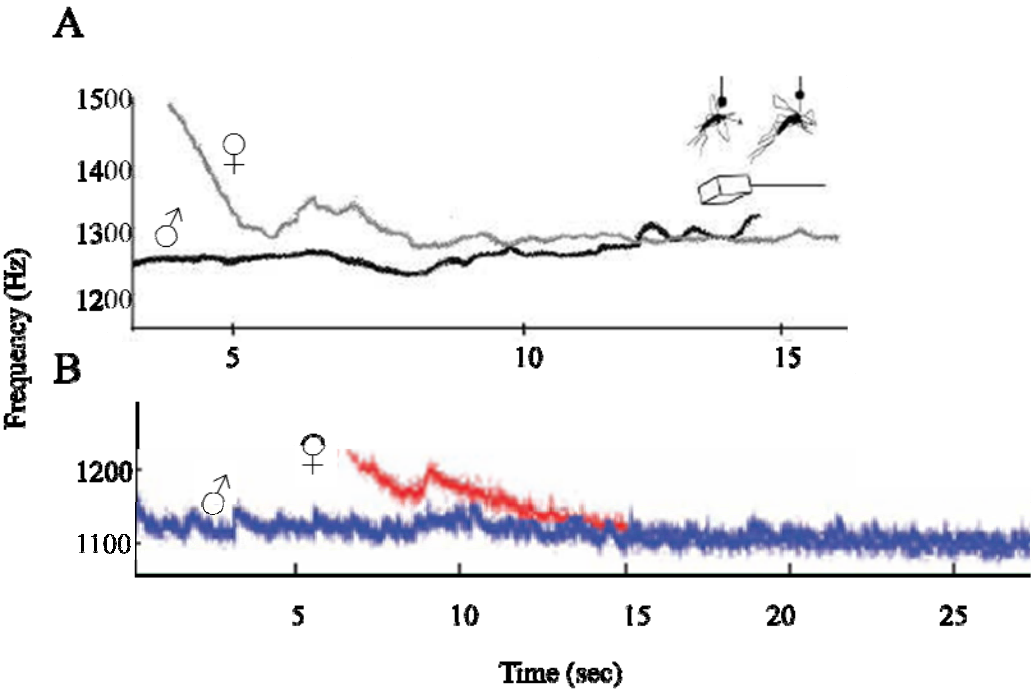


Figure 2: A; Match between male 1st and female 2nd harmonics in a male-female pair of *An. gambiae* female flight tone trace is in grey and male trace in black. B; Matching of male 2nd (blue) and female 3rd (red) harmonic in *Ae. aegypti*.

Playback Experiments in Ae. aegypti

Preliminary experiments**.** The results of preliminary male and female frequency optimization curves can be seen in Figure 3. Males were most likely to respond to playback with carrier frequencies at 400 Hz and females were most likely to respond to playback spectra with 700 Hz carrier frequencies. The playback screen revealed that males were able to modulate their flight tones in the absence of a carrier frequency (n=5 out of 10).

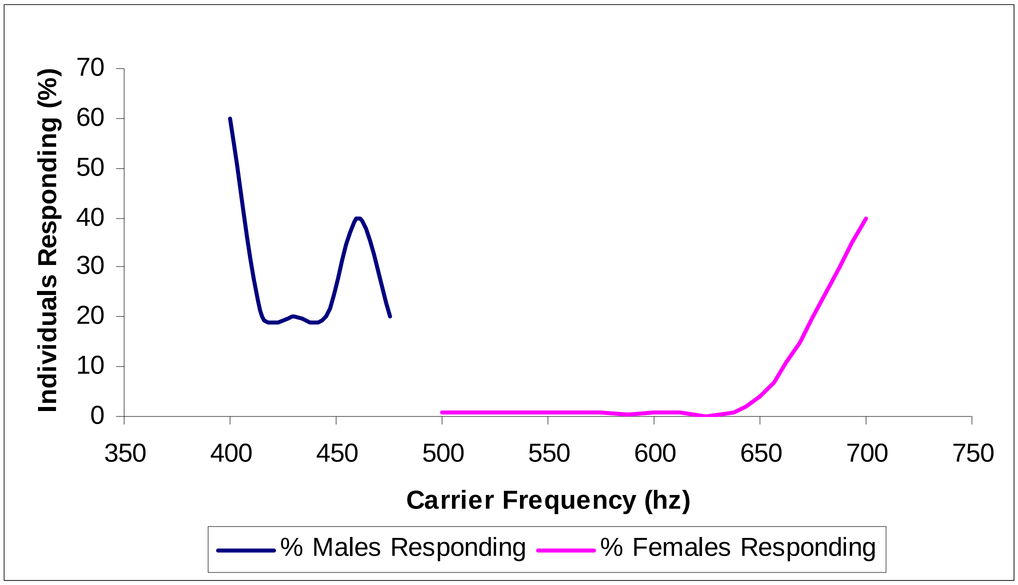


Figure 3. The majority of females responded to playback with 700 Hz based stimulus (40%). Most males responded to 400 Hz based stimulus (60%).

Playback experiments demonstrated that males were able to synchronize their 2nd harmonic to the female's 3rd harmonic in the presence of the 2nd and 4th harmonic without the carrier (39%,n=11 out of 28). Males were also able to match the female 3rd harmonic (1200 Hz) when played alone (22%, n=12 out of 54) (Figure 4). Females demonstrated the ability to converge their 2nd harmonic with the male 3rd harmonic when playback consisted of the entire male harmonic stack (30% n=6 out of 20) (7000, 1400, 2100, and 2800 Hz) and the male 2nd harmonic (1400 Hz) alone (35%, n=7 out of 20). Mated females matched full male playbacks at a lower frequency (11%, n=2 out of 18).

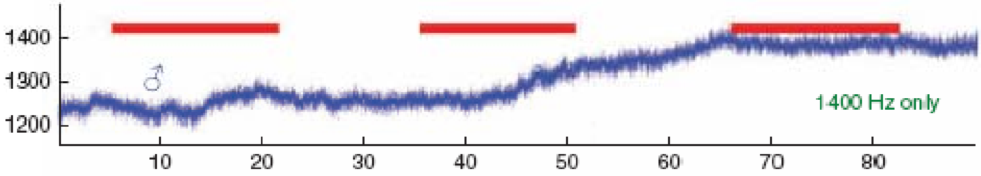
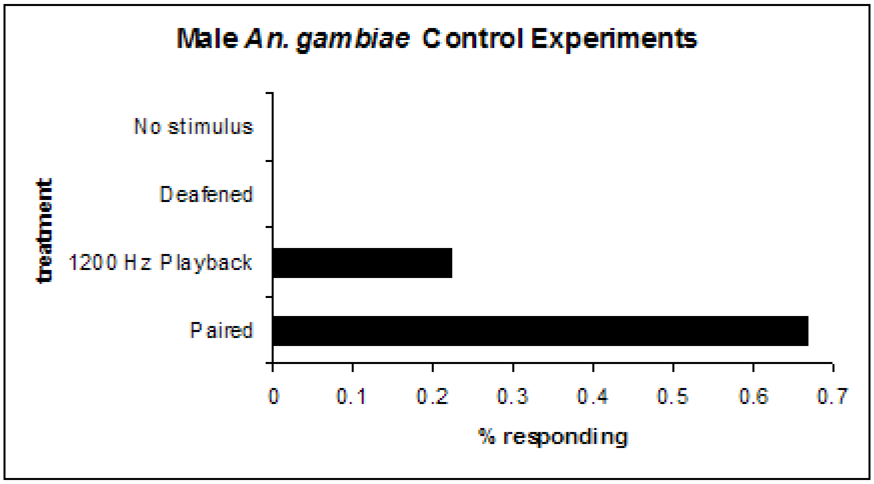


Figure 4. Synchronization of male 2nd harmonic to computer playback simulating female 3rd harmonic alone.

The intensity dependent behavioral tuning curve revealed a significant impact of stimulus intensity on response level (Binary Logistic Regression, P=0.008). Winglength was also a significant predictor of converge response (Binary Logistic Regression, P=0.037). Larger males were more likely to modulate and synchronize with playback frequencies.

Control experiments are summarized in Figure 5. Control experiments indicated that both males and females responded to stimuli with 7/20 females modulating to playback and 0/10 modulating with no play back (Fisher's Exact Test, P= 0.001); 11/38 males modulated to playback compared with 0/9 modulating with no playback (Fisher's Exact Test, P< 0.001). Both sexes perceived stimuli with their antennae (11/38 males modulated with antennae vs. 0/8 modulating with antennae removed, Fisher's Exact Test, P<0.001; 7/20 females modulated compared with 0/10 with treated antennae, Fisher's Exact Test, P=0.001). Frequencies observed were not the product of tether resonance (n=10, Student's t test, t=31.44, d.f.= 9, P< 0.001). Field collected *Ae. aegypti* demonstrated synchronization in paired flight (n=4 out of 10) and 3 out of 14 pairs of field collected *Anopheles* demonstrated synchronization behaviors.

I recorded 250 free flight interactions between pairs of large *Ae. aegypti*. I found that the presence of harmonic convergence significantly predicted the formation of a successful copula (Wald Chi Square, W=14.54, d.f.=1, P<0.001). Synchronization between both the male 1st and female 2nd harmonic and the male 2nd and female 3rd harmonic was observed.



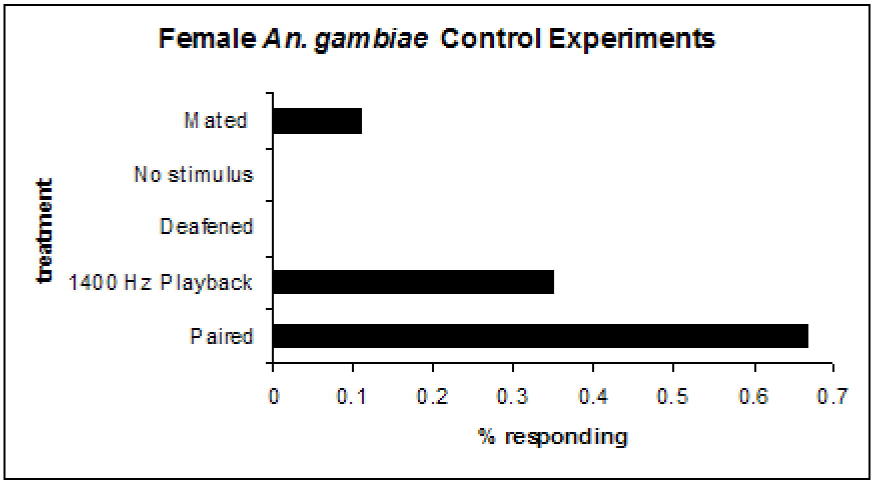


Figure 5. Controls from playback experiments. Controls were conducted to ensure that behavioral responses were in response to the stimuli and that stimuli were perceived by the Johnston's organ. Additionally, female controls in the lower panel revealed a decrease response in mated females.

Flight Tone of An. gambiae found in copula

Virgin 3-5 day old male and female *An. gambiae* were recorded in paired flight. I modified the tether design described above to allow for free orientation. I attached a 1 cm long section of a single strand of human hair to the end of an insect and attached the free end of the hair to the dorsal surface of the thorax of anesthetized adults with super glue.

Discussion

When flown in tethered opposite sex pairs *Ae. aegypti* and *An. gambiae* actively altered their flight tones to converge at harmonic frequencies. This is the first description of "harmonic convergence" in mosquitoes. Previous work on acoustic signals in mosquitoes assumed the females were deaf and therefore, unable to utilize acoustic stimuli. Both behavioral and physiological (Cator et al. 2009) measurements demonstrate that both males and females are able to perceive sound and that the range of this sensitivity extends above 1 kHz.

Both males and females adjust their flight tone to converge at harmonics. In tethered flight, 67% of *Ae. aegypti* and 40% of *An. gambiae* converged at harmonic frequencies. The differences in the occurrence of harmonic convergence in paired flight may be due experimental factors such as the smaller sample size of *An. gambiae* (n=20 pairs) or the presence of the experimenter (and their host stimuli) which may have altered Anopheline mating behaviors. Additionally, the cues leading to successful copulation and insemination are likely multimodal. While acoustic stimuli may be utilized in both species, *An. gambiae* may rely more heavily of cues in other modalities for mate assessment. Given the vast differences in the ecology and mating biology of these two mosquito species, it is likely that they weigh the importance of cues differently.

Free flight experiments confirmed that in *Ae. aegypti* harmonic convergence is not a product of tethered flight. I also found that successful harmonic convergence significantly predicted the formation of a copula. Females were less likely to reject males who synchronized to their harmonic frequencies. Based on these results, I am confident that harmonic convergence is involved in precopulatory mating behavior.

It should be noted that paired flight does not accurately mimic one aspect of the mating system of these insects. One-on-one interaction between males and females typically take place in the context of a swarm with multiple interactions occurring between different individuals sometimes simultaneously. However, in order to methodically dissect swarm behavior it is critical the rules which govern one-on-one interactions are established. Future studies should explore interaction involving more than two individuals.

The frequencies of the harmonic convergence observed in paired flight exceeded the known range of hearing in male mosquitoes and suggested that females were processing acoustic stimuli. To confirm this I conducted a series of playback experiments which, when coupled with electrophysiological measurements from the Johnston's organ (Cator et al. 2009), demonstrated the existence of female audition and extends the frequency range of both sexes over 1 kHz. Control experiments confirmed that the behaviors observed in playback experiments were triggered in response to acoustic stimulus, that the stimuli was detect by the antennae, and that the behaviors were not phenomena of tether resonance or laboratory culture.

I built frequency and behavioral tuning curves using harmonic convergence as the measured behavioral response. The male intensity curve indicated that males were sensitive from 0.0122 to 0.800 m/s^2 to a 1400 Hz stimulus. Male frequency tuning curves suggested that they responded to fundamental and harmonic stacks from 400-475 Hz. This is within ranges reported in previously work in which male orientation was used as the response measured (Roth, 1948, Wishart and Riordan, 1959, Njihout and Craig, 1967). Results of the female tuning curve indicated a response to fundamental and harmonic stacks based on 650- 700 Hz. I did not test flight tone signals above 700 Hz and is possible that females may respond to even higher frequencies.

The function of harmonic convergence is unclear. It may serve as a prezygotic isolation mechanism. It appears that flight tone modulations are widely conserved across mosquito groups. This indicates that they may be important for species recognition. *Toxorhynchites* is the only genus within its subfamily of Toxorhynchitinae, whereas *Ae. aegypti* and *An. gambiae* are part of the large mosquito subfamiles. The uses of harmonic components, as observed herein are more complex than the use of fundamentals in *Toxorhynchites.* Perhaps, this complexity is required to discriminate between many closely related species. *Ae. aegypti* and *An. gambiae* have parasympatric distributions with several other closely related species. Pennetier (2010) observed the males and female from different molecular forms of *An. gambiae* were less likely to converge at harmonics than pairs from the same form. This would explain the Duhrkopf and Hartberg's (1992) observation that *Ae. albopictus* did not seem to respond to fundamental frequencies. The timing and duration of synchronization at harmonics may instead supply species specific information. In future studies it would interesting to examine flight tone interaction between a sympatric mosquitoes species to determine the utility of modulations in species discrimination.

An alternative explanation, for the complexity seen here, is that modulations are used in mate selection. It occurs directly prior to mating, there is an intimate relationship between flight tone production and mating behavior, and both males and females are in possession of highly sensitive hearing apparatus ideal for receiving these signals. In the remaining chapters of this dissertation, I focused on testing this hypothesis. First, I presented evidence that there is variation in flight tone signals and that this variation is correlated with an important fitness characteristic. I also demonstrated that this variation is perceived by conspecifics and further, that behavioral difference in response to signal variation is suggestive of preference. Next, I presented data on the material and genetic benefits associated with individuals possessing certain signaling characteristics. Finally, I reported on the broad sense heritability of harmonic convergence behavior.

CHAPTER 3

THE EFFECT OF BODY SIZE ON FLIGHT TONE FREQUENCY AND CONSPECIFIC ACOUSTIC INTERACTIONS

Introduction

In numerous animal mating systems the sexes have evolved the ability to discriminate the quality of their potential partners before copulation. Thus, signals have evolved so that males and females may indicate fitness status to conspecifics and increase mating success (Searcy & Nowicki 2005). Signals are valuable if they provide the receiver with adequate content to inform mating decisions. It is widely believed that these signals should vary with fitness traits and elicit different responses based on their information content (Smith 2003; Searcy & Nowicki 2005). Thus, the information communicated in courtship signals can provide researchers with valuable insight into mate choice and fitness traits.

In flight, mosquitoes produce a humming sound known as flight tone. The flight tone signal produced by mosquitoes is composed of both fundamental and higher harmonic frequencies (Clements 1999). Harmonics occur at integer multiples of the fundamental frequency and decrease exponentially in amplitude. Roth (1948) was the first to describe the role of flight tone in male mating behavior and attraction to females in *Ae. aegypti*. Since that time, numerous studies have explored the hypothesis that the fundamental frequency of female flight tone signals species identity in mosquito swarms (Roth 1948; Wishart & Riordan 1959; Belton & Costello 1979; Belton 1994; Wekesa et al. 1998; Tripet et al. 2004; Gibson & Russell 2006). Recently, we demonstrated that male and female *Ae. aegypti,* flying in pairs, alter their flight tone to match each other at harmonic frequencies (male 2nd and female 3rd harmonics) instead of fundamental frequencies, a behavior termed “harmonic convergence” (Cator et al. 2009). Previous to this study, males and females were thought to be deaf to these higher harmonic frequencies and even the ability of females to hear conspecifics was under debate (Clements 1999; Göpfert et al. 1999; Göpfert & Robert 2000; Gibson & Russell 2006). More recent work has suggested that harmonic convergence is involved in intersexual communication, but the adaptive significance of these signals in mosquito mating interactions is uncertain (Cator et al. 2009; Warren et al. 2009).

The fundamental frequency and its overtones in the mating songs of many animals appear to be linked to body size, and are used by conspecifics to assess size of animals producing sounds (insects, Gwynne (1982); fish, (Machnik & Kramer 2008); frogs, (Ryan et al. 1990). While some mosquito studies have reported no relationship between body size and mating success (Charlwood et al. 2002), others have suggested that both larger male and female mosquitoes have higher fecundity and mating success (Yuval et al. 1992; Okanda et al. 2002; Ponlawat & Harrington 2007; Ponlawat & Harrington 2009). Therefore, the ability to discern the body size of a potential mate may be advantageous for mate assessment in swarming mosquitoes.

In this study, I tested the latter hypothesis and explores the possibility that these two medically important species use acoustic signals to assess fitness of potential mates. If flight tone in mosquitoes provides reliable information about body size, and if body size is correlated with fitness (Yuval et al. 1992; Okanda et al. 2002; Ponlawat & Harrington 2007; Ponlawat & Harrington 2009), then flight tone might be a key signal for mate assessment.

First, I measured the effect of body size on flight tone in *Ae. aegypti* and *An. gambiae.* Next, I measured signaling behaviors of tethered mosquitoes in response to both the flight tone of live mosquitoes as well as synthesized tones that mimicked potential mates of varying body size. I predicted that harmonic convergence behavior would vary with body size of the computer-generated perceived sender and live receiver of the signal, conspecifics would be able to perceive this variation, and response of tethered mosquitoes would be modulated by the perceived size of a virtual conspecific.

Methods

Creation of Size Cohorts

Ae. aegypti. I created large and small body size cohorts by varying crowding conditions during the larval stage. Large cohorts were created by transferring 75 1st instar larvae to a 3.9 L tray containing 1 L of distilled water. Small body size cohorts were reared in 3.9 L containers with 750 larvae and 1 L of water. Medium body size was created by group 200 larvae in 1 L. Amounts of 38, 75, 113, 150 and 113 mg of *Aedes* diet were added to trays on days 1, 2, 3, 4 and 5 respectively. In order to confirm that significantly different body sizes had been created in experimental groups, a right wing from each mosquito was removed and measured (Nasci 1990).

An. gambiae I created large and small body sizes by rearing larvae under different feeding regimes. First instar larvae (IHDRC colony; established from 1996 collection from Njage Village, Tanzania) were sorted into two groups of 1000. Larvae were held in large 15 L basins containing 4 L of water and were fed a diet of ground fish food (Tetramin, Melle, Germany). The large cohort received 400 mg diet/day and the small cohort received 100 mg diet/day (Lyimo et al. 1992).

Pupae were removed from larval trays and transferred them to small (12 x 12 x 12 inch) mesh cage for emergence. I separated males and females into cartons within 24 hrs (prior to the rotation of the male genitalia) (Clements 1999). Adults were held in cages for three-days and supplied a 10% glucose solution. *An. gambiae* were held in the IHI center insectary at approximately 25-29°C with 40% humidity. Mosquitoes held in colony conditions received 12 hours of dark and 12 hours of light from florescent bulbs.

Tethering and Recording Procedures

Male and female *Ae. aegypti* were immobilized with wet ice for 15 min at which time individuals were tethered to size 02 insect pins (Bohemia ®, Czech Republic) using super glue (Ross Products, Inc., Columbus, OH). *An. gambiae* is more sensitive to cold than *Ae. aegypti*, and therefore were only immobilized for 2-6 mins. I tethered each mosquito to a blunted sharp end of the pin and positioned subjects in an upright orientation conducive to flight with the tether fastened to the anterior end of the thorax. The tether was positioned so as not to interfere with either the thoracic flight muscles or antennae (Figure 6). For *An. gambiae*, I recorded the average flight tone of 25 each large and small males and females (for a total of 100 mosquitoes) and replicated this experiment twice. In *Ae. aegypti*, I recorded a minimum of 20 of each large and small male and female *Ae. aegypti* and completed three replications.

Figure 6: Example of tethered mosquito. Note that the tether does not interfere with either the antennae or thoracic flight muscles of this female.

Recordings were taken using a particle velocity microphone (NR-21358; Knowles, Itasca, IL) positioned with alligator clips 1.5 cm below the subject's abdomen.

Experiments on *Ae. aegypti* were completed at Cornell University. All recordings were taken in a quiet, light and temperature controlled environment (24 ±4˚C and fluorescent lighting). Human heat, chemical, and other sensory stimuli were provided by the proximity of the researcher during observations (no farther than 31 cm from the test site). Experiments with *An. gambiae* were completed at the Ifakara Health Institute in Ifakara, Tanzania. Experiments were conducted between 1730 and 2330 hrs with natural lighting and temp/humidity conditions. In order to control flight initiation in both species, small balls of paper were placed in the grip of the tarsi. Flight was induced by removing the ball of paper and gently blowing on the subject.

Solo Flight

A particle velocity microphone (NR-21358, Knowles, Itasca, IL) was positioned 2cm below the test mosquito. Average flight tone was calculated by recording individuals for up to 2 min. The data points taken every 0.001 secs were averaged. I removed the first and last 5 secs of each recording to ensure that I was not averaging startle flight responses. Next, I exported peak frequency readings as calculated by gPRIME (Lott 2007) into an ExcelXP spread sheet (Microsoft Corporation, Redmond, WA) and calculated mean flight time.

Playback Experiments

I used artificial playback to determine the effect of body size on signaling parameters of the sender and receiver in *An. gambiae*. I constructed electronic playbacks using Koé and used them to test for synchronization response. Each playback stimulus consisted of the fundamental frequency as well as the first three components of the harmonic stack. I exposed 2-5 day old large virgin females to stimulus modeled after either a large (708 Hz) or a small male (654 Hz) as determined in the first replicate of solo flight recording. I also exposed 2-5 day old large virgin males to playbacks modeled after either a large (462 Hz) or small female (433 Hz). A group of twenty individuals of each sex was exposed to large and small stimulus.

I exposed each mosquito to 10 sec of stimulus with 5 sec rest periods. Each subject was recorded for the duration of their sustained flight up to 60 sec. Recordings were taken using gPRIME and analyzed using Raven. Only test mosquitoes that produced a minimum of 20 sec of sustained flight were included in the analysis. Synchronizations were identified as modulation of a female or male harmonic after stimulus introduction and maintenance of matching frequency with test stimulus for at least 2 sec. If synchronization was observed, I noted time (sec) of first synchronization after introduction of the stimuli (latency of response) and the rate of synchronization (Hz/s). Rate of synchronization was calculated as the change in frequency (Hz) divided by the time (sec) the subject was exposed to the stimulus (Figure 7).

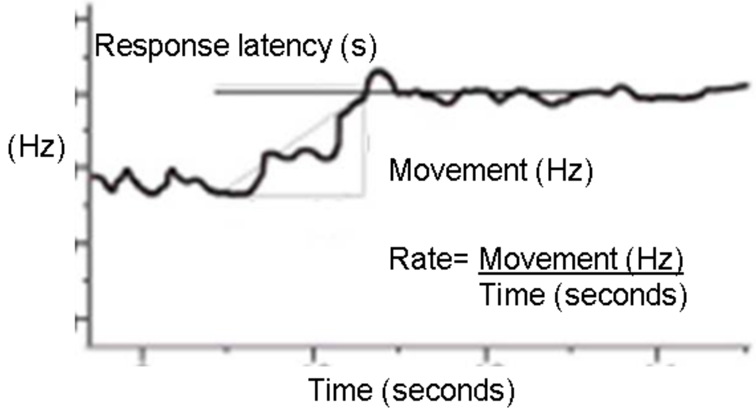


Figure 7: Schematic of harmonic convergence with important parameters such as rate and response latency labeled.

Statistical Analysis

All statistical analyses were conducted in SPSS (Version 16, SPSS Inc., Chicago, IL). In instances where there was no replicate effect, data were combined. Normality of data allowed me to confirm significant differences in wing length and fundamental flight tone frequency between body size cohorts using a Student's t-test.

A general linear model (GLM) was used to determine the importance of size and sex in predicting relevant harmonic frequencies (male 2nd and female 3rd). The significance of size, sex, and playback frequency in predicting presence of a synchronization response was determined using a binary logistic regression. Analysis of variance was used to determine the effect of playback frequency (433, 462, 654, and 708 Hz) on the rate of synchronization and Tukey's HSD was used to clarify relationships between cohort and rate of synchronization. The parameters of start time and synch duration were not normally distributed. A generalized linear model was applied to determine the effect of size and sex on these variables as well as the effect of playback stimuli.

Results

The Effect of Body Size on Flight Tone Frequency

Ae. aegypti Large males were significantly larger than small males (Student's t test, t=-15.34, d.f.=155, P<0.001). Size cohorts of females were also found to be significantly different (Student's t test, t= =21.38, d.f.=117, P<0.001). There was no significant difference between the flight tones of large and small females (Student's t test, t=-0.26, d.f.=117, P=0.797). Large males produced a significantly higher average flight tone than small males (Student's t test, t= -2.38, d.f.= 155, P= 0.019) (Table 2).

Table 2: The winglengths and fundamental flight tone frequencies of large and small cohorts of *Ae. aegypti* and *An. gambiae.*

Species	**Cohort**	**Winglength (mm± SE)**	**Fundamental Flight Tone (Hz ± SE)**
An. gambiae			
	Large Males	3.14 ± 0.03	695.68 ±7.94*
	Small Males	2.71± 0.04	639.4 ±10.6*
	Large Female	3.38± 0.02	465.53 ±8.06**
	Small Female	2.82± 0.03	433.11± 5.19**
Ae. aegypti			
	Large Males	2.36 ± 0.03	734.6 ± 20.30**
	Small Males	1.77 ± 0.03	676.0 ± 13.90**
	Large Female	3.00 ± 0.03	549.7 ± 20.10
	Small Female	2.15± 0.03	541.1 ± 26.60

* significant at P <0.001, ** significantly different at P = 0.001.

An. gambiae. Large males and females were significantly larger than small males and females (Females; Student's t test, t=12.96, d.f.=79, P<0.001, Males; Student's t test, t= 8.83, d.f.= 91, P<0.001). The flight tone of large individuals was significantly higher than that of small individuals in both sexes (Table 2). The behaviorally relevant male 2nd harmonic and female 3rd harmonics for *An. gambiae* were significantly affected by the body size of the individual (Generalized Linear Regression, P<0.001), but not sex (Generalized Linear Regression, P=0.52) (Figure 8). Large males and females produced higher harmonic frequencies than smaller individuals (Tukey's Post Hoc, P=0.05), but no difference between males and females of the same body size (Tukey's Post Hoc, males, P=0.887, female, P=0.998).

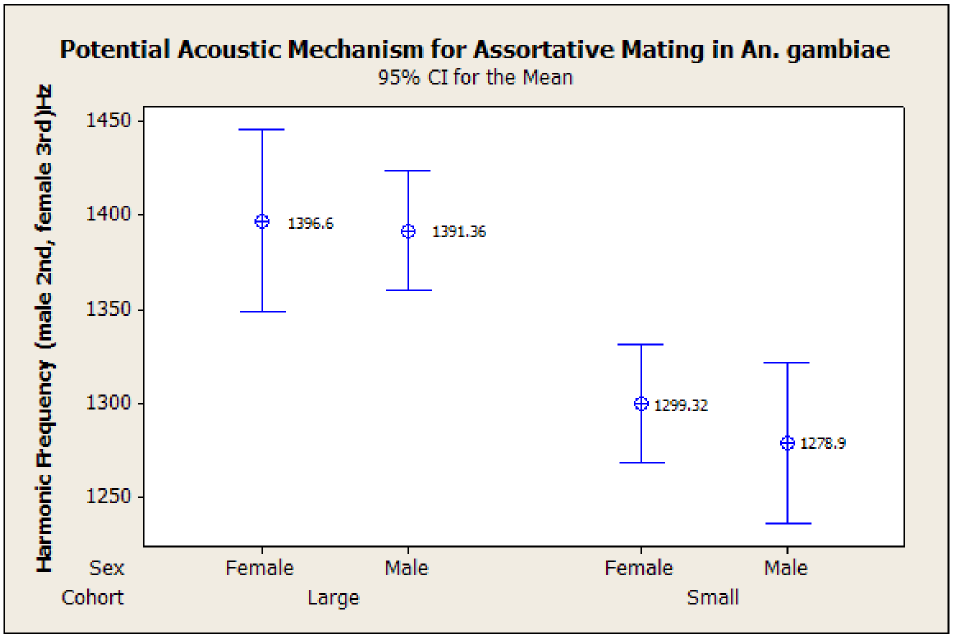


Figure 8: Comparison of female 3rd and male 2nd harmonic for two replicates of large and small body size cohorts of *An. gambiae*. Replicate were not significantly different and were pooled together.

Playback Experiments in An. gambiae

The frequency of playbacks did not alter how often males or females responded to stimuli (Binary Logistic Regression, P=0.62). A total of 344 individuals (175 females, 169 males) were used in playback experiments. Responses to these 'virtual" large and small conspecifics in playback experiments are summarized in Table 3. There was a significant interaction between sex and size of test individual in our ability to predict the presence or absence of synchronization (Wald Test, W= 11.38 d.f.=3, P=0.01). Using large and small males as test subjects, the occurrence of synchronization did not differ significantly between the two body size cohorts (Chi Square Test, $\chi 2$ = 0.247, d.f.=1, P=0.62). Large females, on the other hand, synchronized more frequently to playback stimuli than small females (Chi Square Test, $\chi 2$= 9.75, d.f.=1, P=0.02).

Table 3: Percentage of individuals from large and small cohorts responding to playback simulating large and small conspecifics

Playback Stimuli (Hz)	**Test Individual**	**Sample size**	**Percent Responding**
433	Large Male	44	34.1
433	Small Male	35	28.6
654	Large Female	47	48.9
654	Small Female	50	20.0
462	Large Male	45	22.2
462	Small Male	37	24.3
708	Large Female	48	35.4
708	Small Female	35	17.1

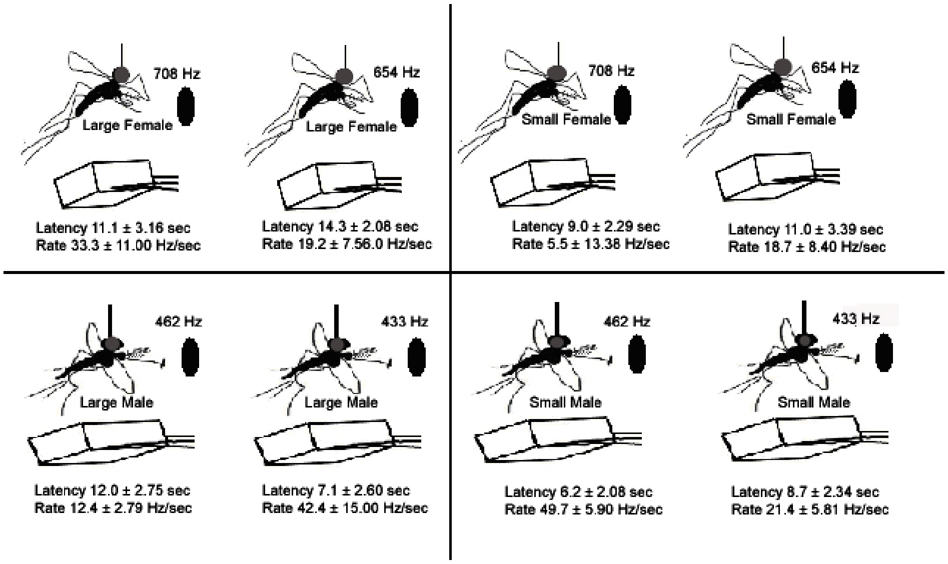


Figure 9. Results of playback experiments. Test individuals were stimulated with computer-produced pure tone acoustic stimuli. A) Results showing the response characteristics (response latency and rate) in 8 playback treatments. B) There was no difference between response to playback stimuli within treatment group, but when males and females were grouped together, males responded to 433 Hz stimuli, mimicking large females, at a faster rate and males and females had decreased response latencies to playback simulating large members of the opposite sex (*= significant at P< 0.01)

The frequency of playbacks did alter the nature of the response. When both receiver size and sex were taken into account, there was no difference between responses between test groups (rate; ANOVA, F=1.42, P=0.242, latency; ANOVA, F=1.69, P=0.175). However, when the responses of males and females were pooled I found a significant effect of playback stimuli. Playbacks imitating large conspecifics elicited shorter response latency times in males and females (Wald Chi Square Test=283.76, d.f.=1, P<0.01) and males responded to large females at a faster rate (Tukey's HSD test, P=0.05). Both sexes modulated their flight tone frequencies by similar amounts with males moving an average of 124.5 ± 13.6 Hz and females moving 107.3 ± 10.5 Hz (Student's t test, t=1.00, P=3.20, d.f.=83).

Discussion

Solo flight measurements indicated that flight tone frequency varied with body size. Larger males of *Ae. aegypti* and *An. gambiae* produced higher flight tone frequencies than small conspecifics. This relationship was also found in *An. gambiae* females, but not in female *Ae. aegypti*. Playback experiments in *An. gambiae* revealed that large females responded most frequently to playback stimuli. The frequency of playback, and therefore perceived size of a potential mate, did affect the nature of response to stimuli in all groups tested. Both male and female *An. gambiae* responded earlier and more quickly to stimuli mimicking large conspecifics.

One other study has measured the effect of body size on flight tone and also found a positive relationship between these variables in *An. gambiae* (Wekesa et al. 1998). No such study has been conducted using *Ae. aegypti* , but average flight tone calculations from this experiment were similar to those of Göpfert et al. (1999) and congruent with Nijhout & Craig (1971) finding that males respond to frequencies in the 400-450 Hz range. Brogdon (1994) found slightly higher frequencies, but this is likely due to difference in how average frequency was calculated. In this study, I sampled flight tone every one thousanth of a sec to get averages where as Brogdon (1994) only used six random points.

In order for flight tone to be involved in mate selection signals it must reliably vary with fitness characteristics. Body size has been implicated as a fitness characteristic in both species used in this study (Yuval et al. 1992; Okanda et al. 2002; Ponlawat & Harrington 2007; Ponlawat & Harrington 2009). In *An. gambiae*, signal frequency varied in both sexes, whereas in *Ae. aegypti* this variation was only found in females. There are likely different ecological and evolutionary forces shaping the mating systems of these two species, and it may be than in *Ae. aegypti* males do not use female flight tone in mate assessment and thus there is no selective pressure for flight tone to vary with body size in females.

I also found evidence that *An. gambiae* can discern between acoustic stimuli produced by large and small mates. When males and females encountered playbacks of conspecifics they actively altered aspects of acoustic response depending on the perceived body size of playback stimuli. In both sexes, playback simulating large mates elicited a response with shorter latency than playbacks mimicking small mates. Our data show that males modulate their flight tone frequencies at a faster rate (Hz/ sec) to the songs of larger females. The nature of the differences in behavioral response to acoustic stimuli is consistent with the hypothesis that flight tone functions in mate assessment.

Decreased response latency to large individuals would be advantageous if the mating system privileged larger individuals as better mates. This “size-latency principle” would also be important where mating occurs in flight swarms and the ability to respond quickly to a preferred individual is advantageous. Females could initiate synchronization and determine male quality by presence and characteristics of his response. While it is hypothetical, in this scenario there would be increased competition between conspecifics for large individuals and this competitive pressure would drive shorter response latency for such individuals.

If the increased fecundity of large females make them more desirable to males and harmonic convergence is a component of mate assessment, males able to identify large females and modulate their flight tone frequencies at a faster rate would likely enjoy a fitness benefit. I predict that engaging in harmonic convergence is metabolically demanding, and hence costly for males to accomplish. If so, males may allocate energy to harmonic displays based on the perceived benefit of mating with females of greatest fitness. A male mating investment with higher costs is predicted to produce this type of "choosiness" in males (Bonduriansky 2001). Such adjustment of courtship investment has been described for other insect species (Markow et al. 1983). In the fruit fly *Drosophila mojavensis*, males presented with a choice between virgins and previously mated females courted virgins more often and mating with these females increased male fitness through sperm precedence (Markow 1982). Investment in the courtship of large females may provide greater benefits to males (Okanda et al. 2002). If the energetic costs to the male of increasing synchronization rate are outweighed by the benefit of mating with a larger more fecund female, then I would predict that males will produce energetically costly mating behavior preferentially in response to large females. This prediction is consistent with our findings; males exposed to large "artificial" females did modulate faster to converge at harmonic frequencies.

Females may also benefit from this male signal. Furthermore, females may use the rate of male synchronization as a criterion to assess males as potential mates. In our study, synchronization rate was not limited by male body size. This suggests that convergence behaviors are not index signals that are inextricably linked to body size. Alternatively, these behaviors may act more like quality handicap signals where large males may be able to complete modulations without incurring as high a cost as small males (Zahavi 1975). Future studies will assess the costs incurred by males during signal production and benefits received by females that mate with males who are able to complete harmonic convergence behaviors.

In an alternative scenario, in which females practice passive females choice instead of active assessment and rejection, females may obtain benefits by synchronizing with male harmonics as a method for inciting male-male competition. A female can ensure that the quality of her mate by increasing the amount of intrasexual competition that the male has to endure to gain access to her. Females have been shown to encourage male competition in other insect species including the love bug, *Plecia nearctica* (Thornhill 1980). I found that large female *An. gambiae* matched playbacks more often than males. Large females may be able to modulate more frequently by drawing on a greater reservoir of energetic resources.

It should be noted that all interactions described here were recorded under experimental conditions. All recordings were taken under optimal humidity and lighting conditions for natural swarm behaviors.

These findings support the hypothesis that flight tone is used to communicate information to conspecifics within a mating swarm. Many studies (Roth 1948; Wishart & Riordan 1959; Belton 1994; Warren et al. 2009), have demonstrated that acoustic stimuli is sufficient to elicit behavioral responses associated with mating. It is likely that multiple cues are used by these insects to assess the status and quality of mates (Allan et al. 1987; Polerstock et al. 2002; Cabrera & Jaffe 2007) and it is likely that this explains the maximum response of only 50% of mosquitoes to acoustic stimuli.

It cannot be denied, that both male and female mosquitoes possess functioning Johnston's organs, which are highly complex and sensitive for audition (Göpfert et al. 1999; Göpfert & Robert 2000; Göpfert & Robert 2001). Mosquito acoustic physiology combined with newly described behavioral interactions (Gibson & Russell 2006; Cator et al. 2009; Warren et al. 2009), and the intimate relationship between mating and flight, provide strong support for the notion that acoustic communication is a significant component of mating interactions.

This is the first demonstration that flight tone signals may communicate more than species identity to conspecifics. We have long known that flight tone varies with body size in *An. gambiae*. Here I present evidence of a similar relationship in male *Ae. aegypti* and the first behavioral evidence that *An. gambiae* can discriminate between these signals. In general, the current lack of information about mosquito behavioral ecology, including the role of sexual selection is unfortunate, especially in light of the immense medical and economic importance of these disease vectors. Even recent reviews stipulate that there is no discernable courtship behavior in the mosquito species studied here (Yuval 2006). However, the data presented here indicate otherwise. The role of communication signals in mosquito mating behavior is reminiscent of other insect mating systems in acoustically active insects and surely warrants further investigation

Currently, the existence of female choice in mosquitoes is under debate. In female choice systems, females must be able to use a combination of prior knowledge, direct assessment and signal information from the opposite sex for mate selection (reviewed by Searcy & Nowicki 2005). I have demonstrated that male flight tone characteristics and behavior are capable of providing females with such cues and that conspecifics are able to perceive signal variation. As one would expect in a sexual communication system, signal characteristics vary with male fitness characteristics and female response varies with male signal characteristics. In future studies, I will focus on measuring the genetic and material benefits associated with signal variation. This relationship is critical for the evolutionary maintenance of signaling systems (Andersson 1994).

CHAPTER 4

HARMONIC CONVERGENCE OF MALE AND FEMALE PAIRS PREDICTS THE MATING SUCCESS OF MALE OFFSPRING IN *AEDES AEGYPTI*

Introduction

In many organisms, female mating preferences are thought to have evolved in response to variation in the fitness benefits offered by different males (Andersson 1994). Males may offer females direct fitness benefits such as increased fecundity (Simmons 1987; Moore 1994) and decreased risk of parasites (Borgia & Collis 1989; Able 1996). Female preference for males offering these benefits is maintained through natural selection (Neff & Pitcher 2005).

In many instances, however, males do not offer females any of these resources and yet, females in these mating systems still display preferences among males. In this case, many have postulated that female preference traits are maintained via indirect selection (Andersson 1994). Males offer female indirect or genetic benefits which impact the fitness of the female's offspring (and therefore, her own indirectly). Theoretical models have shown that these benefits are likely to take the form of increased survivorship or mating success of offspring (Neff & Pitcher 2005). Offspring of females mating with preferred males may enjoy higher survival and growth rate (Moore 1979; Simmons 1987; Moller 1990; Reynolds & Gross 1992; Petrie 1994; Hasselquist et al. 1996; Welch et al. 1998). Females may also choose to mate with males that will increase offspring mating success (Reynolds & Gross 1992; Gilburn & Day 1994). In many instances, males signal these benefits to females and females use these signals to distinguish between males (Searcy & Nowicki 2005). Males use indicator traits to provide information to potential mates about their quality or genetic resources (Neff & Pitcher 2005).

In cases where signals indicate an advantage for offspring mating success, the indicator trait is expected to be found in offspring. For example, in crickets, females are attracted to the calls of large males (Gray 1997), these males experience higher mating success (Shaw & Herlihy 2000), and body size is heritable (Simmons 1987). In populations of seaweed flies, large males are preferred by females and sire large sons which are also preferred by females (Gilburn & Day 1994). Similarly, in guppies, females preferred large males and the offspring of these males were large and were preferred by females over the smaller offspring of smaller males (Reynolds & Gross 1992b).

Here, we examine the role of direct and indirect benefits in the flight tone signaling system of *Ae. aegypti*. When flying male and female mosquitoes come into within a few body lengths of each other they alter their fundamental flight tone frequency to match at harmonic components of their flight tone (Cator et al. 2009; Warren et al. 2009; Cator et al. 2010; Pennetier et al. 2010). The function of "harmonic convergence" behaviors remain unclear, but there is mounting evidence that they may be related to mate choice in mosquitoes.

Materials and Methods

Rearing Procedures

All mosquitoes used in mating experiments were hatched from the *Aedes aegypti* MEXICO strain (Rio Florido, Mexico) and reared under low density (75 larvae/1L water). I fed larvae the following amounts of *Aedes* diet (1:1 brewer’s yeast: lactalbumin) on days 1-6, respectively; 38 mg, 0 mg, 75 mg, 113 mg, 150 mg, and 113 mg. Pupae were placed individually into 15 ml vials containing 3 ml of water and plugged with cotton wool. Each day I transferred newly emerged adult males and females into separate 800 ml cartons. Adults were provided with a 20% sucrose solution *ad libitum* and maintained in an environmental chamber set at a temperature range of 24 ± 4˚C, 80% RH and a photoperiod of 14 h L: 10 h D with a 2 h period of dusk/dawn conditions.

Recording Procedures

Virgin 3-5 day old males and females were used in paired flight experiments. Experiments were conducted between 0630 and 1800 hrs. I anesthetized females on wet ice for 5 mins. I then tethered the female using Elmer's glue to a 1-2 cm strand of human hair, which had an insect pin attached to the opposite end (the "semi"-tether). Females were positioned so that they are within 4 cm of the sensitive face of a particle velocity microphone at all times (NR-21358; Knowles, Itasca, IL). Host stimuli were provided by a human arm. I stimulated flight using tarsal inhibition and gentle puffs of air.

First, I observed the behavior of 50 pairs of virgin 3-5 day of *Ae. aegypti* in the arena. Males were released into a 12 x 6 x 8 cm test plastic arena containing the female and the host stimuli. To increase the likelihood of interaction, males were added in groups of 3. Five of the six arena faces were opaque (covered with white paper) and the front face was clear plastic to allow the researcher to observe mosquito interactions. Acoustic recordings were taken from the release of males to the formation of a successful copulation. I classified female behaviors as kicks, aversion of abdomen, holding away or acceptance (See Figure 10). Trials in which males took longer than 5 mins to initiate interaction with the female were discarded.

In the next experiment, females were tethered as before and 3 males were released into the mating arena. Recordings were initiated with the entry of males into the arena and terminated when mating pairs were removed. Copulas were not interrupted and males were not removed until they had released the female genitalia with their claspers. After mating, I removed males, cataloged them, and removed the right wing for body size estimates. I cut the hair strand as close as possible to the female's thorax using dissecting scissors r and gently transferred them into a clean 800 ml carton using a mouth aspirator.

Recording Analysis

Recordings were analyzed using Raven 1.0 (Cornell Lab of Ornithology, Ithaca, NY). The definition of convergence was a matching of harmonic frequencies after encountering a potential mate. If convergence was determined, then I recorded the time of convergence in relation to paired interaction, the movement of male and female fundamental frequencies leading to convergence, the rate of convergence, and the duration of convergence. I recorded the initial flight tone of males and females from all copulas.

Measurement of female benefits: Direct Benefits

Females were placed in 800 ml cartons and offered a blood meal (LJC, Cornell University, Institutional Review Board Protocol #06-03-043). I provided females with a 20% sucrose solution. Cartons contained 88 mL oviposition cups containing 50 mL of filtered water. I lined the cups with filter paper. Females were offered a blood meal every 3-5 days. Females received blood meals in this manner over the course of their lifetime. Sugar sources were removed and replaced with cotton wicks saturated with water 12 hrs prior to blood feeding. Females were offered a human blood meal until they successfully engorged. Those that did not feed were offered a blood meal on the following day and subsequently until they fed. Egg papers were checked for eggs each day. If eggs were laid, I counted them and the papers were replaced with fresh filter paper. I documented female mortality each day until all females died. Upon death, females were removed from cartons and I measured the right wing as an indicator of body size (Nasci 1990).

After removal, egg papers were dried for 24 hrs and then allowed to embryonate for 10 days in a plastic bag under high humidity. The first batch of eggs from each female was placed in 88 mL plastic cups and flooded with 50 mL of filtered water. Cups were checked daily for larvae. After one week the water from each cup was drained, kept dry and then reflooded one week later. After another week, papers were examined under a dissecting microscope. I counted the number of hatched (operculum open) and unhatched eggs visible on the paper. In some cases eggs completely shattered upon hatching, fortunately this occurred rarely and in isolated positions on the papers. These counts were used to calculate the hatching success for each female's first batch of eggs.

Measurement of female benefits: Indirect Benefits

The remaining batches of eggs laid by experimental females were allowed to embryonate at 28 ºC for a minimum of 1 week. I pooled egg papers into two groups. One group contained offspring of parents that demonstrated harmonic convergence behaviors prior to mating and the other group contained offspring from non-converging parents. Pooled egg papers were hatched under a vacuum for 15 mins. I provided newly hatched larvae with 0.3 mg of *Aedes* diet and held them at 28 ºC overnight.

Pilot study to optimize stressed rearing conditions.

My aim was to measure the indirect or genetic benefits males may have passed to their offspring. In order to observe genetic effects I reared larvae under stressed conditions. A pilot cohort of extra larvae were separated into groups of 100 and placed in 0.5 L of water in a 12 cm x 12 cm x 7.5cm plastic container. I fed these larvae 28, 0, 60, 90.4, 120, and 90.4 mg of *Aedes* diet on days 1-6 of development. Larval trays were kept at 28 ºC for the majority of development time. Upon reaching 4th instar, one tray each was placed for 0, 1, 2, or 3 days in a 20 ºC incubator and then returned to the 28 ºC condition. Pupae were collected and allowed to emerge individual in 3 mL vials plugged with cotton wool. Percent mortality was calculated by subtracting the number of emerged adults from 100 larvae and multiplying by 100. I compared the mortality of the 0 day treatment (optimal conditions) to the 1, 2, and 3 day treatments. The protocol that yielded 10% greater mortality than optimal conditions (2 days) was used selected as the "stressed" rearing protocol to use in this study.

Larvae were reared under the stressed protocol conditions and pupae were isolated in tubes. Males were placed into cartons and provided with 20% sucrose solution *ad libitum* and held under the environmental conditions described previously. A large and small cohort of female *Ae. aegypti* was reared synchronously with experimental males. Cohorts were reared using the procedures previously described (Chapter 3).

Five male offspring from "converging" and "non-converging" mothers were used in each trial of a mating competition experiment. One-day old males taken from each condition were marked with an identifying color of florescent dust one day prior to the experiment. For each trial the identifying color was rotated between green, pink and orange. Five "converging" males and five "non-converging" males were aspirated into a bucket cage (22 cm diameter x 26 cm height) for a total of 10 males in each mating trial. One large and one small female were aspirated into the bucket cage (for a 1:5 ratio of males and females). Copulas were collected and aspirated into individual tubes. The color/group of the males found in copula was recorded and the right wing of both males and females was removed and measured. We replaced males and females removed in copulas with fresh males and females of the appropriate color and size. I wanted to ensure that newly released males did not have an advantage over males who had been in the arena previously. In order to mitigate this effect, I replaced all individuals (male and female) every 15 mins. At the conclusion of the experiment, I placed the mating bucket into a 4 ºC freezer, collected unmated males and females and removed the right wing to be measured.

Statistics

In the first experiment, a Generalized Linear Model with a Binary Logistic distribution was used to test for the significance of male and female body size, initial flight tone, convergence, and replicate effects in predicting the formation of a successful copulation. In the sec experiment, I determined the difference between the duration of convergence between pairs converging at different harmonic combinations using a Mann-Whitney test. When data were normally distributed I tested for difference in wing length between converging and non-converging individuals using a Student's t-test. The life table values of net reproductive rate (R_0) of converging and non-converging groups of pairs was compared using a Cox regression. I used a General Linear Model to determine the effects of female body size and convergence behavior on the number of eggs each female laid. Female survival from individual data was compared using a Cox Regression. Male mating competition was compared using a Generalized Linear Model with a Binary Logistic distribution. A GLM with a Binary Logistic distribution was used to determine the effect of parental convergence behavior on offspring behavior.

Results

Preliminary Behavioral Observations

I recorded 250 free flight interactions from 56 pairs with 41 of these forming successful copulas. The presence of harmonic convergence significantly predicted the formation of a successful copula (Wald Chi Square, W=14.54, d.f.=1, P<0.001). Synchronization between both the male 1st and female 2nd harmonic and the male 2nd and female 3rd harmonic was observed (Figure 10).

Figure 10: Harmonic convergence and mating behaviors of *Ae. aegypti*. A. Examples of some observed mating behaviors in this study. In panel 1, female tilted abdomen away from male to prevent genital contact and semen transfer. In panel 2, female was holding male away from her body with her legs. In panel 3, the female and male were in successful copula. Females were tethered to strand of human hair (shown in 1) In order to produce clear images, we used hard tethers for some photographs and videos. B. Example of converging pair. Spectrogram trace of a male (blue trace) converging with a female (red trace). Convergence occurred between the male 1st and female 2nd harmonic. C. Example of a non-converging pair. Male1st harmonic (blue trace) and female 2nd harmonic (red trace) did not converge prior to mating.

Acoustic Recordings of Pairs Used to Measure Benefits

In the next experiment, I measured female daily egg laying and survival to determine whether benefits were associated converging with a male prior to mating. I recorded 141 pairs with 77 not converging prior to mating (54.61%) and 64 converging pairs (45.39%). Male flight tone averaged 711.98 Hz ± 13.82 (SE) and responded to females at an average rate of 92.37 Hz/sec ± 14.99 (SE). Convergence occurred between two different harmonic combinations. In the majority of interactions (81.2 %, n=52), males and females converged at the male 1st and female 2nd harmonics. Convergence between these harmonic occurred at 833.13 Hz ± 7.15 (SE). In 12 of the interactions (18.8%) convergence occurred between the male 2nd and female 3rd harmonics at 1506.08 ± 32.65 (SE). Pairs converging between at the male 1st and female 2nd harmonics stayed together longer than the pairs converging between the male 2rd and female 3rd (Mann-Whitney, P=0.004).

There was no significant difference between the wing lengths of converging and non-converging males and females (Students t-test; females, t=0.984, d.f.=47, P=0.182; males, t=0.06, d.f.=77, P=0.182). Replicate did not affect the wing length of females (ANOVA, F=0.150, d.f.=1, P=0.70). There was a significant difference between the wing lengths of males across replicates (ANOVA, P=4.206, d.f.=1, P=-.044). Males in replicate 2 were significantly larger than males in the other replicates (Tukey HSD, P=0.05).

Direct Benefits

I compared the net reproductive rate (R_0) of converging and non-converging females using a Cox regression (Begon et al. 1996). There was no significant effect of convergence behavior on R_o over all days in any replicate (see Table 4).

Table 4: Life table parameters for 3 replicates of direct benefits experiment. Net reproductive rate (R_0)= lxmx, Generation time (Tc)= xlxmx/ R_0, and Intrinsic rate of increase (r)= ln R_0/Tc. P-value is for the difference between the R_0 of converging and non-converging pairs, Wald Chi-Squared Test, d.f.=1.

Replicate	Convergence	N	R_0	Tc	r	P-value
1	Yes	24	42.60	14.86	0.32	

	No	16	52.81	15.23	0.26	0.165
2	Yes	14	73..46	15.07	0.29	
	No	28	57.52	23.89	0.62	0.133
3	Yes	11	29.36	35.47	0.01	
	No	21	52.24	20.86	0.19	0.540
Overall	Yes	49				
	No	65				0.774

When I analyzed all three replicates together, there was no significant effect of converging on R_o controlling for replicate (Wald Chi-Squared= 0.082, d.f.=1, P=0.774). There was no significant difference in the survivorship of females from the two groups (Cox Regression, P=0.331) (Figure 11a).

I also analyzed my data at an individual level. Data for the total number of eggs laid by each female were square root normalized. There was no significant effect of convergence behavior on the total number of eggs laid by each female controlling for wing length (General Linear Model, F=1.04, P=0.310) (Figure 11b). Controlling for female wing length, replicate, and male wing length there was no significant effect of convergence on female survival (Cox Regression, Wald test statistic=0.938, d.f.= 1, P=0.333).

Figure 11: Direct benefits of females mated with converging and non-converging males. A. Survival of females which mated with males after successful harmonic convergence (▲) and females which mated with males without converging at harmonics (■). B. Number of total eggs laid by these females.

Indirect Benefits

The outcome of mating competition experiments is recorded in Table 5. The successful convergence of parents significantly affected whether or not male offspring obtained matings (Binary Logistic Regression, d.f.=1, P=0.003). Male offspring of converging pairs were more likely to achieve matings. There was no significant interaction between the dust color on males and parental convergence behavior (Binary logistic regression, d.f.=3, P=0.491).

Table 5: Male mating success of offspring. Number of males from parents that did converge or did not and the number of male offspring that achieved successful copulation.

Replicate	Converging		Non-converging	
	mated	not mated	mated	not mated
1	26	154	10	170

2	21	164	16	169
3	24	141	9	156

There was no difference in the wing lengths of non converging and converging male offspring used in competition experiments (Mann-Whitney test, z=-0.302, P=0.763). The mean wing length of the females mated by converging and no-converging males was also not different (Mann Whitney test, z=-0.411, P=0.651). There was no significant difference in the number of eggs laid in the first clutch by females mated to converging and no-converging males (Mann-Whitney test, z=-0.308, P=0.780.

Discussion

Females that converged with their mates prior to mating enjoyed a significant indirect benefit in the form of increased male mating success in offspring. In other systems, courtship signals communicate information about this type of benefits to male offspring. Wedell and Tregenza (1999) found that in the field cricket, *G. bimaculatus*, sons of successful males obtain significantly more copulations than sons of unsuccessful fathers. The mating success of male sandflies is positively correlated with their fathers (Jones et al. 1998). Several other studies have found evidence for other types of indirect benefits including offspring survival (Petrie 1994; Hasselquist et al. 1996), offspring development rate (Moore 1979; Reynolds & Gross 1992a), and offspring viability, such as offspring size and development (Alatalo & Lundberg 1986) and increased resistance to parasites (Moller 1990). Benefits associated with increased mating success (Fisherian model) are often separated from benefits associated with other fitness characteristics (Good-genes model). Recent theoretical work suggests that in the view of evolution these types of genetic benefits are not distinct. In both instances females are choosing males based on the traits that they will pass onto their offspring (Kokko et al. 2002).

The finding that harmonic convergence behavior are associated with indirect benefits has important implications for our understanding of mosquito mating behavior and for mosquito control strategies. This is the first clear evidence of a courtship signal in a medically important mosquito. Currently, swarming mosquitoes are thought to have no discernable courtship (Yuval 2006). It is assumed that mate choice does not exist in these species (Howell & Knols 2009). The results of this experiment indicate otherwise. Females experience variation in the indirect benefits offered to them by males. Further, that variation is associated with a pre-mating signal. At a minimum, these two findings suggest that females exercise choice among males. It will be important for our general understanding of mosquito life history and behavioral ecology to further characterize the role of female choice and its mechanisms in this mating system. This work may lead to novel control targets. Control strategies targeting the reproduction of mosquitoes have been disappointingly ineffective (Reisen 2003). The avenue of research created by these findings may lead to the development of novel targets.

More immediately, transgenic control programs depend on the ability of laboratory reared and genetically modified males to outcompete wild-type males for mates. Broadly, these results indicate that female choice must be considered as a factor affecting these females. Intriguingly, the benefits measured here are indirect. If there is a genetic component to both signaling and offspring mating success, then this must be considered in the design of constructs used in transgenic mosquito lines. In the following chapter, I will present data on the heritability of harmonic convergence.

I did not measure any differences in the direct benefits associated with harmonic convergence. There are two explanations for this finding. First, it may be that there are no direct costs or benefits associated with harmonic convergence. This does not mean that direct selection is not involved in the mating system of *Ae. aegypti*. It simply means that males and females do not communicate about these types of costs and benefits using harmonic convergence.

A second explanation for these results, is that there are differences in the direct benefits associated with harmonic converge and I was simply not able to measure them with my experimental design Females were kept under ideal humidity and temperature conditions. Sugar was available to them for much of their lifespan and they did not experience many of the metabolic and ecological stressors that they may in the field. It could be that, under field conditions, differences in fecundity and longevity would become apparent.

It should be noted that females in these experiments were tethered to a human hair and thus unable to exercise a complete range of behavioral responses to males. Females were not able to avoid flight and had limited ability to outmaneuver males, behaviors that have been described in previous experiments (Jones & Pilitt 1973). If females were not able to exercise the full range of their rejection behaviors and if convergence is preferred by females, there may have been instances in which females mated with undesirable males. In fact, tethering may have dampen the ability to measure differences. Therefore, I do not believe that female tethering affected the outcome of these specific experiments.

Future studies are needed to clarify the total effect of mate choice by harmonic convergence on the total behavioral ecology of mosquitoes. For example, Brooks & Endler (2001) found a negative genetic correlation between male sexual attractiveness and survival in guppies. There may be a trade-off between harmonic convergence traits and other fitness traits in offspring. To fully understand the importance of harmonic convergence signaling in male fitness a cost-benefit analysis must be conducted. Additionally, evidence of inheritance of the convergence signal would greatly strengthen the hypothesis that harmonic convergence is linked that an indirect fitness benefit to offspring.

CHAPTER 5

THE HERITABILITY OF HARMONIC CONVERGENCE

Introduction

The evolution of indirect selection is likely widespread as there is evidence for positive correlation between preference and display traits in animals (Kokko et al. 2002). In some instances indirect benefits are facilitated by male parental care or provisioning, but in non-resource based systems lacking male parental care, males only pass genetic benefits to offspring. Sexual selection for indirect benefits is hallmarked by female preference for variable signal characteristics that are linked with an indirect fitness benefit. Further, signal traits must be inherited by the offspring (Kokko et al. 2002).

There are several examples of these types of signaling systems. In birds, plumage characteristics that were correlated with male viability (Norris 1993) and parasite resistance (Moller 1990) were heritable. Hasselquist et al. (1996) used cross-fostering experiments to determine that male great reed warblers inherit song repertoires from their fathers, which has been linked to male mating success.

Similar evidence has been reported in insects. Boake (1985) used genetic techniques to measure the heritability of male mating success and male pheromone traits in cockroaches. Male pheromone characteristics were inherited and males with preferred pheromone traits not only produced more grandchildren, but also passed on genes related to pheromone characteristics. In an elegant experiment, Moore (1994) presented evidence that genes associated with decreased nymphal development time were positively correlated with genes associated with increased male attractiveness in cockroaches. Perhaps the best example of the association between signal heritability and indirect fitness benefits comes from the Orthoptera. Multiple studies have demonstrated female preference for signal characteristics (Gwynne 1982; Tuckerman et al. 1993; Gray 1997) that are associated with indirect benefits (Zuk & Simmons 1997) and that both of these traits are inherited (Simmons 1987; Hedrick 1988; Gray 1997; Shaw & Herlihy 2000).

In the mosquito, *Ae. aegypti,* males and females encounter one another in aerial swarms which form around the host (Hartberg 1971). Mating occurs in flight with males and females meeting, forming a copula in mid-air, and mating all in a matter of secs (Hartberg 1971; Yuval 2006). Cator et al. (2009) demonstrated that when males and females are within 2-3 body lengths of one another that both parties actively modulate their flight tone frequency so as to converge at harmonic frequencies. Successful convergence at harmonics in this species also predicted the formation of a successful copula (Chapter 2), suggesting female preference for mates with converging mates. Further, the male offspring of converging pairs successfully outcompeted male offspring of non-converging pairs to achieve copulas (Chapter 4).

These findings indicate that male *Ae. aegypti* may inherit a genetic trait which allows them to be more efficient at achieving successful copula formation. Additionally, the presence of this trait appears to be associated with harmonic convergence behaviors of parents prior to mating. Here, I tested whether harmonic convergence behavior is inherited. I addressed this question using the broadest definition of heritability: the similarity between offspring and parental phenotype (Lynch and Walsh 1998). I determined whether harmonic convergence behavior of male offspring was correlated with the harmonic convergence of their parents.

Methods

I repeated the recording procedure for copulating pairs of *Ae. aegypti* described in Chapter 4. Briefly, females were tethered to a 2-3 cm strand of human hair and placed over a particle velocity microphone (NR-21358, Knowles, Itasca, IL). We determined whether harmonic converge occurred just prior to mating in these pairs by visualizing spectrograms in Raven 1.0 (Cornell Lab of Ornithology, Ithaca, NY). Females were removed from semi-tethers using dissecting scissors and held in 0.5L cartons. Females were offered 3 blood meals from a human host (LJC) at 3-5 day intervals and provided 20% sucrose solution and oviposition sites as previously described. The right wing of each male and female was removed and measured as a proxy for body size. Eggs were collected, labeled by adult pair ID, and held for embryonation as described previously for a minimum of 1 week. The egg papers from each female were placed into a separate plastic dish (4.5 cm diameter x 4 cm high) and flooded with water. Dishes were placed into larger plastic containers (22 cm x 22 cm x 8 cm) with sealed lids and vacuum hatched for 15 mins.

Larvae from each pair were reared separately. The day after hatching 50 randomly selected 1st instar larvae from each pair were placed in a 12 x 12 x1.5 cm plastic container with 500 mL of filtered water. On days 1, 2, 3, 4, and 5, these trays received 30 mg, 60 mg, 60 mg, 90 mg, and 120 mg of diet, respectively. Larvae were held in an environmental conditions as described above. Upon reaching 4th instar, larvae were moved for 2 days to a 21 °C incubator to induce stress. Pupae were individually held in 15 ml tubes containing 3ml of filtered water and plugged with cotton wool. Emerged males were released into a large bucket cage and provided with a 20% sucrose solution.

A large cohort of *Ae. aegypti* taken from the original colony (Mexico Strain, Cornell University) were reared synchronously with the offspring from recorded pairs. Pupae from these trays were sorted into individual tubes to obtain virgin females. Females were held with a 20% sucrose solution

When sons were 2-5 days old, I measured the harmonic convergence behavior of 10 randomly selected sons. I tested for harmonic convergence using the identical experimental approach as described above for the parents. Females used in these experiments were obtained from the MEX colony and reared synchronously with experimental males. After recording, the right wing of both males and females was removed and measured.

Data analysis was conducted with SPSS (Version 16, SPSS Inc., Chicago, IL). I used a mixed model to determine the effect of parental convergence behavior (converged/did not converge), replicate, day of recording (random effect), family identity (random effect), and both parental and son wing length. Factors shown to have no significant effect on offspring convergence behavior were removed from the final model. We determined the relationship between parental and offspring signaling characteristics by measuring the fundamental frequency of males, the convergence frequency of pairs, and the rate of male and female convergence using a generalized linear model. Initially, we tested for the effect of replicate, day of recording, and wing length on these parameters. Non-significant predictors were eliminated from the model.

Results

I included 46 non-converging pairs and 47 converging pairs and the behavior of their male offspring (total of 741 mosquitoes) in the analysis. There was no significant difference between the body size of converging and non-converging parents (males, females). The harmonic convergence behavior of parents significantly predicted the harmonic convergence behavior of their male offspring (Binary logistic regression, Wald chi-square= 8.391, d.f.=1, P=0.004). There was no significant effect of day (Wald chi-square= 11.476, d.f.=16, P=0.779), replicate (Wald chi square= 0.144, d.f.=2, 0.930), male offspring wing length (Wald chi square=

17.298, d.f.=71, P=1.00), or the wing length of the female found in copula (Wald chi-square=12.85, d.f.=85, P=1.00).

When I compared the rate of convergence (Δ frequency Hz/ time sec) no significant correlation between offspring and their fathers was detected (R^2=0.00, P=0.424).

Discussion

The harmonic convergence behavior of male offspring was significantly predicted by the harmonic converge behavior of their parents. The likelihood of successful copulation of males is also significantly predicted by harmonic convergence behavior. These results suggest that inheritance of these traits are linked. It may be that females are selecting mates carrying genes for the harmonic convergence signal alone and that harmonic convergence traits may be completely unlinked with another trait. Females increase the mating success of their sons by mating with males carry the signal trait. The physical mechanism of harmonic convergence is not known. Alternatively, it maybe that this offspring inherit a sensory, metabolic, or physiological trait that makes them more likely to converge and that this trait is also correlated with increased mating success. The subtle difference between these two scenarios is more important from a theoretical and evolutionary stand point, than a practical one, but determining the exact mechanisms that dictate how convergence is accomplished would greatly improve our understanding of the precise trait being inherited, its functional significance in the physiology of males, and how it relates to male mating success.

Further, convergence of offspring in this experiment was related to the convergence of both parents and not necessarily the convergence of their fathers. It would be interesting to conduct experiments to determine the relative importance of maternally and paternally inherited genes in determining convergence behavior. In addition, we measured convergence behaviors of sons and not daughters. Future experiments should be conducted to determine the costs and benefits to daughters of converging pairs.

The rate at which fathers converged did not predict the rate at which their sons converged. It may be that convergence rate is dictated by interactions between males and females. Females may only attend to that fact that a male is able to match at harmonics and not how he accomplishes this matching. Alternatively, convergence rate may be largely dictated by the actions of the males. In *An. gambiae* (Cator et al. 2010 and Chapter 3), male convergence rate was a function of the body size of perceived mates. In these series of experiments, we used large female *Ae. aegypti* only. Lack of variation in female size and signal characteristics may have decreased variation in male convergence rate, masking any correlation between the rate of fathers and sons. A more simple explanation is that *An. gambiae* are highly divergent from *Ae. aegypti* and that while harmonic convergence plays a role in both of these species, the nature of that role is different.

This study demonstrates that convergence appears to have a heritable component. There may be single or multiple genes involved in regulation ofinheritance for the convergence trait. If convergence is an important predictor of mating success, as my previous work suggests, then it will be important to characterize these genes. If transgenic constructs disrupt expression of these regions of the genome, the mating success of released males may be hampered.

CHAPTER 6

BEHAVIORAL OBSERVATIONS AND SOUND RECORDINGS OF FREE-FLIGHT MATING SWARMS OF *AE. AEGYPTI* (DIPTERA: CULICIDAE) IN THAILAND.

Introduction

Although mosquitoes are widely studied in their capacity as disease vectors, there are surprisingly little data on their basic life history and behavior (Ferguson et al. 2010). Mating behavior is one of the most neglected areas of mosquito biology (Ferguson et al. 2010; Takken et al. 2006). Control strategies based on disruption of mosquito reproduction, especially those relying on the mating success of genetically modified males, require a better understanding of the mating systems of these insects (Scott et al. 2002).

Aedes aegypti (L.), form swarms in response to host cues (Hartberg 1971). These swarms are composed predominately of males, with females entering singly to be mated (Hartberg 1971, Yuval 2006). Mating occurs on the wing, with males approaching the female from behind and then rotating 180 degrees to position themselves venter-to-venter (Roth 1948, Hartberg 1971).

Sound has long been known to be important for male localization and orientation to females in *Ae. aegypti* (Roth 1948). The male Johnston's organ actively amplifies the fundamental flight tone frequency of females (Göpfert and Robert 2001). Recently, new pre-copulatory acoustic interactions have been described. In *Ae. aegypti*, tethered males and females alter their flight tone frequency to converge at harmonic components of their flight tone signals (Cator et al. 2009). This behavior, which we call “harmonic convergence”, now has been described in several mosquito species (Cator et al. 2009, Warren et al. 2009, Cator et al. 2010, Pennetier et al. 2010). Harmonic convergence also has been implicated as a potential mate choice mechanism (Cator et al. 2009, Cator et al. 2010, Pennetier et al. 2010).

To date, the majority of studies investigating mosquito bioacoustics have been conducted in the laboratory using males and females tethered into position. Cator et al. (2009) conducted recordings of field collected *Ae. aegypti* and found that they also displayed convergence behavior and Warren et al. (2009) described the neurophysiology of field collected *Cx. pipiens*. Despite laboratory investigations of field collected insects, there are very few data on free-flying mosquitoes. Durkopf and Hartberg (1992) collected recordings of a free-flying laboratory population of *Ae. aegypti* and *Ae. albopictus*. Wekesa et al. (1998) used short clips of free-flying mosquitoes held in small plexiglass tubes to determine average flight tone frequencies of *Anopheles gambiae* and *An. arabiensis*. Some studies have measured free-flight frequency using light-deflection (Moore et al. 1986, Tripet et al. 2004). Again, these methods required that the test mosquitoes be held individually in small plastic cages. There is no record of the acoustic behavior of swarming mosquitoes in nature.

In order to clarify the role of bioacoustics in mosquito mating, it is important to observe swarming dynamics in the field. Here, I describe an approach for recording the natural bioacoustics of mosquito swarms. I also present data collected using this method from naturally forming swarms of *Ae. aegypti* in Thailand.

Methods

Recording Apparatus

Six particle velocity (Knowles NR-23158, Itasca, IL) and two pressure sensitive microphones (Knowles EK-23132, Itasca, IL) were positioned around the perimeter of a funnel

(15 cm diameter) to create a spatially separated microphone array (Figure 12A). A custom electronic circuit was designed to power the microphones as well as amplify and filter their output signal (B.J.A., unpublished). An analog-to digital converter (779676-01 NI USB-6211 Bus-Powered M Series, National Instruments, Austin, TX) was used to transfer data to be stored on a laptop computer.

Recording Procedure

Recordings were taken from residential homes in Nai Muang subdistrict, Muang district, Kamphaeng Phet Province, Thailand (16^o 27’ 48” N, 99^o 31’ 47” E). Recordings were taken between 1300-1530 hrs ICT. A total of 3.5 hours of observation were conducted over two days. Temperature and humidity was recorded using a data logger (Hobo Pro Series, Onset, Pocasset, MA). I stood on a 0.6 m x 0.6 m white cloth to facilitate visual observations. The microphone array was attached to a 1 m long wooden pole. Swarms of *Ae. aegypti* formed naturally within the house around the observer. I moved the microphone array in and out of the swarming area. The array funnel was positioned so that the funnel was under the flying individual (Figure 12B).

Figure 12: Recording procedure. A. I acted as the swarm marker. The microphone array positioned along the funnel was moved in and out of swarming *Ae. aegypti* B. Overview of microphone array. Six particle velocity and two pressure sensitive microphones were positioned equidistantly around a 15 cm diameter funnel. Microphone positioning alternated between being pointed into the center of the funnel and upward (parallel with the ground).

Recording Analysis

Each of the eight simultaneously recorded channels from the array was analyzed individually in Raven (Version 1.0, Cornell University Laboratory of Ornithology, Ithaca, NY). Recordings were initially viewed in 17.2 Hz resolution in order to distinguish the very short fly-by clips. After clip identification, I configured the analysis software to enhance resolution of frequency information to 4.95 Hz. At the time of the original recording session, the observer dictated behavioral observations in real time. Females and males recorded while flying alone were classified as female or male solo fly-bys. I identified paired flights visually. Paired flight was classified as either male with male paired flight, when two males were in the acoustic scope of the array simultaneously, or as male-female paired flight. Male-female paired flight was reported anytime males and females were in contact including pre-copulatory, copulatory and post-copulatory flight. I did not observe paired females.

Statistical Analysis

The average flight tone frequencies of individuals engaged in different types of flight (male-female paired, male-male paired, or solo) were compared using a Student's T-test when appropriate, and nonparametric tests when data were not normally distributed.

Results

Behavioral Observations

Males appeared around me within 5 min. Males began flying in a "figure eight" pattern as reported previously for this species (Hartberg 1971). Swarms formed within 1 m of the my legs (in the horizontal plane) and approximately 30 cm from the ground. Swarms consisted of 12-30 individuals, the majority of which were male. The average temperature during the recording period was 32.6 ± 0.5 (SE) °C with a R.H. of 49.9 ± 1.9 (SE) %.

I observed 23 copulating pairs using the methods described above. Once a pair formed a copula, both males and females continued beating their wings in order to stay aloft. Interestingly, once copulating pairs had assumed the venter-to-venter position, they moved rapidly away from the host. It was not clear whether this movement was a function of male behavior, female behavior, or both. I described pairs that are in the venter-to-venter orientation in flight as being in copula.

Frequency of Flight

I captured recordings of 114 separate fly by events (Figure 13). These included 23 instances of solo female (Figure 13A), 82 instances of male solo flight (Figure 13B), and 13 instances of paired flight (Figure 13C). Three of the paired flights were between males and females and the remainder between males (Figure 13D).

Figure 13: Power spectra of natural flight behavior within a swarm of *Ae. aegypti*. A. Female solo flight. B. Male solo flight. C. Two males in paired flight. Fundamental frequencies are indicated by black arrows. D. Paired flight between male and female. Male 2nd and female 3rd harmonic appear to be approaching convergence.

The average duration of sound clips was 365 ± 6 ms. Average fundamental components of the flight tone frequency of these groups are presented in Table 6.

Table 6: Average flight tone frequency measured in Hz ± SE of free-flying *Ae. aegypti* in swarms in Kamphaeng Phet, Thailand.

Recording	N	Flight Tone
Male solo	12	982.0 ± 1.0
Female solo	27	664.3 ± 4.6
Male paired	78	989.3 ± 7.4
Female paired	3	609.1 ± 48.5

There was no significant difference between the flight tone frequencies of paired and unpaired males (Mann Whitney U-Test, *P*=0.102). Males in paired flight with other males had slightly lower flight tone frequencies (975.08 ± 8.09 Hz) than males recorded in paired flight with females (1010.9 ± 45.51 Hz). This difference was not statistically significant (Student's t Test, df=11, *t*=-0.582, *P*=0.572). In one recording clip, I was able to observe a male in pursuit of a female (Figure 14).

Figure 14: Spectrogram of male engaged in an in-flight pursuit of a female. Female flies past the microphone first and male follows 0.8 secs later.

The male crossed the array approximately 0.8 s after the female. In another clip (Figure 13D), I observed a paired flight in which a male and female appeared to be in the process of convergence. The clip only lasted 0.04 s, but even in this short interval, we saw the female's 3rd harmonic and male's 2nd harmonic were at frequencies near to convergence.

Discussion

I was readily able to observe mating swarms of *Ae. aegypti* and recorded flight tones from swarming individuals in their natural habitat in Thailand. The average flight tone frequencies from males and females were higher than those typically described in laboratory experiments (Brogdon 1994, Duhrkopf and Hartberg 1992, Wishart and Riordan 1959). This is likely because of the higher temperatures experienced in the field. The wingbeat frequency of Diptera has been found to increase with temperature (for review see Belton 1986). Some data also suggest that tethering may decrease the flight tone (B.J.A., unpublished). Others have found that tethering increases the load on the flight mechanism and actually increases wing beat frequency (Belton and Costello 1979; Chadwick 1953). Further studies to confirm that laboratory and field populations produce similar flight tone frequencies under identical conditions would clarify whether there are any differences between these populations. Differences would be important because future studies will undoubtedly use laboratory data to evaluate the behavior of males used in transgenic or sterile male release programs.

These behavioral observations largely support those of Hartberg (1971). Male *Ae. aegypti* were attracted to human host stimuli and flew in a characteristic figure-eight pattern. Females paired with males as they came to the host to feed. It has been suggested that swarming is a vestigial behavior and not associated with mating (Nielsen et al. 1960). However, I was able to readily observe mating in swarms.

In one instance, Hartberg (1971) described a male-female pair moving away from human hosts in flight. I observed this behavior in all pairs in the venter-to-venter position in the field (n=23). Similar behaviors have been reported in field observations of *Culex* (Reisen et al. 1985) and *Anopheles* (Reisen and Aslamkhan 1976). Among these *Ae. aegypti* is unique in that its swarms occur in close proximity to a defensive human host. Rapid movement away from the host and swarm may serve as an avoidance response evolved in *Ae. aegypti* to limit exposure to host-defensive behaviors while in copula. Pairs flying in copula appear to be less agile than individuals. This type of response has been reported in the water strider, *Gerris remigis*, threatened by sun fish predation. Mating durations were shortened to increase the speed of escape (Sih et al. 1990). Alternatively, movement away from aggregations may be a male-driven action. Males may be able decrease interference from other males and increase the likelihood of successful insemination. This type of interference was been described in other swarming mosquito species (Reisen et al. 1985) and similar behavior has been reported in several swarming Dipteran species (for review see Thornhill and Alcock 1983). I have observed male interference in the laboratory (L.C.H., unpublished data). While we did observe males flying in proximity to one another (male-male paired flights), I did not observe male-male interference during copula formations in the field.

Due to the rapid retreat of pairs, it was difficult to capture the entire acoustic sequence of paired flight between males and females. My low sample size of male-female pair auditory data collected (n=3) did not allow me to conclude with confidence that harmonic convergence was occurring in these pairs. I did however, observe at least one intriguing instance in which convergence may have been occurring (Figure 13D). Future field studies with greater sampling may lead to recordings of convergence between mating pairs.

I observed 23 pairs in copula, but were only able to acoustically record a small proportion of these because of the speed of the mosquitoes in free flight. *Ae. aegypti* flight movement is temperature dependent and can be very fast during hot periods (Belton and Costello 1979, Sotavalta 1947, Tamarina et al. 1980). To compensate for the increased speed of flight, the size and scope of the recording array should be increased in future studies. This could be accomplished by increasing the number of microphones and decreasing the spatial separation of microphones.

Recent laboratory studies have revealed a greater level of complexity of mosquito mating behavior (Cator et al. 2009, Cator et al. 2010, Pennetier et al. 2010, Warren et al. 2009) than was previously known (for review see Yuval 2006). Here, I have described an innovative new technique for recording acoustic data in field observations of mosquito swarms. This methodology can be used to more accurately assess the role of acoustics in laboratory, as well as be used in further applications in the field.

Currently, most of the work on mosquito acoustics has involved measurements of behavior for two tethered individuals. Working with free-flying laboratory and field populations will enable better comparisons between acoustic behaviors of these two groups. Cator et al. (2009) reported lower harmonic convergence response to playbacks in mated compared with virgin female. Measuring such differences in free flight, in which not only acoustic, but mating attempt outcomes can be measured would be particularly interesting. Finally, coupling this type of recording array with a video data, would allow us, for the first time, to dissect the timing of convergence in relation to male pursuit and copula formation. Innovation of this kind will enhance our ability to study field behavior and will ultimately lead to a better understanding of mosquito mating behavior.

CHAPTER 7

HARMONIC CONVERGENCE IN MEDICALLY IMPORTANT MOSQUITOES: SYNTHESIS AND FUTURE DIRECTIONS

Summary of Major Findings

When male and female *Ae. aegypti* and *An. gambiae* meet in flight, they alter their flight tone to converge at harmonic components of their flight tone signal. Convergence occurs between either the male 2nd and female 3rd harmonics or the male fundamental frequency and the female's 2nd harmonic. Harmonic convergence behavior appears to only occur between members of the opposite sex.

I demonstrated that large male and female *An. gambiae* produce a higher flight tone frequency than small individuals supporting previous reports (Wekesa et al. 1998). In addition, I found that harmonic convergence behavior is affected by the perceived body size of potential mates. Males and female encountering playbacks mimicking the flight tone of a large potential mate responded with convergence earlier than those played recordings of small individuals. This study also demonstrated that males and females are able to perceive differences in body size, an important fitness characteristic, based solely on acoustic stimulus.

Harmonic convergence significantly predicted the successful formation of a copula in *Ae. aegypti*. My mating competition experiments demonstrated that male offspring of females, which had converged with their mate prior to mating, were superior to male offspring from non-converging pairs. Additionally, the male offspring of converging pairs were more likely to converge with mates.

In conclusion, these results suggest that harmonic convergence may be used to signal indirect benefits to females. These findings challenge the current paradigm among mosquito biologists that there is no courtship in swarming mosquitoes. Further study and clarification of this behavioral phenomena will not only provide us with insight into the behavioral ecology of mosquitoes, an area that is severely lacking in empirical study, but also may provide us with novel tools to combat mosquito borne diseases. In order to develop control strategies based on this work, physiological, mechanistic, and evolutionary aspects of harmonic convergence behavior must be further defined. Below, I describe three key areas for future research.

Clarification of the mechanism of convergence

There is still a considerable amount of work to be done on the mechanisms of harmonic convergence and flight tone production in mosquitoes. It appears that flight tone signals are a product of both the wing beat frequency and the tuning of thoracic box (Belton & Danthanarayana 1986). We do not have any data on whether mosquitoes converge their tones by altering how fast they are beating their wings or by making subtle changes to the stiffness of the thoracic box. This question could be answered relatively easily by taking slow speed footage of males responding to electronic playbacks in free flight.

Clarification of how harmonic convergence is physically achieved could offer some insight into what factors are important for being successful at converging. The research presented in this dissertation indicates that harmonic convergence is associated with at least one benefit to offspring. I measured increased male mating success in offspring of converging pairs, but I have not identified what leads to this increased success. Results indicate that a genetic factor is involved. The mechanism leads to increased convergence rates in these males is linked with, contributes to, or is evidence of, the traits that make them more successful at forming copulas.

Thus, identifying the mechanism of convergence will help to illuminate the factors that contribute to male mating success in mosquitoes.

Further Characterize Harmonic Convergence

The current definition of harmonic convergence is an acoustic interaction in which either or both members of a flying male-female pair alter their wing beat frequency to converge at a harmonically related frequency. The exact sequence and timing of convergence behavior in relation to the midair approach and copulation is still vague. Detailing this sequence will provide information about how convergence facilitates successful copulas. For example, if convergence occurs prior to proper alignment, then it would support the hypothesis that convergence may be used by male and female to coordinate the formation of a copula. Simultaneous acoustic and slow speed video measurements would allow for clarification. Given that different mosquito genera (ie. *Anopheles* and *Aede*s) copulate in different positions, it would be particularly interesting to determine if convergence occurs at the same step of the copulation sequence across mosquito taxa or if there is variation.

Investigation of the evolutionary significance of harmonic convergence

All of the experiments conducted on harmonic convergence thus far have been in the laboratory. As the field moves forward, it will be important to measure acoustic interactions and their fitness consequences in the field. I have begun working on a portable swarm recording system as described in Chapter 6. Continued development of such techniques will greatly improve our understanding natural swarming behaviors.

In addition to describing harmonic convergence in natural mosquito swarms, we must also further investigate the costs associated with this behavior. If convergence behavior is genetic, then there may be trade-offs associated with this genotype. It is extremely difficult to mimic the costs imposed by nature in the laboratory. It may be that male offspring suffer a cost that I did not measure. Also, I did not measure direct fitness costs to females mating with converging males, but this may be because these females were not under the same stress that they might experience in the field.

Field studies on the genetic and life history factors that affect convergence would also clarify the role of convergence in mosquito mating systems. For example, I found a positive correlation between flight tone and body size in *An. gambiae* and males responded to females producing higher frequencies faster. In addition, females converged with tones mimicking large males earlier. These results suggest that large mates produce more attractive signals. Further, in many insects larger males have higher fitness than smaller cohorts (Reviewed by Thornhill and Alcock). If swarming mosquitoes are under selective pressure to be larger, then why do we observe such a large range of body sizes in the field? It may be that there are costs associated with large body size that counterbalance the preference for this trait by females (Reynolds & Gross 1990). In some instances, this type of conflict between the pressures of selection on the sexes results in negative genetic correlations between male and female traits (Andersson 1994). Clearly, clarifying the role of mate choice and understanding the costs imposed on mate choice in mosquitoes would help to inform our understanding of the mating system.

Concluding Remarks

Mosquito mating behavior is not a black box. The studies presented in my dissertation in combination with the work of my peers (Charlwood & Jones, 1967; Hancock et al. 1990; Yuval et al. 1992; Okanda et al. 2002; Ng'habi et al. 2005; Zsemlye et al. 2005; Ferguson et al. 2005; Gibson & Russell 2006; Ponlawat & Harrington 2007; Ng'habi et al. 2008; Helinski & Knols 2008; Ponlawat & Harrington 2009; Ferguson et al. 2010) has provided evidence that mating success in mosquitoes is not random. Instead male-male competition (Yuval et al. 1992; Yuval et al. 1994; Ng'habi et al. 2005; Ponlawat & Harrington 2007; Ng'habi et al. 2008; Ponlawat & Harrington 2009) and female choice (Charlwood & Jones, 1967; Cator and Harrington *In Review*) may be important.

I believe that mosquito swarms can best be described as "mobile scramble" arenas in which males lack fixed display sites and move among females to court them (mating on the run) (Reynolds & Gross 1990). There is evidence of genetic benefits to females that are able to exercise choice. This choice has been clearly documented as the female's acceptance or rejection of potential male mates. Male mosquitoes transfer proteins in addition to sperm to females (Sirot et al. 2008, 2011) and thus the potential for an unknown material or economic benefit still exists.

For too long, mosquitoes have been studied as only as disease vectors and their basic biology has been ignored (Ferguson et al. 2010). In light of their medical importance, it is amazing how little we know about them. If we want to control mosquitoes, we must develop a greater understanding of their basic ecology, habits, and behaviors.

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