MAKING WAVES: THE USE OF SOUND BY A MOSQUITO AND THREE MOTH SPECIES

by

Melanie Hart
MSc, Simon Fraser University 2006
BSc, University of Victoria 2003
BA, University of Victoria 2003

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

In the Department of Biological Sciences
Faculty of Science

SIMON FRASER UNIVERSITY

Summer 2012

APPROVAL

Name: **Melanie Hart**

Degree: **Doctor of Philosophy**

Title of Thesis: **Making waves: the use of sound by a mosquito and three moth species**

Examining Committee:

Chair: **Dr. David Lank**
University Research Associate and Adjunct Professor,
Simon Fraser University

Dr. Felix Breden
Senior Supervisor
Professor, Simon Fraser University

Dr. Peter Belton
Supervisor
Retired Faculty, Simon Fraser University

Dr. David Green
Supervisor
Assistant Professor, Simon Fraser University

Dr. Carl Lowenberger
Public Examiner
Professor, Simon Fraser University

Dr. Ian Russell
External Examiner
Professor of Neurobiology
University of Brighton

Date Defended/Approved: August 10th, 2012

Partial Copyright Licence

ABSTRACT

Mosquitoes hear wingbeat sounds with antennal Johnston's organs. In males, their sensitivity is thought to be enhanced by long fibrils on the antennae; males of many species erect these fibrils before they swarm with females. I investigated the anatomy and acoustic properties of antennae of male and female *Aedes togoi*, a species whose males erect these long fibrils before swarming. Many moths also hear, but several species that communicate with wingbeat sound have no tympanal organs, sensitive ears thought to have evolved in moths to detect the ultrasonic echolocation of bats. I therefore investigated potential ears in three species known to use sounds, tympanate Indianmeal moths, *Plodia interpunctella* (IMM) and atympanate peach twig borers, *Anarsia lineatella* (PTB), and webbing clothes moths, *Tineola bisselliella* (WCM). These investigations used microscopy, laser vibrometry and electrophysiology.

Male and female *A. togoi* antennae vibrate best at 385 and 252 Hz, respectively, males near the female wingbeat frequency (wbf) of 306 Hz; females are unlikely to hear male wbfs of 523 Hz. In contrast, both sexes of IMM had wbfs near 50 Hz, with associated synchronous ultrasonic clicks spanning 25-80 kHz. Male tympana vibrated best at 90 kHz, females at 70 kHz, whereas the antennae of both sexes vibrated best near 150 Hz, the 3rd harmonic of their wbf. Similarly, both sexes of PTB had wbfs near 56 Hz, with associated ultrasonic clicks spanning 25-80 kHz. Their antennae vibrated in response to wingbeat sound, and an air-backed circular area on the metepisternite of both sexes vibrated best at 90 kHz but did not meet all the criteria for an ear. Finally, male WCM had a wbf of 58 Hz but females never flew or fluttered. Male and female antennae vibrated in response to the wbf and had a best frequency at 100 Hz, near the 2nd harmonic of the wbf.

I conclude that these moths, like mosquitoes, have mechanically resonant antennae sensitive to sounds. Like many other flies and bees, the moths may use their antennae to detect wbfs. Because they have little acoustical effect, I suggest a different function for fibrils of male mosquitoes.

KEY WORDS: Johnston's organ; tympanal organ: mosquito hearing: moth hearing; antennal fibrils.

DEDICATION

For Drs. Elspeth and Peter Belton, who helped me achieve this goal.

ACKNOWLEDGEMENTS

My greatest thanks go to Drs. Elspeth and Peter Belton. Their down to earth natures, extensive and seemingly exhaustless knowledge, and amazing support ensured I achieved my goal of getting my PhD.

I also want to thank my supervisory committee. Each person brought their different areas of expertise to the table and helped with the writing and statistical analyses of the data. Dr. Carl Lowenberger took the time to read carefully through the thesis, catching the typographical and grammatical errors that the rest of us missed, and raised interesting ideas at the defence about how the findings might be used for pest control. Finally, I'd like to thank Dr. Ian Russell. Despite having a very busy schedule, he took the time to be my external examiner. His comments and suggestions provide direction for future research and give a different perspective from which to view some of the results. I wish more time could have been spent at the defence discussing all the topics he brought up during the question period.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF EQUATIONS

GLOSSARY

Acoustic	Relating to sound or ultrasound
Best frequency	The frequency that results in the greatest amplitude of an oscillating system (also called resonance frequency in a resonant system)
Chordotonal organ	A mechanoreceptor that can detect movement (stretch) or vibration (infrasonic, sonic and/or ultrasonic)
Decibel (dB)	Logarithmic unit of a power ratio used here to measure sound pressure relative to the approximate human threshold of hearing (20μPa rms) [10dB represents a 10-fold power difference, 20dB a 100-fold, and so on]
Diapause	Delayed development in a life cycle
Ear	A sense organ that can detect acoustic waves
Electrical response	Potential changes recordable from a cell or cells in response to a stimulus
Electrophysiology	Study of the electrical activity of (mostly) sense organs, nerves or muscles
Fibrils	Long setae radiating from the flagellomeres of the antennae (of a male mosquito)
Flagellomeres	The segments of a flagellum
Flagellum	The 3rd section of an insect antenna, attached to the scape and pedicel
Frenulum	A group of bristles (fused together in many species) on the front edge of the hind wing that locks the hind wing to the fore wing in flying moths

Generator potential	Graded electrical activity in a sense cell related to the strength of the stimulus
Hearing	Following Hoy and Yack's 2008 definition, the ability to detect small time-varying movements in a medium
Hz: Hertz	Cycles per second
Infrasound	Acoustic waves below 20 Hz (the lower limit of human hearing)
Instar	One of the growth stages of an insect between moults
Johnston's organ	A chordotonal organ with radially arranged scolopidia in the distal pedicel, stretched by movement of the flagellum
Ku	Defined by Raven software as thousands of digital samples (u). In digital recordings, the amplitude of a sound
Laser vibrometry	A technique to measure the vibration of an object
Mechanical response	The displacement of a structure in response to a physical stimulus
Metepisternite	A sclerite on the metathorax above the hind leg
Patagium(a)	One (of 2) inflated scales on moths on the anterodorsal thorax between it and the head
Pedicel	The second antennal segment housing Johnston's organ
Phase	The position in a sound cycle relative to an arbitrary point
Photophase	The light portion of a light/dark cycle (artificial or natural)
Quality factor (Q)	Describes how sharply tuned a resonating system is; the higher the Q, the lower the damping of the system. A tuning fork vibrates virtually undamped at only one frequency and thus has a very high Q
Receptor potential	Electrical activity in a sense organ that can include generator potentials and nerve impulses

Resonance	A physical property of an oscillating system causing it to vibrate with greater amplitude at a particular frequency or frequencies
Retinaculum	A catch for the frenulum, on the moth forewing
Scape	The first antennal segment
Sclerite	A cuticular plate forming part of the exoskeleton
Scolopale	A cell containing rods that surrounds the cilium of a mechanoreceptive neuron
Scolopidium	The fundamental unit of an arthropod mechanoreceptor
Scotophase	The dark portion of a light/dark cycle (artificial or natural)
Setae	Hair-like outgrowths from the cuticle, most are sensory
Sound	Acoustic waves between 20 Hz and 20 kHz (the range of human hearing). In physics, the mechanical displacement of particles in a medium
Sound pressure level	The amplitude of the rapid pressure changes in a sound wave usually measured in decibels (dB)
Spectrum analysis	The separation of complex waves into their component frequencies
Stenogamous	Mating where no nuptial flight is necessary: mating can occur in a confined space
Subgenual organ	An array of scolopidia in the tibia that detects substrate vibrations
Tegula(e)	Structure(s) that cap the articulation of the forewings and thorax
Tymbal	An air-backed, often ridged/corrugated part of the exoskeleton used to produce sounds
Tympanum(a)	An air-backed, innervated, and thinned cuticular area in insects that vibrates in response to sound or ultrasound.

Ultrasound	Acoustic waves above 20 kHz (the upper limit of human hearing)
Verticillate	Arranged in whorls – correctly used to describe the bottlebrush shape of a male mosquito antenna

1 INTRODUCTION

1.1 Insect sounds

There are five main mechanisms by which insects produce sound (Drosopoulos & Claridge, 2005), and five different ways they receive it (Haskell, 1961). Yack (2004) later combined two of Haskell's categories, so today only four are accepted. A summary of these is given below (Tables 1.1 & 1.2). The number of groups of insects known to use acoustics has increased since the 1960's from 10 (Haskell, 1961) to at least 14 orders (Coleoptera, Dictyoptera, Diptera, Hemiptera, Hymenoptera, Isoptera, Lepidoptera, Mantophasmatodea, Neuroptera, Odonata, Orthoptera, Plecoptera, Psocoptera, and Thysanoptera). Most early research focused on the loudest insect singers, grasshoppers and crickets (Orthoptera) and cicadas (Hemiptera) (Pierce, 1948). Most male grasshoppers and crickets produce loud, high-pitched sounds by rubbing hard cuticular files against ridges on the wings whereas male cicadas emit even louder sounds by clicking a pair of membranes (tymbals) on the underside of the first abdominal segment (Moulds, 2009). The use of sound for communication by mosquitoes and moths (Diptera and Lepidoptera) is much less obvious, but because it has recently become the object of an increasing number of important studies, I chose to concentrate on these two groups.

1.1.1 **Low frequencies (50-1000 Hz)**

Mosquitoes (Diptera: Culicidae) are claimed to have among the most acute ears in the animal kingdom in their antennae (Göpfert *et al*., 1999). Johnston's organ (JO), in the large spherical pedicel (second segment) of the antenna, is their sensory component. In both sexes, the organ is a dense radial array of thousands of mechanosensory organelles (scolopidia), each containing two or three neurons that generate nerve impulses in response to sound-mediated

nanometer deflections of the flagellum. The antennal flagella vibrate in time with the minute displacements of air molecules that occur in all sound waves and stretch those scolopidial neurons in line with the vibration (Chapter 2). This property makes the JO inherently sensitive to the direction and frequency of wingbeat sounds, unlike the eardrums (tympana) of vertebrates and other insects, most of which respond to the minute non-directional pressure changes in sound waves at higher frequencies (Christensen-Dalsgaard & Manley, 2005). The JO's of male mosquitoes contain about twice as many scolopidia as females of the same species, and there is little doubt that they are used to locate flying females in those mosquito and midge (culicomorph) species that mate in swarms. The JO's of females, although smaller than those of males, are almost as sensitive, (Gibson *et al.,* 2010) but how females behave in response to the sounds they hear is still disputed. I discuss the use of sound by mosquitoes in more detail in Chapters 2 and 3.

Johnston's Organ is found in the antennae of all ~28 orders of insects (Regier *et al.*, 2010) and it is surprising that they have only been described as detecting sound in species of Diptera (Göpfert & Robert, 2002), and Hymenoptera (Dreller & Kirchner, 1993).

Moths produce low frequency sounds when flying or when they wingfan during courtship (Sotavalta, 1952; Spangler, 1985). Some moth species use these wingbeat sounds for communication (Spangler, 1985). Although four moth species are claimed to detect frequencies below 20 kHz with their paired tympana (Spangler, 1984; Rowland *et al*., 2011), the majority have not been investigated for their ability to do this. Moreover, at least two species of moths that evolved early in the history of Lepidoptera with no tympana, clearly use wingbeat sound to communicate (Takács *et al*., 2003; Hart, 2006).

The tobacco hornworm, *Manduca sexta*, is the only moth whose JO's have been examined for the ability to hear. Vande Berg described a JO containing a radial

array of ~650 scolopidia very similar in ultrastructure to the type B scolopidia in mosquitoes (Ch 2 Fig 2.3) in the pedicel, but because he could not record electrical activity in response to sound, concluded that it was a proprioceptor (Vande Berg, 1971). Sane and co-workers (2007) vibrated the flagella of *M. sexta* mechanically (not with sound) and found that the scolopidia in the JO responded maximally at twice the wingbeat frequency. They concluded that the JO allowed the moth to stabilize itself in flight. *Manduca sexta* has not been described as using sound to communicate; however, they are large atympanate moths and would be ideal specimens for a study of antennal hearing using modern techniques.

1.1.2 **Ultrasounds (20 kHz – 100 and more kHz)**

The scolopidia in Johnston's organ are stimulated by stretch and their neurons, like all others, cannot generate more than about 1000 impulses per second (1 kHz) due to limitations of the ionic channels (Gerhardt & Huber, 2002; Field, 2004). A second type of scolopidium that fires nerve impulses asynchronously when vibrated, evidently evolved with paired tympanal organs in the first abdominal segment of some of the first grasshoppers and crickets (Grimaldi & Engel, 2005). These mononematic (single connection) scolopidia have an electron-dense cap at their apex that presumably has enough inertia to stimulate a sensory neuron when it vibrates at frequencies much higher than 1000 Hz (Yack & Dawson, 2008). Similar tympana with mononematic scolopidia have evolved in at least seven other orders of insects allowing them to hear high audible and ultrasonic frequencies (Hoy, 1998).

Species in sixteen families of moths possess tympanate ears. The popular theory is that tympana evolved to detect the ultrasonic echolocating signals of foraging bats (Treat, 1955; Rydell *et al*., 1997). The fossil record shows that tympanate moths appeared after bats in the fauna (Rydell, 1988), and numerous studies illustrate the evasive maneuvers and strategies tympanate moths use when exposed to ultrasound (Roeder & Treat, 1961; Dunning & Roeder, 1965;

Brunel-Pons *et al*., 2011). From this beginning, some species began to communicate with self-generated ultrasounds (Conner, 1999; Skals & Surlykke, 1999). However, although the tympana are highly sensitive to ultrasound, as noted earlier, they respond only minimally (Spangler, 1984; Rowland *et al*., 2011) or not at all to the low frequencies present in wingbeat sounds at biologically relevant sound pressure levels (Svensson *et al.*, 2007).

To produce ultrasound, moths have striated tymbals that buckle to produce a series of clicks (Spangler & Takessian, 1983; Bennett, 1989), castanets on the wings that they click together (Bailey, 1978), specialized scales they rub together (Nakano *et al*., 2008, 2009, 2010), or they can scrape one body part against another (Gwynne & Edwards, 1986; Heller & Krahe, 1994). Several species of noctuid moths produce ultrasonic clicks in time with the wingbeat by methods not completely understood (Waters & Jones, 1994; Lapshin & Vorontsov, 2007). This seems to be the case in two of the moth species I studied in Chapters 4 and 5.

Table 1.1. Mechanisms for insect sound and ultrasound production

Mechanism	Examples	References
Air expulsion	*Elliptorhina chopardi* (cockroaches), *Amorpha juglandis* (caterpillars)	Sueur & Aubin, 2006; Bura *et al*., 2011
Click mechanisms	*Magicicada* (periodical cicadas), *Arctiidae* (moths)	Simmons *et al*., 1971; Blest *et al*., 1963
Percussion	*Xestobium rufovillosum* (death watch beetle); *Pseudacanthotermes spiniger* & *P. militaris* (termites)	Birch & Keenlyside, 1991; Connétable *et al*., 1999
Stridulation	*Atta sexdens* and *Atta cephalotes* (leaf cutter ants); *Aphodius sp*. (dung beetle); *Stenobothrus rubicundus* (grasshopper); *(Gryllus campestris, Oecanthus pellucens* & *Lerneca fuscipennis)* (crickets)	Masters *et al*., 1983; Gerstner *et al*., 2011; Kasper & Hirschberger, 2005; Schütze & Elsner, 2001; Desutter-Grandcolas, 1995
Vibration (including wingbeat)	*Drosophila melanogaster* (fruit flies); *Nezara viridula* (green stink bugs); *Aedes aegypti* (mosquitoes)	Shorey, 1962; Cokl *et al*., 1999; Wishart & Riordan, 1959

Table 1.2. Types of sound and ultrasound receptor based on Yack's 2004 categories

Receptor	Examples	References
Antennae/Johnston's organ	Mosquitoes; *Apis mellifera* (honeybees); *Drosophila* (fruit flies)	Risler, 1955; Dreller & Kirchner, 1993; Göpfert & Robert, 2002
Subgenual organs and substrate vibration receptors	*Nezara viridula* (green stink bugs); *Camponotus ligniperda* (carpenter ants); *Ametrus tibialis* (cricket)	Cokl, 1983; Menzel & Tautz, 1994; Strauss & Lakes-Harlan, 2008
Long sensory setae (Trichoid sensilla)	*Periplaneta americana* (cockroaches); *Barathra brassicae* (caterpillar)	Pumphrey & Rawdon-Smith, 1936; Tautz & Markl, 1978
Tympanal organs	*Arctia caja* (moths); *Ormia ochracea* (Tachinid fly)	Haskell & Belton, 1956; Edgecomb *et al*., 1995

1.2 Thesis goals

I previously found that peach twig borer (PTB) moths, *Anarsia lineatella*, communicate with sound (Hart, 2006), but they are in a family (Gelechiidae) with no tympana. Knowing that mosquito antennae are highly sensitive sound receptors, I compared their form and function with those of the PTB and two other moth species thought to communicate with low-frequency wingbeat sounds. I used both sexes of a local mosquito species, *Aedes togoi*, males of which erect and collapse their fibrils when they swarm and are at rest, respectively, and examined the sharpness of their tuning and the range of frequencies they detect. I recorded their wingbeats, and used laser vibrometry and electrophysiology to elucidate the best frequencies, tuning and sensitivity of their antennae. I compared the mechanical and electrical responses of females with those of males with erect and collapsed antennal fibrils. I then examined the tympanate Indianmeal moth (IMM), *Plodia interpunctella*. Because IMM has tympana that detect ultrasound (Mullen & Tsao, 1971), I wanted to see if its tympana could

also detect low frequency wingbeat sounds. Using behavioural experiments and the methods I used for *Ae. togoi*, I examined the sounds and ultrasounds produced by IMM, and the acoustic physiology of their tympana and antennae. I then turned my focus to atympanate PTB and webbing clothes moths (WCM), *Tineola bisselliella*. Both species use sound to communicate, but their families are thought to have no tympanal organs. They also inhabit different environments that might affect how they communicate with sound. Again, using acoustic recordings and laser vibrometry, I analyzed the sounds and ultrasounds they produced, located potential sound receiving organs and described their vibration in response to the sounds.

1.3 Thesis results

In Chapter 2, I review the morphology and physiology of mosquito antennae. Dr. Helmut Risler, an expert in mosquito hearing, died before publishing a manuscript written in 1983 on the antennae of *Aedes vexans*. In it, he described a hinge that erected the long fibrils on the male antenna identical to that described by Nijhout (1977) from an *Anopheles* species. The chapter reproduces four of his drawings, one of them showing the fine structure of the auditory scolopidia, and I summarize recent important findings on their acoustic physiology for the general reader. In Chapter 3 I illustrate the antennae of female and male *Aedes togoi*, with the fibrils of the male erect and collapsed. I sought to determine if the fibril state of males affected their ability to hear, and in what ways their hearing compared to that of females (lacking long antennal fibrils). I found that erect fibrils significantly altered male hearing, they were tuned to the wingbeat frequency of females, and females could hear about as well as males but their antennae were tuned below their own and well below the wingbeat frequency of males. In chapter 4, I examined the sounds produced and detected by IMMs to see if they heard sound with their antennae in the same manner as mosquitoes. I found that both sexes of IMM detect ultrasound with their tympana, but cannot detect wingbeat sound with them, and that the antennae vibrate as resonant systems in response to sound, exactly like the antennae of mosquitoes,

but are not sensitive to ultrasound. I then investigated the antennae of the atympanate PTB and WCM to see if they could detect sound. I found that the antennae of both species vibrate in response to wingbeat sound and that PTB has an air-backed circular area on a metepisternal sclerite that vibrates in response to ultrasound and may prove to be a functional tympanum.

My research shows that male and female mosquitoes have different wingbeat frequencies and that males hear the sound of females. In contrast, both sexes of the three moth species have similar antennae and wingbeat frequencies. They would be able to hear, but not distinguish between, the sounds of the two sexes. The wingbeat sounds of IMM and PTB contain ultrasonic clicks and these may also be used in communication.

LINKING STATEMENT

When researching mosquito sound production and detection, I came across an unpublished manuscript by the late Dr. H. Risler, one of the first scientists to study mosquito antennae as hearing organs. Because it contained anatomical details that had gone overlooked for almost 30 years and were important for my investigation of *Aedes togoi*, I took the opportunity to publish a summary of Risler's findings in collaboration with Dr. P. Belton and one of Risler's former students, Dr. Roland Kuhn. The publication provides a good, basic introduction to mosquito hearing, so I include it in the next chapter, before my work on *Aedes togoi*.

2 THE RISLER MANUSCRIPT[1]

Melanie Hart[1], Peter Belton[1]and Roland Kuhn[2]

[1] Department of Biological Sciences, Simon Fraser University, Burnaby, BC Canada V5A 1S6; [2] Institut für Zoologie, Johannes Gutenberg-Universität, Mainz, Germany

2.1 Abstract

An unpublished manuscript "The Auditory Organ of Male Mosquitoes (Culicidae) (Studies on Aëdes vexans Meigen 1830)" describes the structure of the pedicels and flagella of male and female *Aedes vexans* (= *Aedimorphus*[2]*vexans*) from scanning and transmission electron micrographs. We reproduce diagrams from the manuscript showing an in-depth section of the pedicel and first few flagellar segments, a hinge involved in extending and collapsing the long fibrils on the male flagellum that has not been described before in the Culicinae and the structure and arrangement of two different types of sensory units (scolopidia), one of which we speculate might be involved in vibrating the flagellum and increasing the sensitivity of Johnston's organ.

Key words: *Aedes vexans, Aedimorphus vexans,* Johnston's organ, hearing, antennae, fibril extension.

[1]This chapter is as it appears in Issue 29 (2011) of the European Mosquito Bulletin: headings have been altered to conform to the thesis format.

[2]Proposed species names follow editorial policy to reflect the creation of new generic and subgeneric rankings in ongoing internal classification of the Culicidae, as published by John Reinert and colleagues in the *Zoological Journal of the Linnean Society* and elsewhere.

2.2 Introduction

During a review of the literature dealing with the acoustic properties of mosquito antennae we came across an unpublished manuscript by the late Professor Helmut Risler (1914-1995). Risler gave a copy of this manuscript to Dr. A.N. Clements in 1984, with permission to use micrographs from it in his upcoming book (Clements, 1999). The manuscript was never published and after Risler's death, Clements deposited it in the library of the Natural History Museum, London (Risler, 1984). Because this subject is changing rapidly and producing very interesting new findings, we consider it important to point out some of Risler's hitherto overlooked results and review the topic for the general mosquito biologist.

In 1955 Risler published his first diagram of a longitudinal section of a male culicid antenna showing long setae (fibrils) extending outward from 3 basal flagellar segments (inset upper right in Fig. 2.1) and it is still one of the most commonly seen illustrations in major references (e.g. Clements, 1999 Fig. 26.5). In 1955 there was good behavioural evidence that males of many species use their highly sensitive antennae to detect and fly toward the sound of the female's wingbeat. In many species this occurs in crepuscular swarms where groups of males fly over visual markers; Marshall (1938) includes several early descriptions of females flying into the swarms, where they are quickly surrounded by a cluster of fast flying males and mate with one of them. Whereas the swarming behaviour was well known, the physiological and physical aspects of the use of sound were not. In many species of several genera the 500 or so long fibrils on the flagellar segments of the male antennae are folded parallel to the flagellum (as they are when they emerge from the pupae) except when they are swarming. There is a blood vessel in the flagellum of the species so far examined which led Downes (1969) to suggest that males extended and collapsed their fibrils through changes in blood pressure. This was disproved, at least in *Anopheles stephensi,* by Nijhout & Sheffield (1979) who showed convincingly that a deeply-grooved crescent of protein becomes hydrated and opens to extend the two groups of

fibrils on either side of the first 12 segments of the male flagellum. In this paper, the authors caused confusion by concluding that “Mating is limited to the period of antennal hair erection since this is the only time males can perceive the female” but Wishart *et al*. (1962) had already shown the contrary, that removing half the fibrils made no significant difference to the electrical activity they recorded from Johnston’s organ (JO) of males in response to sound.

Until the beginning of this century it was believed that females, although possessing JO’s only slightly smaller than those of males with identical sensilla that respond to sounds, did not change their behaviour in response to them. Now it is known that the females of several mosquito species are attracted to the sounds of their amphibian hosts (Borkent & Belton, 2006; Toma *et al.,* 2005; Bartlett-Healy *et al.,* 2008). There is also convincing evidence that in at least four genera, both males and females can hear and adjust to each other’s wingbeat frequency (Gibson *et al*., 2010).

A third recent discovery may also be relevant to Risler’s description of Johnston’s organ. That is the finding that the neurons, or at least some of them, in the movement-sensitive organelles (scolopidia) of both males and females can vibrate the flagella (Göpfert & Robert, 2000). Risler made careful counts and comparisons of the four different types of scolopidia in male and female *Aedes vexans* and we point out here some of their characteristics that may be related to vibration of the flagellum.

2.3 Material and methods

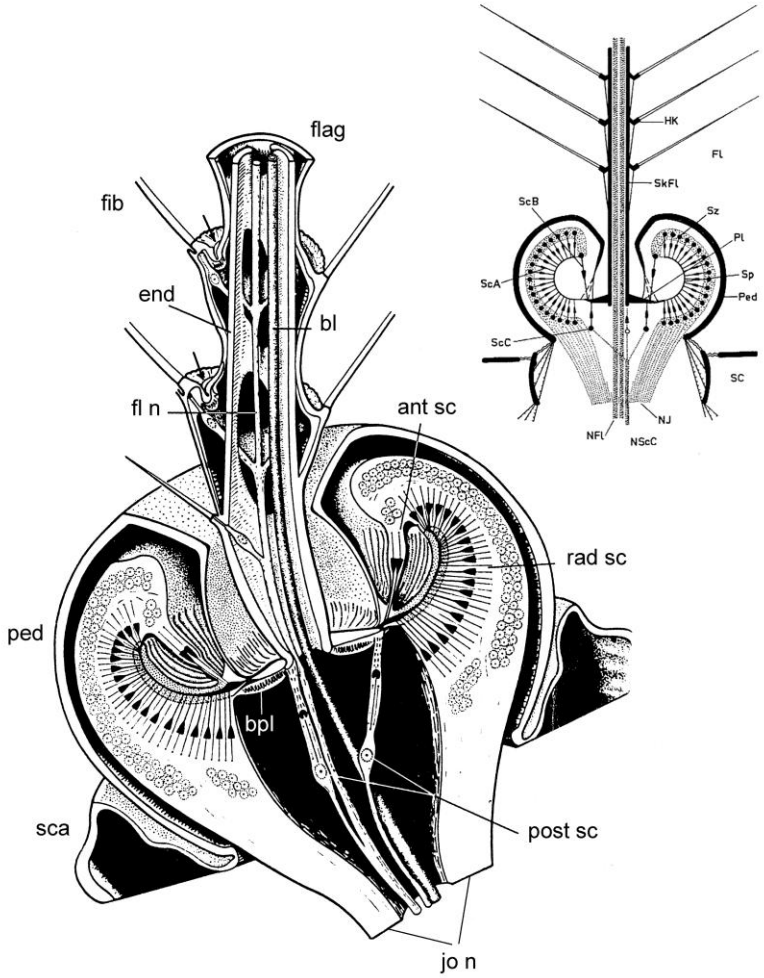
Mosquitoes were from a stenogamous strain of *Aedes vexans* from the Rhein River Valley, near Mainz, Germany. They were reared for the Institute of Genetics, Johannes Gutenberg University (Kuhn, 2002). Adult mosquitoes were fixed and sectioned conventionally with osmium tetroxide fixation and lead citrate staining. For scanning electron microscopy, partly dissected heads were dried at the critical point and sputter-coated with gold. Micrographs were made with a

Zeiss EM9a or a Siemens Elmiscop 1A for transmission and a Cambridge Mark II for scanning microscopy. Some of the micrographs were taken of partly sectioned heads with the Araldite resin partly removed (Mayor *et al.,* 1961).

2.4 Results

We will restrict this summary to descriptions of the diagrams in the Risler manuscript because the original transmission and scanning micrographs have not been found. We replaced the original abbreviations in the diagrams with more usual ones corresponding to the terms used by Clements (1999) and show the position of the fragmented inner wall of the pedicel in Fig. 2.3 to make our explanation of its probable function clearer.

Figure 2.1. Diagram of a section of the male pedicel and basal flagellar segments of *Aedes vexans*, it is clearly related to Risler's (1955) earlier version of the same region (inset upper right).



The first diagram shows the interior of the pedicel (ped) attached to the scape (sca) and the basal segments of the flagellum (flag) in depth. The hollow nerve from JO (jo n) is nearly 70µm in diameter and probably contains over 30,000 axons. Separate nerves run from the single scolopidia (post sc) attached to the underside of the basal plate (bpl) and the sensilla on the flagellum. A blood vessel (bl) runs alongside the inner nerve into the flagellum and small tracheae are present here and between the neurons and outer pedicel wall (Risler, 1953) but not shown in this diagram. The inner surface of the pedicel shows vertical grooves corresponding to the 58 (mean) prongs that curve out and up from the basal plate. Only 19 radial (rad sc, type A) scolopidia are shown diagrammatically

attached to the outer surface of each of the two opposite prongs. Risler estimated that there are actually more than 250 scolopidia of this type attached to the outside of each prong, a total of 15,000 arranged radially in each JO.

The suspension of the basal plate must be extremely flexible because the antennae of the males are thought to be among the most sensitive hearing organs in the animal kingdom. More detail is given in Clements' clear description (1999) but the thin septa that separate the prongs and the fine connecting distal filaments of the scolopidia are shown on the inner surface of the relatively massive 'doughnut' of scolopidia and neurons in this figure. Anatomically distinct scolopidia (ant sc), the anterior or type B series are drawn within the prongs. Two are shown on each prong but in the electron micrographs there are four filaments running down (in this orientation) to the inner surface of the base of each prong where it thickens and attaches to the margin of the basal plate. Risler estimated 232 of these scolopidia (exactly four per prong with a mean of 58 prongs). There is the same arrangement of this type of scolopidium attached to the inner surface of each prong near the basal plate in the female JO.

An endoskeleton (end) is shown originating midway up the first flagellar segment. Oval windows in each segment allow axons from the sensilla to pass through the skeleton and join one of the two flagellar nerves (fl n). The basal 40-50μm of the long fibrils (fib) is shown in deep sockets, their length decreases from 600-300μm toward the apex of the flagellum and those on the inner (medial) side are shorter than those on the outer. There are up to 60 fibrils on each segment arranged in two equal groups dorsal and ventrally on raised crescents of cuticle. Each group has a deep groove on its inner surface that Risler terms 'cuticular ring' shown on the left-hand fibrils in this figure (arrows). This is shown in detail in the next figure.

Figure 2.2. Diagram of the articulation of a fibril and the structure of its mechanoreceptive sense cell.

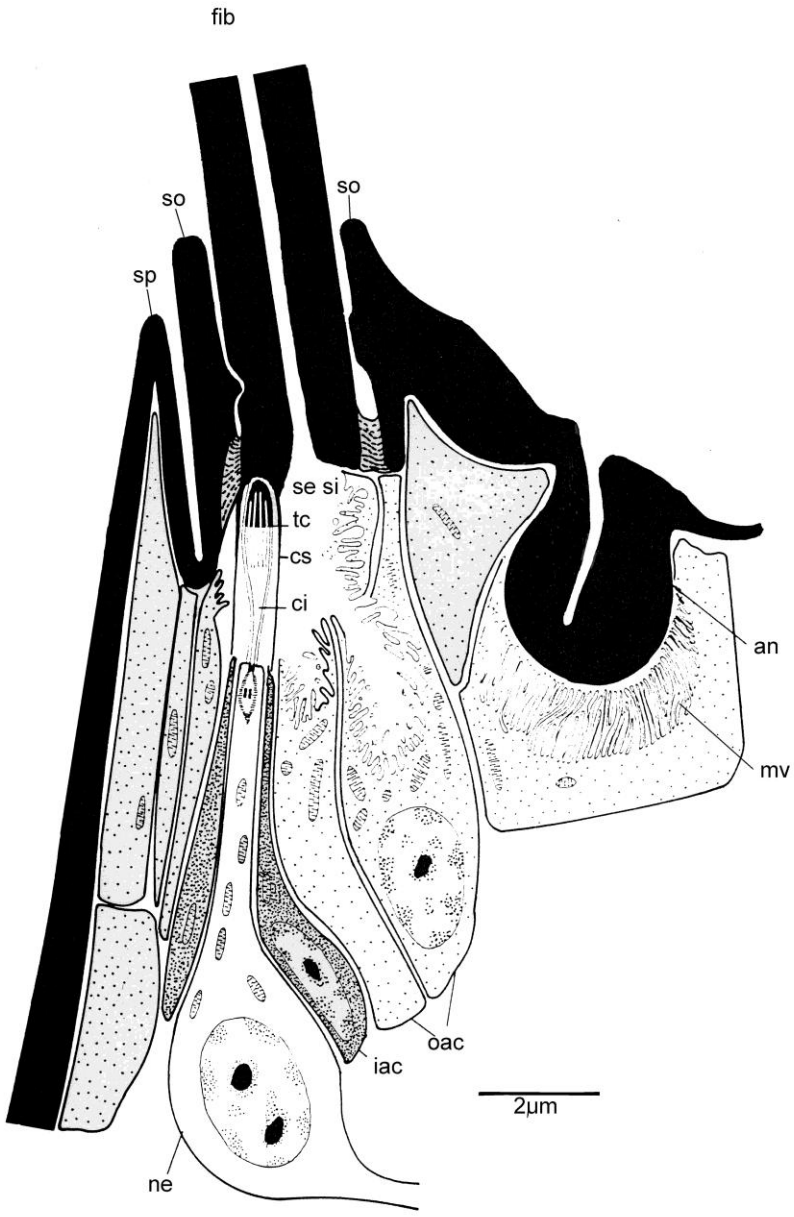


Figure 2.2 shows the base of a typical long fibril with its mechanoreceptive neuron. The sensory neuron has a typical structure at its apex with a cilium (ci) ciliary sheath (cs) and terminal cap (tc). Below the fibril (fib) is a sensory sinus (se si) and the neuron has inner and outer accessory cells (iac and oac). On the

right is the deeply grooved electron-dense annulus (an) and below it a large cell with dense microvilli (mv) and numerous mitochondria.

Risler's scanning micrographs show a scaly process (sp) that overlaps the socket (so) of the adjacent fibril and presumably keeps the fibrils in line as they extend and close. He suggests that the large cell below the inner electron-dense groove of the annulus is secretory.

Figure 2.3. Diagram showing the differences between the radial (A) and anterior (B) scolopidia

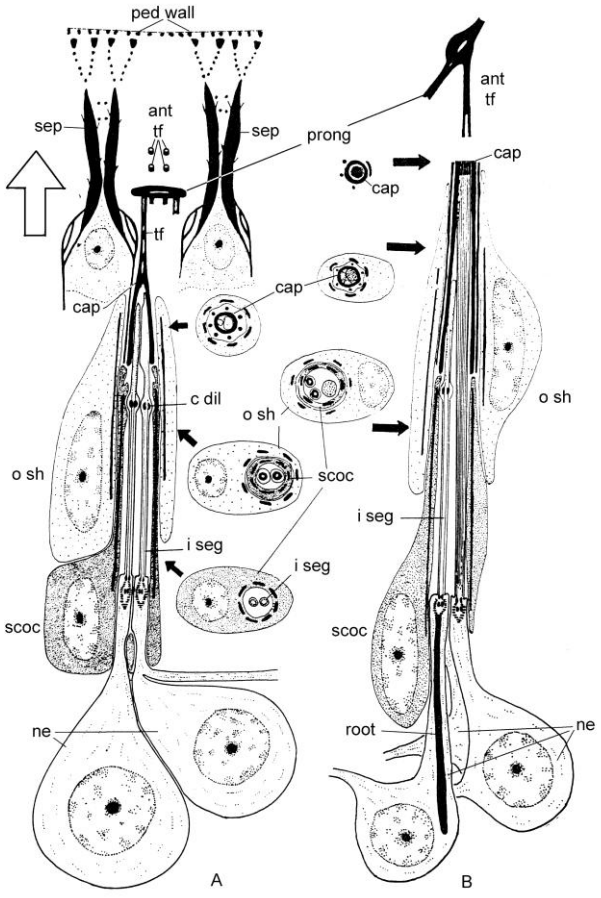


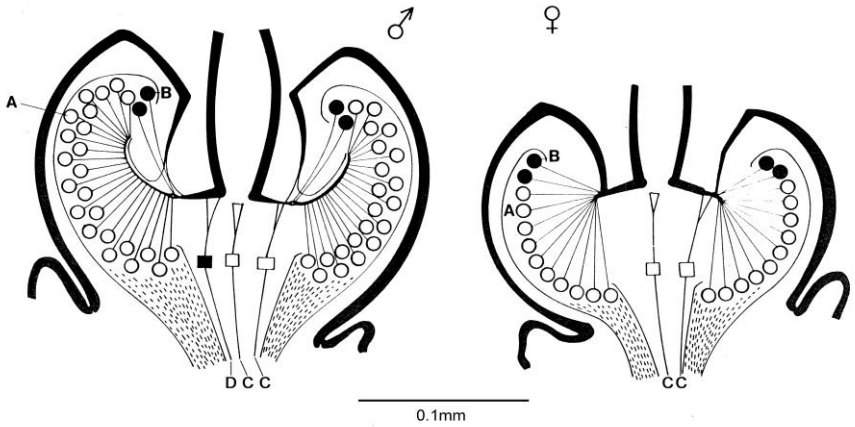
Figure 2.3 shows a radial scolopidium on the left with two similar neurons (ne) enclosed in a scolopale cell (scoc). The scolopidia are shown in transverse section at the arrows in the centre of the figure. The scolopale cell contains a ‘cage’ of seven electron-dense rods, and is enclosed distally by an outer sheath cell (o sh) with a similar arrangement of overlapping rods. The outer sheath envelops a third cage of rods, (bars of Boo & Richards, 1975) that connect to the septa (sep) and the inner wall of the pedicel (ped wall) The two neurons have typical ciliary inner segments (i seg) and distal ciliary dilations (c dil) and appear to be firmly attached apically to a conical cuticular cap (cap). The apical cap tapers to a fine terminal filament (tf) that attaches to the outer surface of the flattened prong that curves up (anteriorly) from the basal plate. Three neurons are contained in each anterior (B) scolopidium, two of them are similar to the ciliated type in A, but the dendrite of the third is packed with microtubules. One of the ciliated neurons has a long electron-dense root process (root) extending close to the nucleus of the neuron. The anterior scolopidia have much longer terminal filaments (ant tf) (broken on the right) joining the inner surface at the base of each prong very close to its origin at the margin of the basal plate. The electron-dense scolopale rods are shown in sixes rather than sevens around the transverse sections of the three dendrites but the number is variable from scolopidium to scolopidium. Curiously, the number of rods in the sheath and envelope cells always seems to be the same as in the scolopale cell.

Risler has clearly combined the results of his former student Schmidt with features taken from the literature and his own micrographs in this diagram. We include them because they allow a side-by-side comparison of the two main types of scolopidium. It is hard to visualize the arrangement of prongs and septa in two dimensions and there is a more complete description in Clements (1999) for those interested in more detail. We will simply point out that when sound moves the prongs in the direction of the open arrow on the left, the terminal filament pulls the tips of the cilia with them and away from the scolopale rods that evidently remain fixed to the interspersed septa. The scolopale rods of the B

units are presumably also attached to septa, but this is not yet clear from the micrographs.

Figure 2.4 shows the very different arrangement of scolopidia along the prongs in the two sexes. Risler points out that the number of B scolopidia is similar (about 220 in females and 232 in males) but that the type A are much fewer in the female (about 2,750 compared with 14,500 in males). The distal filaments of the A and B units in the female are similar in length, but as in the male the B scolopidia are attached closest to the basal plate. The single type C and D scolopidia are probably not involved in hearing (Clements, 1999) although the type D has only been seen in males. The drawings are to the same scale.

Figure 2.4. A scale diagram comparing male and female Johnston's organs. Cell bodies of the type B scolopidia in black, the more numerous type A clear. As in Fig. 1, two opposite prongs are shown with only a few of the scolpidia, but in their correct alignment. The much longer terminal filaments of the B scolopidia in the male are shown attached to the inner surface of the prongs close to the basal plate. Two septa are shown extending from the inner surface of the pedicel above the prongs of the male. The prongs of the female are tiny by comparison and curve downward. The single D (black) and C (clear) scolopidia are shown below the basal plate.



2.5 Discussion

Considering that the males of many species of mosquito in at least five genera keep their long antennal fibrils closely appressed to the flagellum except when they swarm and mate, it is surprising that the method of their movement has not been followed up since Nijhout & Sheffield's study of *Anopheles stephensi* in 1979. In his study of *Aedes vexans,* Risler shows the large cells on the inner margins of the fibril sockets that Nijhout investigated in *Anopheles* very clearly (Figs 1 & 2) and later realized their function. At a meeting in 1989 Risler describes in *Ae. vexans*: "einer dicken, polsterartigen Gelenkfalte am innenrand der Kränze" – 'a thick bolster-shaped hinge on the inner margin of the fibrils' (our

translation), (Risler, 1990). We have evidence that the hinge is composed of a protein similar in properties to that of *An. stephensi* in another aedine that extends its fibrils, *Aedes* (=*Tanakaius*[1]) *togoi*, (Hart *et al.,* 2010). Their fibrils are closely appressed in heads immersed in saline with low pH and expand in alkaline (pH 8) saline, exactly like those of *An. stephensi*. Evidently, this protein absorbs water when alkaline and increases in size, widening the deep groove in the hinge and extending the groups of fibrils. The large cells below the hinge, with their mitochondria and mass of microvilli evidently secrete the liquid. This is the only known hydration device in the animal kingdom (Vogel, 1988) and it would be interesting to make comparisons with other genera of mosquitoes that collapse and extend their fibrils.

The arrangement and structure of the two main types of scolopidia, A and B, were also of great interest to Risler. He believed that the type A, more numerous in the male and arranged radially, was involved in hearing and finding the female. The B scolopidia, with similar numbers in both sexes but less numerous than type A he thought had a more general function, perhaps detecting air currents. This is a logical conclusion, but the discovery that both male and female Johnston's organs can vibrate their flagella and perhaps change their tuning lead us to suggest a different function. Little is known of the movement of sensory cilia, but as there are no muscle fibres in the pedicel that could move its flagellum, some or all of the scolopidia must be motile. We believe the type B scolopidia with their third dendrite packed with microtubules might be prime suspects. If they can contract, their distal filaments are attached closest to the basal plate in both sexes and would thus produce a greater amplitude of vibration than if they were attached to a more distal region of a prong. It may also be significant that the type A and B scolopidia are attached to opposite sides of a prong. With that arrangement, if they both respond to stretch, movement of the flagellum in one direction would excite the type A ciliary dendrites and relax the type B and movement in the other would excite type B and relax type A. If the type B scolopidia included a stretch sensitive microtubular motor, it is not difficult

to visualise how they could cause oscillation at the resonant frequency of the antenna. The radially arranged scolopidia of both types are stimulated maximally in the direction of vibration and those at 0° longitude will move in the opposite direction to those at 180°. Different hypotheses involving this phase difference are possible and have already been proposed to explain spontaneous vibration (Avitabile *et al.,* 2010). There is an interesting parallel in the mammalian ear, where there are two sets of hair cells, the inner with a sensory function and the outer capable of vibrating and increasing the sensitivity of the ear. The outer hair cells have a motor innervation however, and there is no such nerve supply known in insects but the physiological similarity is remarkable. Warren *et al.,* (2010) have shown that dynein and tubulin, molecules found in cilia, are involved in the movement, and that when they are blocked, some sensory cells can still respond to sounds.

The arrangement of the electron-dense and presumably rigid rods around the dendrites of neurons in the scolopidia is still not completely understood, but is more clearly shown in Risler's diagram (Fig. 2.3) than elsewhere. Several recent models of the scolopidia seem to assume that the whole organelle shortens and stretches (Avitabile *et al*., 2010 for example). It should be obvious that the sensory cilium can only be stretched relative to the surrounding arrays of rods, and that the rods are firmly connected to the filamentous septa and inner wall of the pedicel. The same applies to movement. If the cilia move they can only shorten by movement of the distal filament of the cilia with respect to the scolopale rods and septa.

The attachment of A and B scolopidia on opposite sides of the prongs has so far been described in *Anopheles*, *Culex* and *Aedes* (*Stegomyia*) (Risler, 1955); *Aedes* (*Aedimorphus*) (Risler, 1984) and probably also in *Toxorhynchites* (Göpfert & Robert (2001). Of these, male *Anopheles* (Gibson *et al*., 2010) and male and female *Culex* (Warren *et al*., 2010) and *Toxorhynchites* (Göpfert & Robert, 2001) vibrate their flagella spontaneously, and it seems likely that these

characteristics and the ability to fold the fibrils on male antennae evolved with the Culicidae and are plesiomorphic.

Risler's ambidextrous artistic ability has influenced generations of researchers interested in mosquito hearing. His simple diagram of Johnston's organ has been reproduced in almost every text that mentions the subject, sometimes attributed wrongly to Autrum (1963), one of the first to use and acknowledge it. We believe this description of his manuscript could stimulate further investigations of the use of sound by mosquitoes using histological, molecular, or electrophysiological techniques and perhaps their systematic relationships within and outside the culicid family.

2.6 Acknowledgements

We thank Drs Reiny Brust, Alan Clements, August Dorn, Franz Romer and Konrad Schmidt for their help in saving, researching and explaining the Risler manuscript.

2.7 Addendum

Warren has since been unable to detect spontaneous oscillations in female *Culex* (personal communication, 2011).

LINKING STATEMENT

In Chapter 2, I used Dr. Risler's drawings of mosquito antennae to review antennal morphology and physiology. The drawings showed the fine structure of the scolopidia, which I then related to recent literature on their acoustic physiology.

In Chapter 3 I examined the external morphology and function of the antennae of female and male *Aedes togoi*, when the fibrils of the male were erect and collapsed. I investigated whether or not the position of the fibrils affects their ability to hear, and in what ways their hearing compares to that of females (lacking long antennal fibrils). My results show that fibril state affects the hearing ability of males, and that the antennae of females are almost as sensitive as those of males.

3 THE USE OF SOUND BY *AEDES TOGOI*

3.1 Abstract

This study addresses sound production and hearing of both sexes of *Aedes togoi,* a mosquito whose males extend the long setae (fibrils) on their antennae before they swarm and mate. Males of most species of mosquitoes are attracted to the sound of flying females and, until recently, it was thought that the long fibrils on their antennae enhanced their hearing. However, extension of the fibrils on the antennae of *Anopheles gambiae* did not increase their mechanical sensitivity, but did increase their electrical sensitivity. Furthermore, the antennae of female mosquitoes are mechanically resonant systems and vibrate like those of males although they have no long fibrils and females were not thought to respond behaviourally to sounds. I therefore tested both male and female *Ae. togoi* when the male fibrils were recumbent and extended for changes in function. I studied differences in the morphology of the antennae with light and scanning microscopy, recorded the flight sounds of tethered females and males and used laser vibrometry and electrophysiology of Johnston's organ (JO) to compare the sensitivity and hearing range of males and females. I investigated the best frequency of the antennae with sound sweeps and broad frequency clicks. I determined the sharpness of tuning (Q) of the antennae using both sound sweeps and clicks and expected that if fibrils were affecting tuning, males with erect fibrils would be more broadly tuned than those with recumbent fibrils, whereas females would not change with time of day. The morphology of the female and male antennae is very similar to other species in the genus. They also have the usual differences in wingbeat frequency; females with a mean of 306 Hz and males 523 Hz, never overlapping. Using frequency swept sine waves, female antennae vibrate best at 257 Hz during the day and at 252 Hz at swarming time with Q's of 3.68 and 3.98, respectively, neither difference being statistically significant. Male antennae with recumbent fibrils have a significantly

lower best frequency (309 Hz) than those with erect fibrils (385 Hz) with similar sensitivity. The antennae of males were also more broadly tuned than those of females (Q = 2.66 recumbent and 2.10 erect). Electrical responses had maxima at 302 Hz and 227 Hz for males and females, respectively, lower than the mechanical best frequency, similar to the differences found in other species. I conclude that males can hear flying females whether their fibrils are erect or not, although the fibril state alters their best frequency, and that females lacking long fibrils cannot hear the fundamental wingbeat frequency of males. The tuning of both sexes (Q values ranging between 2 to 4) would allow them to hear over the expected range of female wingbeat frequencies.

3.2 Introduction

In the Culicomorpha, an infraorder of mosquito-like flies that evolved early in the history of the Diptera, the 2nd antennal segment (pedicel) is enlarged and contains Johnston's organ (JO), a complex, radially symmetrical array of sensory neurons. A more complete description of the internal anatomy of Johnston's organ in *Aedes spp.* and differences in its structure between the sexes was given in the previous chapter. The JO detects airborne sound through the deflection of the attached flagellum. Although JO's are present in all flying insects (Regier *et al*., 2010), in this Dipteran group they have evidently evolved as a highly sensitive and directional pair of ears because males of many of its species are known to use their antennae to locate flying females based on their wingbeat sounds (Clements, 1999).

In five families of culicomorphs, the mosquitoes (Culicidae), phantom midges (Chaoboridae), frog-biting midges (Corethrellidae) human-biting midges or no-see-ums (Ceratopogonidae) and dancing midges (Chironomidae), there is a striking difference in the structure of the antennae between the sexes. Whereas all flagellomeres on the male and female flagella possess setae (Clements, 1999), the male flagella of most species in these 5 families carry up to a thousand long articulated setae (fibrils), giving them a bushy (verticillate)

appearance in sharp contrast to the females which have many fewer and much shorter (hardly noticeable) setae. The males of most species in four of these five families fly together in swarms and mate with females that fly into the swarms (corethrellids have not been observed swarming).

In many mosquito species, the long fibrils on the male flagellum are erect when the insects swarm at dusk and dawn, and recumbent during the rest of the day (Clements, 1999). Nijhout (1977) studied the movement of the fibrils of *Anopheles stephensi* and found them to be under the direct nervous control of an adrenergic receptor stimulated by alpha-adrenergic agonists (Nijhout & Martin, 1978). Further work showed that an annulus bearing the fibrils contains a protein-rich pad that absorbs water and unfolds the fibrils (Nijhout & Sheffield, 1979; Vogel, 1988). Risler described a very similar pad in *Aedes vexans* (Chapter 2) although the mechanism of fibril erection in this species remains unexplored.

A persistent belief has been that when male fibrils are recumbent, either because of time of day (for species that erect and collapse their fibrils) or because they are too young (for species that erect their fibrils permanently soon after emergence), males cannot hear (Roth, 1948; Nijhout, 1977; Nijhout & Sheffield, 1979; Vogel, 1988). A recent study on *Anopheles gambiae* clearly showed that the antennae of males with closed fibrils vibrate in response to sound much like those that are open, although there is an electrical difference between the fibril states (Pennetier *et al*., 2010). Also, removing half the fibrils from a male *Aedes aegypti* antenna made no significant difference to the electrical activity recorded from its JO (Wishart *et al.*, 1962). In contrast to the males, the short setae of females stay erect regardless of time of day in all species examined.

For many years, researchers believed that female mosquitoes could not detect sounds or did not respond to them (Tischner, 1953; Downes, 1969; Clements, 1999). This belief continued despite an early paper showing potential changes in

the JO's of *Ae. aegypti* females in response to sound (Wishart *et al*., 1962). However, a number of papers have been published recently showing that females of some frog-feeding species use calls of their hosts to locate blood meals (Toma *et al*., 2005; Borkent & Belton, 2006; Bartlett-Healy *et al.*, 2008). There is now good evidence that females of several other mosquito species can somehow detect the wingbeat sounds of males and adjust their frequency to match (Gibson & Russell, 2006) or converge on a shared harmonic of their wingbeat frequencies (Gibson *et al*., 2010), perhaps to facilitate mating.

It is now clear that the main use of sound by male culicomorph flies is the detection of females that fly close to swarms, and the antennae of males of all species so far examined resonate at a frequency close to the wingbeat frequency of their respective females (Clements, 1999). The wingbeat frequency of mosquito species varies with their size, so sound might also be used to separate genera or species. Clements (1999) discusses this possibility and concludes that frequency differences alone would not be sufficient to do this.

I chose to investigate the physiology of hearing in *Aedes togoi,* because there are no previous studies of this in any genus other than *Anopheles* that erect and collapse their antennal fibrils. I sought to: a) describe and compare the external morphology of male and female antennae; b) determine and compare the wingbeat frequencies of males and females; c) determine the mechanical sensitivity and range of frequencies that males can detect with erect and recumbent fibrils and compare them with the sensitivity and frequency range of female antennae at the same time of the day; and, d) record the electrical activity of the Johnston's organs of both sexes and compare it with the mechanical responses. For hearing, I use the definition of Hoy and Yack (2008): the ability to detect small time-varying movements in a medium (air in this study).

I used light and electron microscopy to describe the morphology of female and male antennae. I then recorded female and male wingbeat sounds, determined

their frequencies, and used laser vibrometry to test my predictions about *Ae. togoi* hearing. Based on the results of Pennetier *et al*.'s paper (2010) on *An. gambiae*, I predicted that *Ae. togoi* males with their fibrils both recumbent and erect would be able to hear females and be tuned to higher frequencies with their fibrils erect. I also predicted that females could detect sound and that their range of hearing would reveal whether they could hear other females, males or even potential hosts. Because the tuning of the neurons in Johnston's organ is at a lower frequency than the mechanical tuning of the flagellum in male *Culiseta incidens* (Clements, 1999), *Culex quinquefasciatus* (Warren *et al*., 2009) and *An. gambiae* (Pennetier *et al*., 2010) I expected to find a similar difference in *Ae. togoi*.

Because the long verticillate fibrils on the antennae of males have so little acoustical effect I briefly discuss an alternative function for them.

3.3 Materials and methods

3.3.1 Natural history

Aedes togoi is a coastal mosquito whose larvae develop in pools of brackish water (Belton & Belton, 1990; Lee, 2001). Its distribution is from Malaysia north to Russia, Taiwan and Japan, and in North America from Washington State to northernmost islands in the Georgia Strait (Belton & Belton, 1990). Although their full complement of hosts is unknown, *Ae. togoi* have been raised on blood from rats, hamsters (Riyong *et al*., 2000), humans (Purnomo *et al*., 1976), jirds (small rodents) (Bosworth & Ewert, 1971; Purnomo *et al*., 1976), cats (Bosworth & Ewert, 1971; Kan & Ho, 1973) and have been reported in nature as biting deer (Yen *et al*., 1982), cattle (anonymous, 2008) and humans (Belton & Belton, 1990).

3.3.2 **Insects**

An *Ae. togoi* colony was established from larvae collected from rock pools in West Vancouver, BC, Canada. Larvae were fed powdered fish flake food, and maintained at 25˚ C on a 16:8 light:dark photoperiod. Adults were fed sugar water and females allowed a human bloodmeal. All adult insects used in experiments were 3 to 5 days old (to ensure they were ready to mate) (Clements, 1999). For laser vibrometer recordings, males with recumbent fibrils were tested during the photophase when their fibrils were closed naturally; they were tested with erect fibrils during the scotophase once their fibrils had expanded naturally due to time of day, not induced. Females were tested at the same time as males in both the light (males not swarming) and dark (males swarming) portions of the photoperiod. For electrophysiological recordings, the insects were tested during the photophase because, regardless of time of day, insertion of the electrodes caused fibrils to erect along half the length of the flagellum.

3.3.3 **Morphology**

Photographs of the insects were taken with a Canon PowerShot SD1000 through the eyepiece of a Carl Zeiss Technival 2 dissecting microscope. A stage micrometer (Meij Techno, JP) alongside the insect allowed for measurements. Images were analyzed using ImageJ (NIH, USA). Wing lengths were taken from the base of the wing to the tip; the flagellum was measured from the distal pedicel to the tip of the flagellum; fibrils were measured from their attachment to the flagellum to their tip; pedicel measurements were of their length and diameter.

For scanning electron microscopy I used standard procedures (Paprican, Vancouver). I coated specimens with 60/40 gold/palladium alloy using an Anatech Hummer VI cold cathode sputtering system with Argon in the chamber. Samples were run in DC plating mode at a current of 8 mA and pressure of 80

mTorr for 7-10 minutes. The environmental scanning electron microscope (ESEM) was an FEI Quanta FEG 400.

3.3.4 **Flight sound recordings and analysis**

I glued each insect to the head of a # 1 insect pin with a dab of Weldbond (Frank & Ross & Sons Ltd., Markham, ON, CAN) on their scutum and flew the mosquitoes approximately 1.5 cm in front of a Knowles NR 3158 microphone. The head of the mosquito pointed at the microphone and the glue did not impede flight. Signals, amplified 800 times with an NI SC 2040 differential amplifier (National Instruments Corporation, Austin, TX, USA) with a sampling frequency of 250 kHz were saved to the hard disk of Dell Latitude D800 laptop equipped with an NI data acquisition card (DAQcard 6062E, 12 bit, 500 kHz maximum sampling rate). I measured sound levels with a C-weighted Realistic sound level meter (SLM) 33-2050 (Radio Shack, Fort Worth, TX, USA) placed ~1.0 cm from the insect in a room with background ambient noise at ~53 dB. The meter was calibrated against a Brüel and Kjær 2204 SLM with a 4133 ½" microphone (Naerum, DK).

Wingbeat frequencies from both sexes were analyzed using a spectrum analysis program developed in LabVIEW 7.1 (National Instruments Corporation, Austin, TX, USA).

3.3.5 **Sound generation**

For laser vibrometry, a sine wave sweeping logarithmically from 100 Hz to 1 kHz in 5.1 s, was generated with a Brunelle Instruments model 3050 sweep generator (Hatley, QC, CAN). This range of frequencies encompassed the wingbeat frequencies of both females and males. Sound pressure was kept constant at 65 dB at the insect with a General Radio 1569 Automatic Level Regulator (MA, USA) that monitored sound level through the Realistic or Brüel and Kjær SLM adjacent to the mosquito. The sound was played from a Sennheiser headphone

(Old Lyme, CT, USA: flat frequency response: 10 to 39.500 Hz, 0.05% THD) approximately 10 cm from the antenna. For the electrophysiology, the sweep was generated with LabView and the level increased from 72 dB to 82 dB with the increase in frequency to prevent overloading the preamplifier at the lower frequencies, or kept at 65 dB for single tones.

I compared the best frequencies determined from sweeps with those measured from the response to clicks made by a toy clicker (Discount Party Supplies, Jackson, MI, USA) ~ 0.5 m from the mosquito. Each click produced frequencies from below 100 Hz to 20 kHz. The peak sound level measured with the Brüel and Kjær in linear mode at 0.5 m from the clicker was 85 dB.

3.3.6 **Laser vibrometry**

Vibration was measured at the base of one flagellum of 16 females and 24 males with a Polytec OFV-2500 Doppler laser using a Polytec Vib-E 220 data acquisition system (Irvine, CA, USA). Laser vibrometry detects the slightest movement of the antenna and thus allows determination of the 1) best frequency, 2) highest velocity of the antenna, and 3) by calculation (Equation 3.1), its displacement at the best frequency. The sampling rate was set at 24 kHz for 10.92 s for the frequency sweep, and for ~3 s to record responses to the clicks both with the 2 mm/s sensitivity range. The stimulus was recorded concurrently with the antennal response by feeding the output of the SLM to the second channel of the Vib-E system. Recordings were done at 24-25˚C. Single sweeps were stored by the Vib-E system and later examined for the velocity at the best frequency. Files were then transferred to a computer running Adobe Audition™ 3.0 (San Jose, CA, USA). The time of the peak and the steepest part of the phase change between the antennal response and the sound stimulus was noted, and the corresponding best frequency determined using Raven Pro 1.4 (Cornell Lab of Ornithology, Ithaca, NY, USA).

Equation 3.1. Calculating displacement from velocity

$$\text{Displacement} = \text{velocity} / (2*\pi*\text{Frequency in Hz})$$

The mean best frequencies and displacements were calculated with three recordings from each of six insects and used to calculate the overall mean for males and females. The response to a click from each male and female was examined with Raven and its frequency measured from the spectrogram feature of the program.

3.3.7 **Electrophysiology**

Electrophysiological recordings were done inside a Faraday cage in an open laboratory with a P18 pre-amplifier (Grass Technologies, West Warwick, RI, USA) set at a gain of 100. One electrolytically-tapered tungsten electrode was inserted into the pedicel, the second into the eye. Signals were recorded with a Picoscope 2203 USB Oscilloscope with version 6 software (Pico Technology, Cambridgeshire, UK), with a sampling rate of 3.268 kHz, and the stimulating sweep taken from the output of the SLM recorded on the second channel of the oscilloscope. Three recordings were made from ten females and ten males and saved to the hard disk of the laptop computer running the Picoscope oscilloscope program.

3.3.8 **Quality factor calculations**

The quality factor Q measures the selectivity of any resonant system, so I used Q to examine how sharply the antennae were tuned (Bennet-Clark, 1999). I calculated it from the response to sweeps by measuring the frequency at the peak amplitude of the antennal vibration and dividing this by the difference between the frequencies at the half peak amplitudes (Figure 3.1, Equation 3.2). The baseline was set where the antennal response was visible above the background noise.

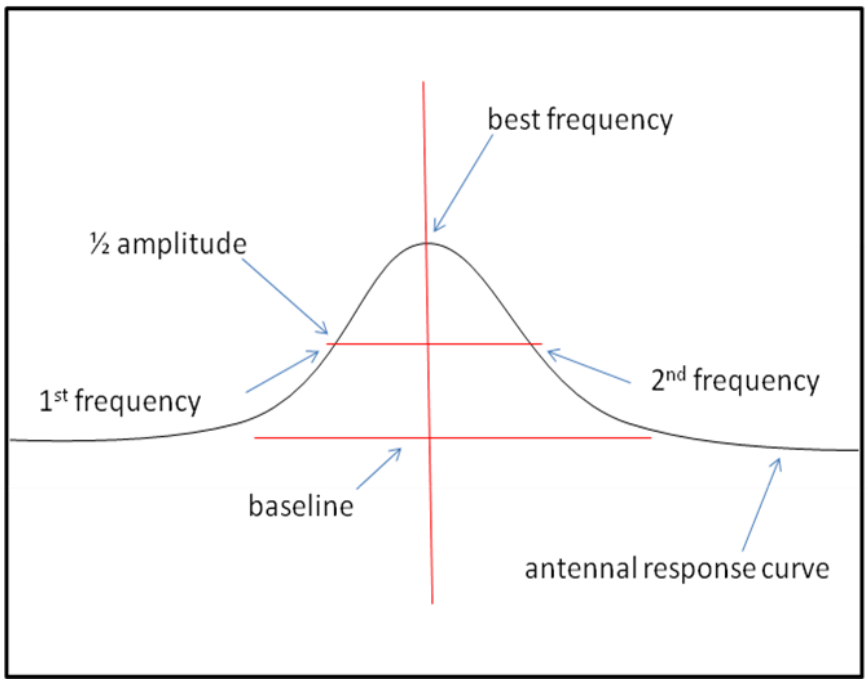
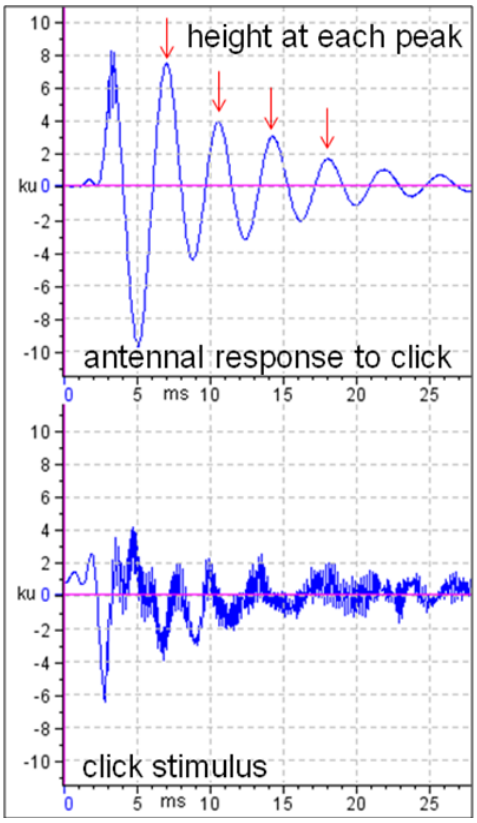


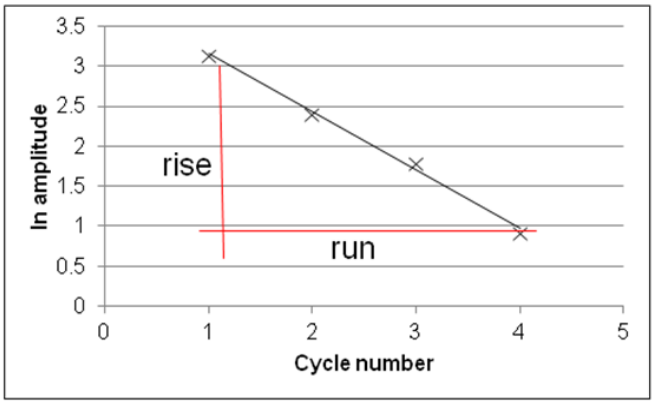
Figure 3.1. Calculating quality factor (Q) from an antennal vibration to sound stimulation

Equation 3.2. Calculating quality factor (Q) from an antennal vibration

$$\frac{\text{best frequency}}{(2^{nd}\text{ frequency at ½ amplitude} - 1^{st}\text{ frequency at ½ amplitude})}$$

For comparison, Q was calculated from the exponential decay of vibration of the antennae in response to clicks: the peak amplitudes of successive waves in the decaying response were measured, the natural logarithm of those heights determined and plotted (Figure 3.2) (Morse, 1948; Fletcher, 1992). A best fit line was plotted using Excel's best fit graphing function, the slope of the resulting line determined, and used in Equation 3.3.





Natural logarithm of transformed heights from the trace in the upper left (antennal response to click), with best fit line fit from Excel to determine the slope. The calculated Q=4.32.

Figure 3.2. Calculating quality factor (Q) from an exponentially decaying wave measured by laser vibrometry. The y axes of the oscillograms are the amplitudes in thousands of samples (ku).

Equation 3.3. Calculating quality factor (Q) from exponential decay of a wave

$$Q = \pi/\text{slope}$$

3.3.9 **Statistical analyses**

Data were analyzed in GraphPad Prism 5.0 and JMP 8 with t-tests, 95% confidence. I present all results as means $\pm$ standard deviations. Ten to 15 records were stored from each sex for each measurement comparing sounds and vibration but some were rejected on analysis because of noise in the laboratory or movement of the insect.

3.4 Results

3.4.1 **Morphology**

Based on winglength, females are significantly larger than the males (3.98 ± 0.29 mm compared to 3.18 ± 0.20 mm, respectively, n=10 for each sex, t=-7.24, p<0.0001).The antennae of female and male *Ae. togoi* have two basal segments: a short, wide scape supporting a larger, almost spherical pedicel housing JO (Figure 3.3). Although females are larger than males the diameter of the male pedicel is significantly larger than that of the female (Table 3.1). Each flagellum is inserted in a socket in the pedicel like those of *Aedes vexans* (Chapter 2, Figure 2.4). The flagella are long: 1.40 ± 0.11 mm and 1.54 ± 0.07 mm, in males and females respectively and strikingly different in structure between the two sexes (Table 3.1 and Figure 3.3). The 13 flagellomeres of the female are uniform with a mean length of 120 $\pm$ 0.015 µm (Table 3.1). At the base of each flagellomere, a ring of 5 or 6 fine setae 220 $\pm$ 47 µm long radiate outwards, roughly perpendicular to the flagellum (Table 3.1). The angle of insertion of these setae never changes after the female has emerged from its pupal case. There are about 72 of these setae on each flagellum but there are also many more, smaller, presumably chemosensory setae on each flagellomere (Clements, 1999). The flagella of females are flexible and bend when moved with a needle or puff of air. The first 11 flagellomeres of the male are short, a mean length of 70 $\pm$ 8 µm and 80 µm in diameter at the base of the flagellum (Table 3.1). At the distal end of the first 11 flagellomeres there are raised crescents of cuticle dorsally and ventrally; each bears a dense array of about 20 long fibrils (630 $\pm$ 0.1 µm) on the first flagellomere, increasing to 900 µm on flagellomeres four and five and shortening to 300 µm on flagellomere 11). These 11 flagellomeres are rigid and remain straight when deflected with a needle or air puff. The 12th and 13th flagellomeres in males are disproportionately long, comprising approximately 40% of the length of the flagellum. They also bear many short setae like the flagellomeres of females and are presumably chemosensory. The appearance of

male antennae in erect versus recumbent fibril position compared with female antennae is shown in Figure 3.3 C, D and E.

3.4.2 **Flight sounds**

Individual males and females had constant wingbeat frequencies, and their means were significantly different (Table 3.1). The sound of the males is almost an octave (1.7 times) higher than that of the females. Female wingbeats were significantly louder than males at 1.5 cm (Table 3.1). Facing the microphone, the sound generated follows the wingbeat in both sexes, with the amplitude of the 2nd harmonic about 1/10th that of the fundamental (Figure 3.4).

3.4.3 Laser vibrometry

The mosquitoes responded to both sweeps and clicks with a single best frequency. In six individuals of each sex, the peak response coincided with the steepest part of the phase change between the antenna and the stimulating sound, consistent with the definition of a simple mechanical resonance (Terman *et al*., 1952). A representative female response is shown if Figure 3.5. With sweep stimuli, males with erect fibrils had a higher best frequency than males with recumbent fibrils (Table 3.2), confirming preliminary results (Hart *et al*., 2010). The displacement of antennae at best frequency in either fibril state was not significantly different (Table 3.2). Measured from the sweeps, males with erect fibrils had a slightly lower Q than males with collapsed fibrils, but the difference was not statistically significant (Table 3.2). Vibration in response to clicks was more variable than to sweeps and for the click stimulus responses (Figure 3.6), there was no statistically significant difference between the best frequencies or Q values of males with erect or recumbent fibrils (Table 3.2).

The best frequencies of females to the sweep stimulus were not significantly different between the different times of day (Table 3.3). Q measured from sweeps decreased from 3.98 to 3.68 from the active time to the inactive time, but

the difference was not statistically significant (Table 3.3). In response to clicks, Q was smaller, but much more variable (Table 3.3).

3.4.4 Electrophysiology: female and male hearing ranges

The electrophysiological responses peaked at lower frequencies than the mechanical responses, and were significantly different between the sexes (Table 3.4). Based on the measurements, male JOs had significantly larger receptor potentials than female JOs (Table 3.4). The best frequency of male antennae, whether from electrical or mechanical measurements, is within the range of the female wingbeat frequency (individuals with 275 to 343 Hz) significantly lower than the male wingbeat of 523 Hz (individuals with 483 to 556 Hz) (Table 3.4).

When the sweep stimulus was run at 72-82 dB, the hearing range of males determined from the electrical activity greater than background noise was 146 to 390 Hz and covered a significantly wider range of frequencies than the females' range of 120 to 244 Hz (Figure 3.7 & Table 3.4). The males also had an upper hearing limit significantly higher than the female's (Table 3.4). The female hearing range did not include the male wingbeat frequency of 523 Hz or the female's own wingbeat frequency of 306 Hz at this intensity (Table 3.4).

3.5 Discussion

3.5.1 **Antennal differences between female and male *A. togoi***

The antennae of *Aedes togoi* are similar in structure to those of most other swarming species of mosquito (Clements, 1999). Like most other species of culicomorphs their size and shape differs significantly between the sexes (Clements, 1999). Female setae are much shorter, fewer and finer than the male fibrils. The male fibrils are closely appressed to the flagellum in many (perhaps most) species during the day and are extended before they take flight and form swarms. Although the mechanism for this extension is understood (Nijhout & Martin, 1978; Nijhout & Sheffield, 1979; Vogel, 1988; Chapter 2), why the males extend their fibrils remains unknown. One other family has this adaptation, the biting midges (Ceratopogonidae), but they are not in the same clade as the mosquitoes. The underlying mechanism in these midges should be investigated to see if it is similar to that of mosquitoes.

The flagella and proximal flagellomeres of the female are both longer than those of males; female antennae do not need to support the numerous, long fibrils that the male antennae carry. The flagella of male *Aedes togoi* are stiff and probably contain an internal skeleton like that described in males of several other species (Clements, 1999); this might prevent the whorls of long fibrils on adjacent flagellomeres from touching each other. An endoskeleton has not been described in female antennae and their flexibility suggests females lack one altogether. Nevertheless, their flagella vibrate similarly to males in response to sound, so an endoskeleton does not appear required for hearing.

The last antennal feature I measured, the pedicel, is the largest segment in both females and males. The pedicels contain a large number of sensory neurons that in both sexes generate receptor potentials when their flagella are vibrated by sound waves. That the male pedicels are significantly larger than the female

pedicels fits well with the male possessing far more scolopidia than females (Chapter 2); more sense cells need more room in which to be housed.

3.5.2 **Wingbeat differences between female and male *Ae. togoi***

Wingbeat frequency is related to size (Costello, 1974), so the smaller males having a higher wingbeat frequency than the larger females makes physical sense. Moreover, the wingbeat frequencies of male and female mosquitoes that mate in swarms never overlap, and males beat their wings 1.33 to 1.59 times faster than females in the nine species listed by Clements (1999, p. 294). The striking difference between the wingbeat sounds of the sexes makes wingbeat sound a reliable indicator of sex, and is essential for males to detect a female as she flies into a swarm of closely spaced males. However, the wingbeat difference in *Ae. togoi* (1.7:1) is greater than any in the literature, and is greater than what would be expected based on winglengths (Belton & Costello, 1979). Very few of the 3,000 or more species of mosquito have been studied, and the ratio of male to female wingbeat deserves more investigation.

3.5.3 **Antennal hearing by male and female *Ae. togoi***

Laser vibrometry of the male antennae in response to the sweep stimulus supports the hypothesis that the antennae of male *Ae. togoi* would vibrate like those of *An. gambiae* (Hart *et al*., 2010). Pennetier *et al*. (2010) found that when the fibrils were extended in *An. gambiae*, a species that also erects its fibrils before swarming, the antennae were less sensitive but tuned to a higher frequency. The amplitude of the sweep and click responses of *Ae. togoi* were not significantly different between fibril states. Their best frequency was higher when the fibrils were erect than when they were recumbent, statistically different when responding to the sweep stimulus but only showing a trend to the click stimulus. Why the best frequency increases with the fibrils erect is not obvious; viscous drag would tend to reduce the best frequency (Fletcher, 1992). Perhaps the contractile properties of the sense cells in JO (Gibson *et al*., 2010;Chapter 2) somehow increase the best frequency, consistent with the hypothesis that an

active tuning process accounts for the difference in frequency between the mechanical and electrical peaks. In *An. stephensi*, an alpha-adrenergic transmitter substance and protein-rich pads below the annuli are thought to cause extension of the fibrils (Nijhout, 1977; Nijhout & Martin, 1978; Nijhout & Sheffield, 1979; described by Risler, Chapter 2). These chemical changes may also affect the sense cells (scolopidia) in JO, perhaps by affecting the permeability of their membranes.

The lack of significant difference between best frequencies of female antennae at any time of day rules out the possibility that a physiological change occurs in both sexes before they swarm. A similar unchanging response with time of day was seen in *An. gambiae* (Pennetier *et al*., 2010). The vibration of female antennae of *Ae. togoi* in response to a click yielded a best frequency 58 Hz greater than that calculated from the sweep stimulus, and closer to their own wingbeat frequency.

The Q values of the antennae in response to the sweeps (2.1 male, 3.9 female) are similar to those measured by Göpfert *et al*. (1999) from *Aedes aegypti* (male 2.1, female 2.4). A low Q value means the males could detect sounds faster over a relatively wide range of frequencies covering the wingbeat frequencies of a larger selection of females. Because wingbeat frequency is associated with size (Costello, 1974), males that can detect a wider range of frequencies can then hear females of varying sizes and thus have a larger selection of mates from which to choose. Limiting mating options does not seem a productive strategy although other males are evidently not heard or responded to because the male wingbeat falls outside their hearing range.

The lack of significant difference between Q values for female antennae at active and inactive times of day is unsurprising, as is their being more sharply tuned than male antennae. I expected females would be more sharply tuned, because: a) they have no long fibrils that should increase their damping; and, b) have

been suspected in the frog-feeding species, *Uranotaenia lowii* to be tuned to specific host frequencies (Borkent & Belton, 2006; Bartlett-Healy *et al*., 2008).

In *Ae. togoi* and the other species so far examined, the displacement of the flagellum and Q of males and females are similar (Clements, 1999, Pennetier *et al*., 2010). These similarities make it unlikely that the physical characteristics of the flagellum and its many fibrils play a major role in the sensitivity and tuning of the antenna of the male.

3.5.4 **Electrophysiology of antennae of male and female *Ae. togoi***

The receptor potentials recorded extracellularly from JO are at twice the frequency of the stimulus, as expected from the flagellum moving back and forth (triggering two responses) with each cycle of the sound. That the peak electrophysiological responses were at lower frequencies (eg. males with erect fibrils, 363 Hz) than the mechanical responses (eg. males with erect fibrils, 385 Hz) is in agreement with similar studies on males of other species (Clements, 1999, Gibson *et al*., 2010; Chapter 2). *Anopheles gambiae* males with erect fibrils had a mechanical best frequency of 540 Hz, and an electrical best frequency of 300 Hz, yet they are known to detect female wingbeat at 467 Hz (Pennetier *et al*., 2010). The significance of this frequency difference remains unclear because the sensory cilia would be stretched most at the mechanical best frequency and presumably generate the largest potentials. This may indicate the ability of the JO to tune the antenna using motile cilia that are thought to be present in the JO's of all species (Chapter 2). It is possible that the ionic channels in the cilia of the scolopidia have a limited rate of activity but this seems very unlikely because males of small species must be able to hear female with wingbeat frequencies near 1 kHz. There are no muscles in the pedicel that could affect the vibration of the flagellum.

The finding that males have a wider hearing range and have more electrically sensitive antennae than females may indicate that males rely more strongly on

hearing than females. The best frequency of males is near the wingbeat frequency of females, furthering support for the hypothesis that male hearing is for detecting potential mates in a swarm. Based on the electrophysiology, it seems very unlikely that non-flying females can hear the wingbeats of males using their antennae because 523 Hz is well outside the upper range of sensitivity of the female JO.

The finding that male and female mosquitoes react to the flight sounds of each other in tethered flight has led to several different hypotheses for how this is possible considering that male flight sound is above the hearing range of females. Arthur *et al*. (2010) suggest that "sustained responses," steady negative potentials recorded when the flagella are vibrating, can somehow inform the female of the male wingbeat frequency. It is not obvious how these steady potentials can be transmitted down the antennal nerves to the brain, and even if they were, I could find no evidence that scolopidia in J.O. are tuned to different frequencies.

Warren *et al*. (2009) and Pennetier *et al*. (2010) have shown that the female JO. can detect difference tones when their antennae are exposed simultaneously to the sound of their own wingbeat and that of a male. It may be significant that the difference in wingbeat frequency between male and female *Ae. togoi* (523-306=217 Hz) is close to the best frequency of the electrical response of females (227 Hz). Recordings from the J.O.'s of flying insects would resolve this question.

I have seen female *Ae. togoi* kicking with their hind legs when another female flies near them, and females of several species do this in response to sounds. Behavioural experiments testing this response could provide additional evidence of their sensitivity to different frequencies.

There is now solid evidence that females of several species that feed on amphibians are attracted to the sound of their hosts and they may use their antennae to hear and locate these hosts (Toma *et al*., 2005; Borkent & Belton, 2006; Bartlett-Healy *et al*., 2008). Visual and chemical attractants can be ruled out because the sounds were recordings. Perhaps females of all species of the frog-biting midge family (Corethrellidae), in the same clade as mosquitoes but believed to have evolved with the amphibia (Borkent, 2008), hear the calls of their amphibian hosts with their antennae. *Aedes togoi* bite humans in the field and lab (Purnomo *et al*., 1976; Belton & Belton,1990), feed on some reptiles and amphibians (Miyagi, 1972) and mammals in the lab (Bosworth & Ewert, 1971; Kan & Ho, 1973; Purnomo *et al*., 1976; Riyong *et al*., 2000) and bite deer and cattle in nature (Yen *et al*., 1982; anonymous, 2008). However, *Ae. togoi's* hosts have not been fully elucidated. Because *Ae. togoi* inhabits the coasts from tropical Malaysia to Siberia and from Washington to British Columbia including uninhabited islands (anonymous, 2008) bloodmeal sources in these areas might include seals, sea lions, and seagulls, just to name a few. Steller sea lion, *Eumetopias jubatus,* vocalizations fall in the range of female hearing *(*Campbell *et al.,* 2002*),* as do those of humans *(*Keating & Buhr, 1978*),* northern elephant seals, *Mirounga angustirostris,* (Insley, 1992), and seagulls, *Larus glaucescens*, (Stout *et al*., 1969). Consequently, it is possible that females can hear their hosts; however, there is no evidence that female *Ae. togoi* are attracted to sound and studies are needed to test this idea.

3.5.5 **Evaluating the click stimulus**

As far as I know click stimuli have not been used before to determine the best frequency or Q values from insect antennae. Because the sweep presents sound over a longer period of time than a click, the JO might adjust its sensitivity to the frequencies it hears during the sweep; whereas a sudden burst of sound might vibrate the antenna closer to its natural frequency. Spontaneous oscillations at the best frequency have been observed from the antennae of several species (Warren *et al*., 2009) and it seems likely that the high Q (5.4)

measured from eight males with erect fibrils in response to the click may be due to oscillation.

Although clicks are commonly used to investigate resonance in other acoustical and physical systems, more research into the response of other well studied mosquito species to click stimuli is needed.

3.6 Conclusion

In *Aedes togoi* the measured mean wingbeat frequencies were 523 Hz in males and 306 Hz in females, the latter close to the best frequency of male JO's. These results are in agreement with studies of female wingbeats and male hearing in other mosquito species (Clements, 1999). Whereas the males can hear female wingbeats, the significance of hearing in the biology of females remains unclear. Because both sexes of *Ae. togoi* were able to detect sound, and males could detect sound regardless of their fibril state, the verticillate antennae are not a requisite for sound detection. Moreover, because there was no significant difference in the tuning (Q) of erect or recumbent fibrils in response to sweeps, verticillate antennae do not appreciably narrow or increase the range of frequencies detected. However, verticillate antennae evidently give male flies that are attracted from swarms by the sound of a female some selective advantage because they have evolved in at least five different families, and in the Ceratopogonid biting midges, not closely related to mosquitoes, the tiny males of most species erect their long antennal fibrils before they swarm and mate.

Perhaps the bushy antennae serve a visual function instead, allowing males to discriminate females that are approaching the swarm from males already in it or for males to avoid each other when swarming. A recent study has shown that the visually acute jumping spider *Evarcha culicivora* preferentially preys upon female *Anopheles* mosquitoes, discriminating them from males based on their antennal morphology (Nelson & Jackson, 2012). If predators can distinguish

male from female mosquitoes based on antennal morphology, it is possible that the mosquitoes too can discriminate the sexes this way. Granted, the eyes of crepuscular and night flying mosquitoes studied do not produce as detailed an image as the day-flying *Toxorhynchites brevipalpis* (Land *et al.*, 1999); however, I believe that even with low resolution, the mosquitoes would still be able to discern the verticillate antennae of males from the comparatively bald antennae of females. A puzzling change that occurs before swarming may also support this idea. In both *An. stephensi* (Nijhout, 1977), *Ae. vexans* (Kuhn, personal communication) and *Ae. togoi* (Figure 3.1) the last segment of the long male palps turns outwards when the fibrils extend on the antennae. It seems likely that this could be a visual signal.

This study is the first examination of changes in the sensitivity of the antennae in an *Aedes* species known to expand its fibrils. Moreover, it shows that the antennae of males with recumbent fibrils vibrate in response to sounds much like those with them erect. Female antennae are tuned much like males but their antennae do not have long fibrils nor do they change their best frequency before mating.

3.7 Figures and Tables

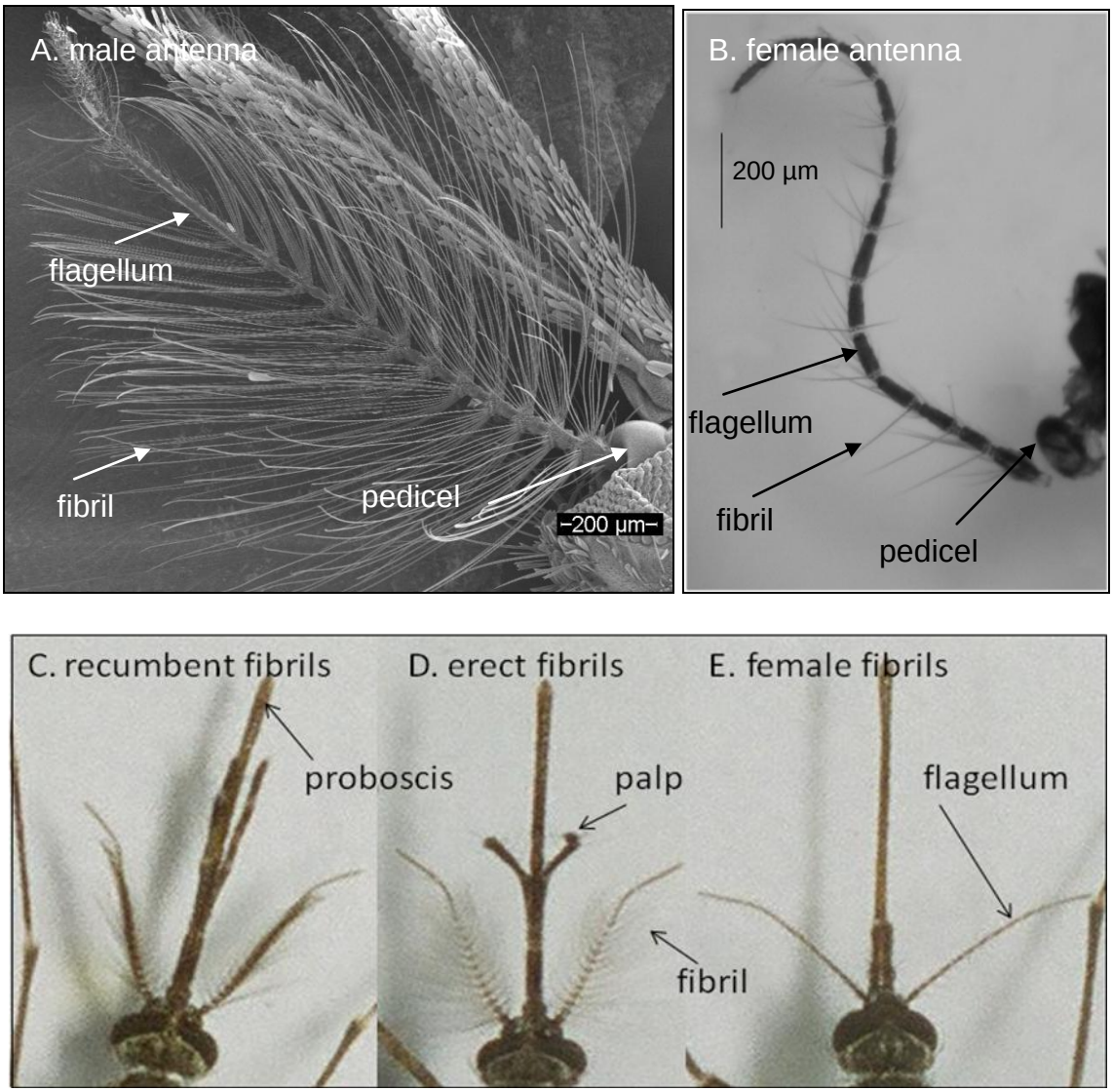


Figure 3.3. Images of female and male *Aedes togoi* antennae. A) SEM image of a male antenna with fibrils extended; photographs of B) a female antenna; C) a male with recumbent/collapsed fibrils; D) a male with erect fibrils; and E) a female with tiny fibrils.

Table 3.1. Comparison of female and male *Aedes togoi* antennal and wingbeat measurements.

Part measured	Female (mean)	Female (SD)	n	Male (mean)	Male (SD)	n	t statistic	*p*-value
Flagellum (length mm)	1.54	0.07	10	1.40	0.11	13	-3.85	<0.01
Flagellomere (length µm)	120	2	6	70	1	7	-6.18	<0.01
Fibril (on 1st flagellomere) (length µm)	220	47	10	630	97	13	12.06	<0.01
Pedicel (diameter µm)	140	2	6	180	3	7	3.01	0.01
Wingbeat frequency (Hz)	306	23	6	523	25	6	15.49	<0.01
Wingbeat sound pressure level (dB)	71	5.8	6	63	5.0	7	-2.62	0.03

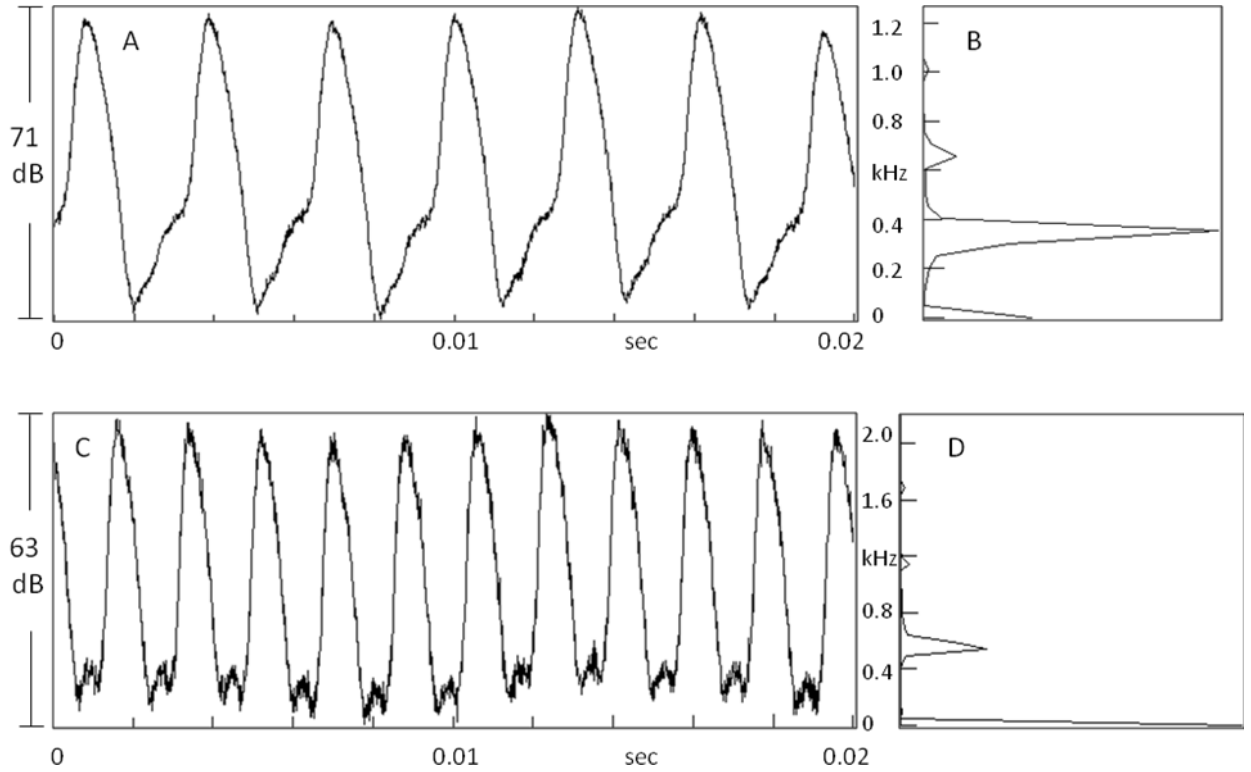


Figure 3.4. ***Aedes togoi*** **wingbeat. A) Waveform of a female at 306 Hz; B) frequency spectrum of the waveform in A; C) waveform of a male at 568 Hz; and, D) frequency spectrum of the waveform in C.**

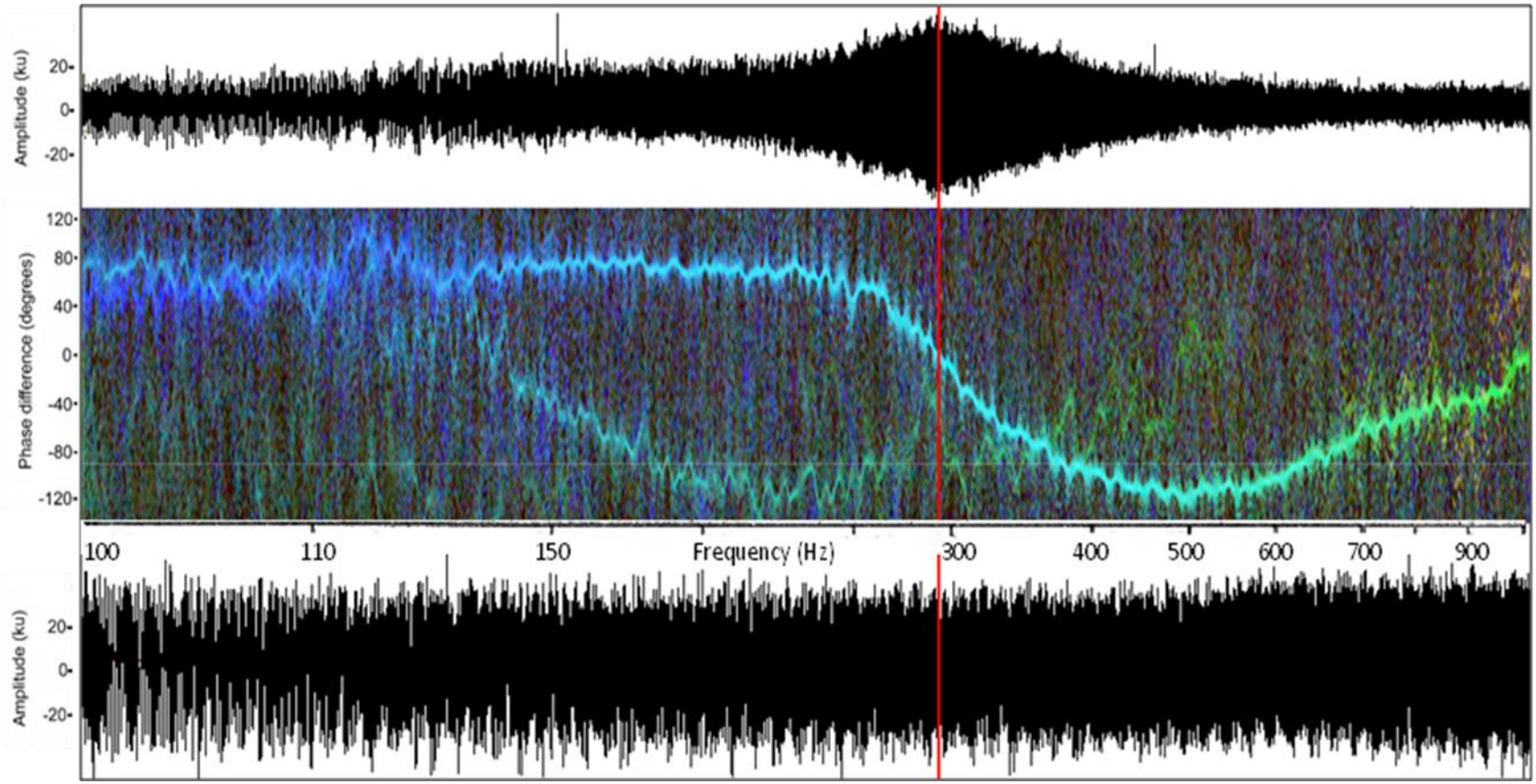


Figure 3.5. Representative vibration of an *Aedes togoi* female antenna in response to a logarithmic frequency sweep (bottom trace). Upper trace shows the best frequency at ~290 Hz and the middle trace shows a typical phase change, steepest at the best frequency of the antenna. (ku = A thousand digital samples)

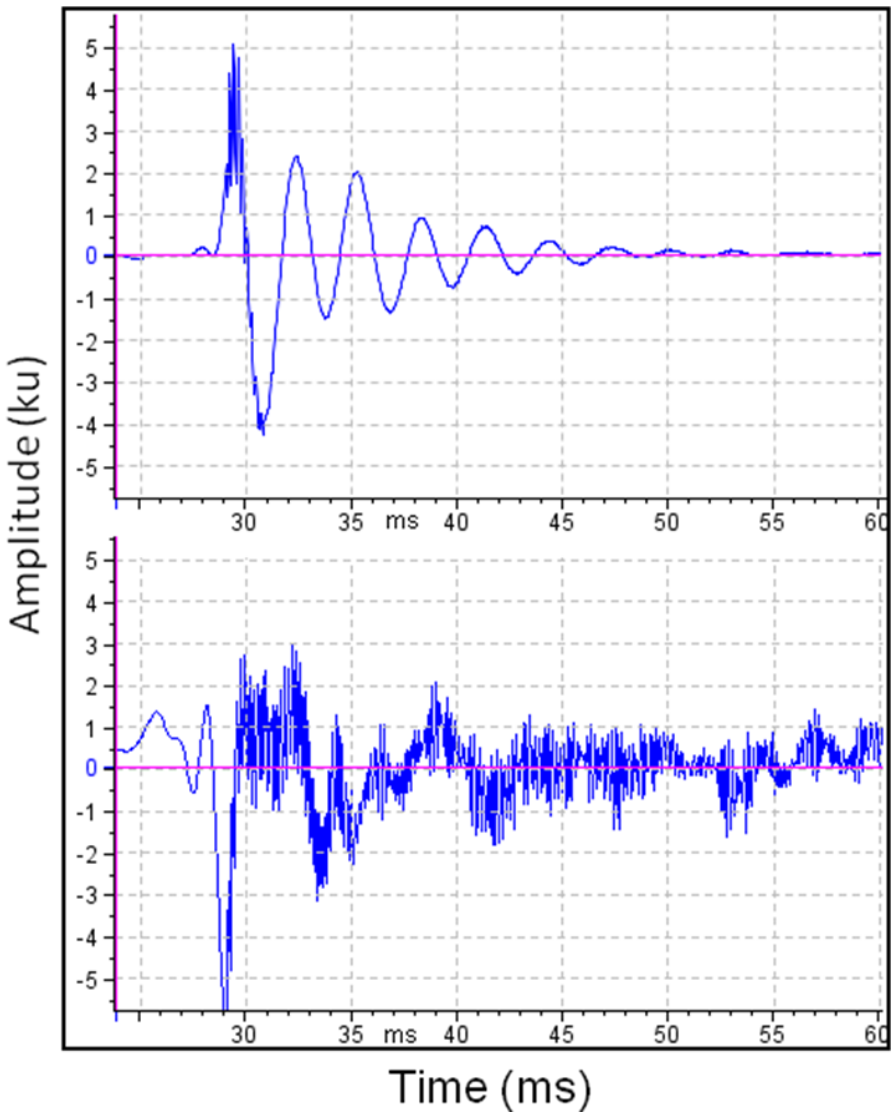


Figure 3.6. A representative male *Aedes togoi* antennal vibration (erect fibrils) (top) in response to a click stimulus (bottom). The displacement of the first negative peak of the upper trace is ~550 nm.

Table 3.2. Measurements of male antennae, in response to sounds, from laser vibrometry.

Stimulus	Parameter	Active Time Erect Fibrils			Inactive Time Recumbent Fibrils			t statistic	*p*-value
		Mean	SD	n	Mean	SD	n		
Sweep	Best frequency (Hz)	385	81	9	309	47	12	2.50	0.03
	Q	2.10	0.74	10	2.66	0.54	10	-1.90	0.07
	Displacement at best frequency (nm)	34.65	8.5	9	52.89	29.5	12	2.03	0.06
Click	Best frequency (Hz)	363	86	8	320	55	12	1.24	0.24
	Q	5.14	2.97	8	3.83	2.48	12	1.03	0.32
	Displacement at the first trough of the decaying wave (nm)	829	572	8	486	360	12	-1.51	0.16

Table 3.3. Measurements of female antennae in response to sound stimuli, from laser vibrometry.

Stimulus	Parameter	Active Time			Inactive Time			t statistic	*p*-value
		Mean	SD	n	Mean	SD	n		
Sweep	Best frequency (Hz)	252	48	7	257	53	8	-0.18	0.86
	Q	3.98	2.00	4	3.68	0.63	6	-0.29	0.79
	Displacement at best frequency (nm)	44.3	20.0	7	78.3	40.5	8	2.09	0.06
Click	Best frequency (Hz)	310	47	4	313	54	6	0.02	0.98
	Q	2.67	2.13	4	3.53	1.13	6	-0.75	0.49
	Displacement at the first trough of the decaying wave (nm)	547	396	7	636	665	7	0.31	0.77

Table 3.4. Electrical activity of female and male *Aedes togoi* Johnston's organs with a sweep stimulus of 100-1000 Hz increasing with frequency from 72 to 82 dB at the antenna.

Parameter	Male			Female			t statistic	*p*-value
	Mean	SD	n	Mean	SD	n		
Lowest detectable frequency (Hz)	146	17.5	10	121	10.7	10	3.88	<0.01
Best frequency (Hz)	302	46	10	227	67	10	2.92	0.01
Highest detectable frequency (Hz)	390	31.5	10	244	31.6	10	10.32	<0.01
Maximum response at best frequency (mV)	2.69	0.085	10	1.42	0.067	10	3.73	<0.01

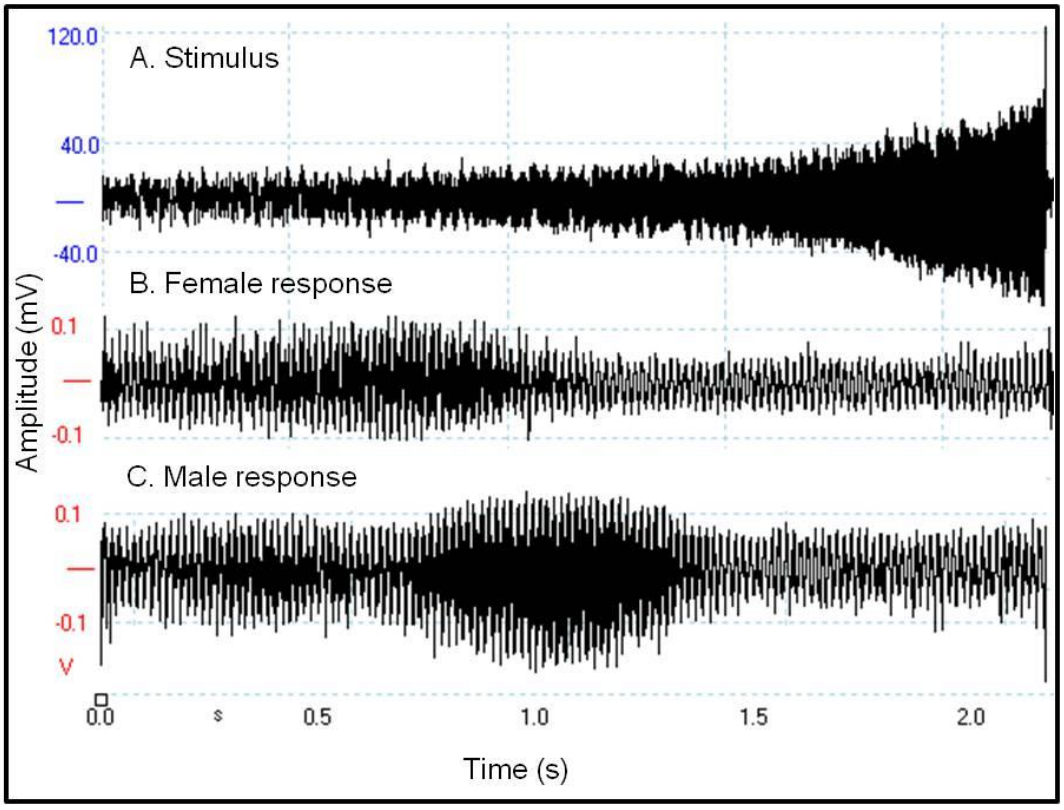


Figure 3.7. Typical electrical activity recorded from JO of female and male *Aedes togoi* to a sine wave swept from 100 Hz-1 kHz in 2.2 s with an pre-amplifier gain of 100. A) Stimulus sine wave; B) typical female; C) typical male.

LINKING STATEMENT

Mosquito hearing and use of sound for mate location has been studied extensively, except in species where males extend and collapse their fibrils. I examined resonance of *Aedes togoi* antennae, a coastal species of mosquito that can transmit *Brugia malayi, Dirofilaria immitis, Wuchereria bancrofti,* and Japanese encephalitis, and may vector West Nile Virus. I developed and tested techniques to record the sound it produces, the range of frequencies it can detect with its antennae.

In the next chapter, I focus on a tympanate pyralid moth, a member of another relatively well-studied group of hearing insects. I examined the sounds produced and detected by *Plodia interpunctella* (the Indianmeal moth). Although the Indianmeal moth is known to have a tympanum suited to detecting ultrasound, I tested the hypothesis that it can also detect wingbeats and that it does this with another sound receptor, the antenna, as mosquitoes do. My results show that the tympanum detects ultrasound, but not the wingbeat sound, and that the antenna can detect frequencies generated by the wingbeat, but not ultrasound.

4 THE USE OF SOUND BY INDIANMEAL MOTHS

4.1 Abstract

Research into sound production and detection by moths has focussed on species with tympana and the ultrasound they produce and detect. How the Indianmeal moth *Plodia interpunctella* (IMM) responds to the ultrasound of bat echolocation and conspecifics occupies the majority of the literature on the acoustics of this species. However, both sexes fly and flutter their wings before mating, generating low frequency sound in addition to ultrasound. Little has been done to determine if and how IMM communicates using wingbeats; thus, the question of how they produce and detect these sounds remains unanswered. I examined the morphology of the antennae, recorded the moth sounds, ran behavioural experiments, and used laser vibrometry and electrophysiology to examine if IMM can hear wingbeat sounds. My results show that females and males had similar antennal morphology; the only statistical difference being the length of the flagellum (male: 4.17 mm, female: 4.56 mm). Furthermore, females and males had similar wingbeat frequencies (50 and 51 Hz, respectively) and, unlike other pyralids, confirming previous research on IMM, females, as well as males, generated broadband ultrasonic clicks ranging from 20-80 kHz with each wingbeat. Behavioural results showed that acoustic traps overall caught more moths than silent traps, but that there was no significant difference between acoustic recordings tested. Laser vibrometry showed that the tympana detect ultrasound (with peak sensitivities at 70 kHz and 90 kHz in both sexes), but not sound, and that the antennae detect sound (males resonantly tuned at 157 Hz, females at 149 Hz), but not ultrasound. The antennae of moths vibrated in response to recorded wingbeat sounds much like those of mosquitoes. Thus, the moths have two possible hearing organs with different tuning, one that can detect ultrasound from predators and clicks of conspecifics, the other sensitive to wingbeat frequencies.

4.2 Introduction

4.2.1 Pyralid sound production and detection

Pyralid moths have tympanal ears for detecting ultrasound (frequencies > 20 kHz). They are one of over 16 (Table 4.1) of the total ~120 moth families described with tympana (Maddison & Schulz, 2007). These paired structures are on the underside of the second abdominal segment (the first segment visible) and contain a scolopidium with four sensory cells attached to the centre of the thin tympanal membrane (Pérez & Zhantiev, 1976; Skals & Surlykke, 2000). They are believed to have evolved to detect the ultrasonic echolocation calls of hunting bats (Skals & Surlykke, 2000; Miller & Surlykke, 2001; Lafaille *et al.*, 2010).

That moths can detect bats is not a new idea, it was put forth in 1877, before people even knew that bats use echolocation (White, 1877). By 1955, Treat published the first behavioural responses of moths to bats, which Belton (1962) followed with electrophysiological recordings from pyralid tympana in response to ultrasound. From these initial papers, research into bat echolocation and moth responses exploded. In pyralids, studies have examined how bat echolocation affects mating decisions (Svensson *et al.*, 2003; Svensson *et al.,* 2004), and pheromone signalling (calling) behaviour (Huang & Subramanyam, 2004; Svensson *et al*., 2003). Despite these defenses, moths with tympana have not thwarted bats in their foraging efforts; studies have shown that those using echolocation signals outside the hearing range of tympanate moths successfully catch both them and atympanate moths (Dunning & Kruger, 1996).

The transition from bat detection to communicating with mates in pyralids would be possible if the moths produced ultrasound. The mechanisms by which they do this have been described for a few species (Table 4.2) and communication itself has only been examined in detail for the lesser, *Achroia grisella* (LWM), and greater, *Galleria mellonella* (GWM), wax moths. In LWM, the males wingfan, generating pulses of ultrasound and emit pheromone that attracts the female and mating ensues (Greenfield & Coffelt, 1983). In GWM, the male wingfanning (wingbeat and ultrasound) triggers females to wingfan, but females will not respond if the

frequency the male produces is too rapid, because they are then similar to the sounds produced by honeybees (Spangler, 1985 & 1987). That more research on communication with ultrasound in pyralids has not been done is disappointing; however, these two examples show that the transition from predator detection to communication between sexes has occurred.

The idea that tympana evolved first to detect bat echolocation then to detect ultrasound produced by conspecifics is established (Conner & Corcoran, 2012). However, this transition fails to recognize that flying and wingfanning pyralids generate low frequencies (Sotavalta, 1963) that might be used in communication. Only two pyralids, LWM and GWM, are so far known to use wingbeats in communication (Greenfield & Coffelt, 1983; Spangler, 1985; 1987). In GWM a proposed manner of detection has been put forward. Spangler (1985) suggested that males hear only when they have their wings extended, coupling their wings maximally with air to stimulate subgenual organs, highly sensitive proprioceptors in the tibia that are known to mediate hearing in crickets and termites (Hoy & Yack, 2008). Spangler's idea has not been tested, and I found no other studies that have examined the use of sound in communication by pyralids.

I therefore investigated sound and ultrasound production by male and female IMM, their response to the sounds, and the acoustic role of the tympana and antennae. Specifically, I investigated: a) the morphology of the antennae, tympana and structures that might produce ultrasound with light and scanning electron microscopy; b) whether sexes produce different sounds by recording moths in flight; c) how the sounds are produced by ablating potential ultrasound generators; d) if the moths exhibit a behavioural response to conspecific sounds and choose the complete signal over its components; e) if the tympana respond mechanically to ultrasound and sound by using laser vibrometry; f) if the antennae respond mechanically to sound and ultrasound and whether they have a mechanical resonance (best frequency response) with laser vibrometry; and g) if the vibrating antennae stimulate the sensory neurons in the Johnston's organ through studying the effect of sound on the antennae with electrophysiology.

4.3 Natural history

IMM uses pheromone and acoustic signals in its mating system. Females are most active during the scotophase (Silhacek *et al*., 2003; Huang & Subramanyam, 2004; Cowan & Gries, 2009) and begin calling (releasing pheromone) upon reaching a suitable resting area (Silhacek *et al*., 2003). Male moths infesting a warehouse wingfanned when many females were calling (Mankin *et al.*, 1999), but flew to them only when a few were doing so (Silhacek *et al*., 2003). During flight and wingfanning, the males emit sound and ultrasonic clicks (Trematerra & Pavan, 1995). Once they have located a female, males wingfan (Mbata & Osuji, 1983; Silhacek *et al*., 2003; personal observation). Courtship then occurs in one of two ways. If approaching the female from the front, males contact female antennae with their own, and if receptive, females allow the male to move forward so that her antennae can be close to the male scent glands at the base of his forewings (Grant, 1974; Grant & Brady, 1975). The female then raises her abdomen so that it is between the pair and the male starts swinging his abdomen dorsolaterally so he can grasp the female genitalia (Grant, 1974; Grant & Brady, 1975). If approaching the female from behind, the male headbutts her abdomen, to which females respond by turning to face the male and courtship follows as described for the front approach (Grant, 1974; Grant & Brady, 1975; Brady *et al*., 1975).

Because of its pest status, IMM has been studied extensively with the goal of managing it. Part of the research on IMM has focused on the moth's tympana and how sound might be used to disrupt its search for a mate (Svensson *et al.*, 2003; Svensson *et al.,* 2004), its calling behaviour (Huang & Subramanyam, 2004; Svensson *et al*., 2003), mating (Svensson *et al*., 2003; Huang & Subramanyam, 2003), oviposition (Huang & Subramanyam, 2004, Sambaraju & Phillips 2008; Huang *et al.*, 2003; Svensson *et al.,* 2003), and fertility (Huang & Subramanyam, 2004; Huang & Subramanyam, 2003). Although the tympana have been thought to detect conspecific ultrasound (Trematerra, 1997; Trematerra & Pavan, 1995), the authors admit that more research is needed to clarify the role of ultrasound in the mating system. Many authors have commented on the wingfanning behaviour that

males exhibit when exposed to female pheromone (Lum, 1974; Trematerra & Pavan, 1995; Silhacek *et al*., 2003). The detection of sound and the effects of sound on IMM is poorly understood. Infrasound has been found to accelerate larval development (Mullen, 1973), whereas four days of exposure to sound from 120 Hz to 2 kHz at amplified levels (dB levels unspecified) led to 75% fewer larvae surviving (Kirkpatrick & Harein, 1965). These two studies and the measurement of male wingbeat sound by Mankin *et al*. (1999) encompass the extent of research into the role and effect of infrasound and sound on IMM.

Table 4.1. Moth families possessing tympana. *abdominal base refers to the first visible abdominal segment

Moth family	Common name	Location	Reference
Arctiidae	tiger moths	Metathorax	Scoble, 1992; Minet & Surlykke, 2003 include them in their statement that all Noctuoid families have tympana
Axiidae (now Cimellidae: Yen & Minet, 2007)	gold moths	7^{th} abdominal segment	Minet & Surlykke, 2003 do not consider it a "typical" tympanum, but do describe it as a hearing organ
Crambidae	grass moths	abdominal base*	Minet & Surlykke, 2003
Doidae	--	Metathorax	Scoble, 1992; Minet & Surlykke, 2003 include them in their statement that all Noctuoid families have tympana
Drepanidae	hooktip moths	Abdomen	Scoble, 1992; not considered a tympanum by Minet & Surlykke,2003
Dudgeonidae		abdominal base	Minet & Surlykke, 2003
Geometridae	winter moths	abdominal base	Minet & Surlykke, 2003
Hedylidae	--	under forewing base	Minet & Surlykke, 2003
Lymantriidae	tussock moths	Metathorax	Scoble, 1992; Minet & Surlykke, 2003 include them in their statement that all Noctuoid families have tympana
Noctuidae	owlet moths	Metathorax	Scoble, 1992; Minet & Surlykke, 2003 include them in their statement that all Noctuoid families have tympana
Notodontidae	Oakworms	Metathorax	Scoble, 1992; Minet & Surlykke, 2003 include them in their statement that all Noctuoid families have tympana
Oenosandridae	--	Metathorax	Minet & Surlykke, 2003
Pyralidae	snout moths	abdominal base	Scoble, 1992; Minet & Surlykke, 2003
Thyrididae	picture-winged leaf moths	under forewing base	Minet & Surlykke, 2003
Tineidae†	fungus/tineid moths	abdominal base	Minet & Surlykke, 2003 † No nerve cells identified
Uraniidae	swallowtail moths	abdominal base	Minet & Surlykke, 2003

Table 4.2. Pyralid species that produce ultrasound

Species name	Common name	Sex	Proposed Mechanism	Reference
Achroia grisella	Lesser wax moth	Male	Tegular tymbal	Spangler & Takessian,1983; Spangler *et al*.,1984
Chrysauginae species		Male	Frenulum scraped on retinaculum	Hannemann in Conner, 1999
Corcyra cephalonica	Rice moth	Male	Tegular tymbal	Spangler, 1987
Eldana saccharina	African sugar-cane borer moth	Male	Tegular tymbal	Zagatti, 1981; Bennett *et al*., 1991
Ephestia cautella	Almond moth	Male	Tegular tymbal	Pérez & Zhantiev,1976
Ephestia kuehniella	Mediterranean Flour Moth	Male	Tegular tymbal	Trematerra & Pavan,1995
Galleria mellonella	Greater wax moth	Male	Tegular tymbal	Spangler, 1985; Bennett, 1989
Aphomia sociella	Bee moth	Male	Tegular tymbal	Kindl *et al*., 2011
Plodia interpunctella	Indianmeal moth	Both	Tegular tymbal	Trematerra & Pavan,1995; Trematerra,1997
Symmoracma minoralis	--	Male	Genital stridulation	Heller & Achmann,1995; Heller & Krahe,1994
Syntonarcha iriastis	--	Male	Genital stridulation	Gwynne & Edwards,1986

4.4 Materials and methods

4.4.1 Insects

IMM for this study were from a colony maintained at Simon Fraser University since 2005. Larvae were reared according to Cowan and Gries (2009), modified to a photoperiod of L16:D8. Virgin insects were used in all experiments.

4.4.2 Morphology

I used a scanning electron microscope (SEM) to examine the tympanum and two of three potential ultrasound producing sites (tegulae, and tymbal-like protrusions on the thorax; wings were not imaged). Due to the cost of imaging, I obtained a limited number of images (from 1-4) per structure. I used dissecting and compound microscopes to show the external anatomy and fluorescent and non-fluorescent stains to show neurons within the antenna.

For scanning electron microscopy, I followed the techniques described in Chapter 3. Sites to examine were located on the stub, focussed, and images of each captured and saved to the hard drive of a computer.

For light microscopy, I used a Nikon digital fluorescence imaging system comprising a Nikon TE-2000 microscope stand with an Orca-ER Camera (Hamamatsu), Lambda-LS xenon light source (Sutter), excitation and emission filter wheels with a Lamba-10-3 controller (Sutter). Digital image acquisition and control of the microscope used SimplePCI software (Hamamatsu Corporation). Phase/contrast and fluorescence images used 20X or 40X extra-long-working-distance objectives (Nikon). Freshly killed or preserved (Bouin's fixative, Sigma-Aldrich, Canada) whole moths of each sex and individual antennae of each sex were stained with methylene blue and Neurotrace (Molecular Probes, Eugene, Oregon, USA). In samples labelled with Neurotrace, fluorescence images used a

490 nm excitation filter, a 536 nm emission filter and a “C-Y-R” dichroic mirror (Chroma Technology).

I measured the antennae with a Nikon SMZ645 microscope. A stage micrometer (Meiji Techno, Japan) was used to calibrate an ocular micrometer, and antennae (n=6 for each sex) were photographed for analysis with ImageJ (Research Services Branch, National Institute of Mental Health, Bethesda, Maryland, USA). I measured the length and width of the scape, length and width of the pedicel, length of the flagellum, and length and width of the most distal flagellomere. As an indication of their size, I measured the length of the mesothoracic femur from males and females (n=6 for each sex) with the same set-up.

4.4.3 **Sound and ultrasound produced by IMM**

I recorded sound and ultrasound from moths (3 or more days old). I tethered them with human hair behind their head and they flew ~ 1.5 cm in front of sonic and ultrasonic range microphones. For sound I used an AKG condenser microphone (AKG Acoustics Nashville Tennessee, USA), signal amplification x 800 with an SC 2040 differential amplifier (National Instruments (NI) Corporation, Austin, TX, USA), with recordings saved to computer with an NI data acquisition board (DAQcard-6062E; 12 bit, 500 kHz maximum sampling rate). Knowles SPM0102ND3 and SPM0404UD5 ultrasonic microphones (specified frequency response: 1-60 kHz $\pm$ 5 dB – my measurement: 10 Hz- 100 kHz (Appendix A) $\pm$ 12 dB; Knowles electronics, Itasca IL, USA) were the others used. Concurrent video confirmed that tethering did not impede flight or cause unwanted sounds from wings hitting the tether. Wingbeat and click frequencies were analyzed using a spectrum analysis program developed in LabVIEW 7.1 (from NI).

Three potential ultrasound-producing structures were altered to determine if they were the source of the ultrasonic clicks. Because both males and females produce identical clicks (Trematerra & Pavan, 1995), only males were used. The body parts were a pair of tymbal-like structures on the dorsal thorax (Figure 2A),

the wings, and tegulae (Figure 2B). I covered the tymbal-like structures with Weldbond glue (n=2), removed both tegulae (n=5) with forceps, and cut the wings to half their length (n=4). Recordings of moths were made before and after the alteration and the interval between clicks was measured in both recordings and the interclick frequency, to compare with the wingbeat frequency, calculated from this.

4.4.4 **Behavioural experiments**

To determine if recorded sound, ultrasound or both would increase the attractiveness of light-traps to females, I ran a two-choice experiment, testing the male ultrasound (click), sound (wingbeat), or sound and ultrasound (wingbeat + click) against a silent control. Two delta traps (PheroTech Inc. Delta, BC, CAN), each containing a blue-violet LED (405 nm wavelength, Roithner lasertechnik, Vienna, Austria), known to attract female IMMs (Cowan & Gries, 2009) were placed in front of speakers at opposite corners of a Plexiglas cage (70x119x50 cm high). One Panasonic WM-R57A speaker (frequency response 50 Hz to 100 kHz $\pm$ 12 dB) played the wingbeat (50 Hz to 3 kHz), click (10 to 100 kHz) or wingbeat + click (20 Hz to 100 kHz), while the other was silent; treatments were randomly assigned. Stimuli were set at a sound pressure level of 65 dB (a biologically relevant dB level) 1 cm from the speaker. For each of 10 replicates per stimulus pair, 10 females were released in the middle of the cage and trap captures recorded after 4 hours, extending the response time of 2 hours established by Cowan and Gries (2009).

4.4.5 **Tympanal vibration**

I used a 2-channel Polytec OFV-2500 Doppler laser vibrometer (Polytec Inc., Irvine, CA, USA). The output of the laser, focused on the attachment point of the scolopidia to the tympanal membrane, went to one channel, and the output of an AKG microphone for sound recordings, or of a Brüel and Kjær 2204 sound level meter with a 4133 ½" microphone (Brüel & Kjær, Naerum, DK) for ultrasound recordings, adjacent to the moth, went to the other. Stimuli were: a) pure tones

at intervals from 40-1000 Hz at 60 dB to cover the range of the fundamental frequency and harmonics of the wingbeat; and, b) pure tones every 10 kHz from 20-100 kHz, 80 dB at the preparation to cover the possible range of ultrasounds from bats or moths. Because the Polytec software did not store ultrasound, the vibrometer output was digitized and stored using a 2-channel digital Picoscope 2203 oscilloscope (Pico Technology, Cambridgeshire, UK) with a sampling rate >200 kHz to avoid aliasing. To determine the frequency response of the tympana, I compared the phase and amplitude of the stimulus to those of the tympanum. A tympanal vibration was considered to be vibrating in response to the stimulus when the sine waves of each were at the same frequency, in phase with each other, and at a measureable level (> 0.2 mV). I took three measurements at each frequency tested for six females and six males and used the means in analyses.

4.4.6 **Antennal vibration**

Laser vibrometry used the set-up described above. The laser was focussed on the base of the flagellum. The stimuli were: a) sine waves swept from 30-1000 Hz to cover the range of the fundamental frequency and harmonics of the wingbeat; and, b) although preliminary tests showed no antennal vibration in response to ultrasounds, one male and one female antenna were tested with pure tones every 10 kHz from 20-100 kHz to cover the possible range of ultrasounds from bats or moths; or, c) recordings of the sound of the male wingbeat played from the computer to examine the response of female antennae. To see if the antennae had a simple mechanical resonance like those of mosquitoes (Göpfert *et al*., 1999), responses to sweeps were analyzed using the 'Spectral Phase' view of Adobe Audition 3.0 (Adobe, San Jose, CA, USA). The time of the steepest phase change between antennal response and the sound was noted, and corresponding frequency determined using Raven Pro 1.4 (Cornell Lab of Ornithology, Ithaca, NY, USA). The mean from the three recordings from each insect was calculated, and those means used to calculate the overall mean for males and females (n= 6 for each sex). From the sweep

responses, I also calculated the quality factor, Q, of the antennae (how sharply tuned they are) using the method described in Chapter 3. I determined the velocity of the antenna at the best frequency from the laser vibrometer output and then calculated the displacement of the antenna at the best frequency from the velocity reading using the equation described previously (Chapter 3: Equation 3.1). I also analyzed responses of the antennae to male wingbeat sound for matching between stimulus and response. I considered a response to occur when the stimulus sine wave frequency matched the response sine wave frequency of the antenna, and scored them as categorical data: yes, no responses (n=6 for each sex).

4.4.7 **Sound playback**

Playback of sound recordings was through Sennheiser headphones (Old Lyme, CT, USA: matched $\pm$ 1 dB, flat frequency response: 10 to 39,500 Hz, 0.05% THD) placed approximately 10 cm from the antenna. I generated ultrasound with a Wavetek 145 20 MHz pulse/function generator (San Diego, CA, USA). amplified by a RAE 1503 12 W amplifier (level frequency response DC-100kHz; RAE Industrial Electronics, Vancouver, BC) and played it through an electrostatic speaker with 200 V polarization voltage (Kuhl *et al*., 1954). A Brüel and Kjær 2204 sound level meter with a 4133 ½" condenser microphone was used as a reference.

4.4.8 **Sound pressure level measurements**

For playback of recordings, I used the Brüel and Kjær 2204 sound level meter with the 4133 ½ " microphone to detect and set ultrasound levels, and a Realistic sound level meter (SLM) 33-2050 (Radio Shack, Fort Worth, TX, USA) to detect and set frequencies below 20 kHz. When analyzing recordings, I determined the sound pressure level (SPL) from the peak to peak amplitude of the microphone output in mV, calculating its root mean square and reading SPL in dB from a nomogram (Appendix B). The calculations from microphone amplitudes matched the SPL from the Brüel and Kjær SLM.

4.4.9 **Electrophysiology of antennae**

Electrophysiological recordings were done in an open teaching laboratory inside a Faraday cage with a P18 pre-amplifier (Grass Technologies, West Warwick, RI, USA). One electrolytically sharpened tungsten electrode was inserted into the scape, the second into the eye. Signals were viewed with the Picoscope 2203 oscilloscope, sampling rate 3.268 kHz, and saved to the hard disk of a computer. Stimuli were sine waves, swept from 100-1000 Hz to cover the range of the wingbeat harmonics, increasing in sound pressure level with increasing frequency from 65 to 82 dB at the antenna, or a sine wave of 150 Hz at 65 dB at the antenna (n=6 for each sex). When the stimulus was a sine wave, the output of a Knowles NR 3158 noise cancelling microphone (Knowles Electronics Co, West Sussex, UK) next to the antenna was also sent to an Edirol R-09HR digital recorder (Roland Corporation, Los Angeles, CA, USA) and saved with the stimulus as stereo .wav files to allow analysis in Raven and Adobe Audition. Serendipitously, when the recordings in response to 150 Hz were reviewed, a very clear burst of nerve activity was noticed when a man was speaking. Because the equipment was needed for teaching, the experiment was not repeated, but because of its importance, the voice was recorded and its frequency spectrum investigated.

4.4.10 **Statistics**

Stastistics were done in JMP software (SAS®, Cary, NC, USA). Throughout, data are presented as the mean $\pm$ standard deviation. I analyzed morphology and sounds produced by IMM with t-tests to look for differences between the sexes, and the ultrasound produced before and after ablation with paired t-tests to see if the ablation eliminated the click. In the sound plus light experiment, I used a repeated measures ANOVA to look for differences between treatment groups. For tympanal responses, I first categorized responses as yes or no to each frequency stimulus. I then eliminated non-responders and used the remaining data in a linear fit model on log-transformed data, setting individual as a random effect, frequency as the independent variable and the amplitude of the

response as the dependent variable to test for differences between the responses of the sexes and to determine if one or more frequencies elicited a greater response than the others. Analyses of antennal responses to conspecific sound were done with contingency tables to see if there were differences between males and females responding to sound, and antennal responses to sweep stimuli used t-tests to look for differences between the sexes.

4.5 Results

4.5.1 Morphology

The antennae of female and male IMMs have a long scape from which extends a flagellum with 49 flagellomeres; those most proximal to the scape are squat whereas the most distal are shorter and narrower. The length of their flagella is the only significant difference between the sexes, despite females being larger than males (based on femur length: 1.94 $\pm$ 0.11 mm females, 1.81 $\pm$ 0.03 mm males n=6 for each sex, t=-2.98, p=0.025) (Table 4.3).

Light microscopy of male and female antennae shows cells resembling a Johnston's organ in the distal pedicel (Figure 4.1A), and staining with Neurotrace confirms that the cells are neurons, forming a ring at the distal attachment of the flagellum with at least 46 cells (Figure 4.1B).

SEM's show a dorsal view of a male thorax (Figure 4.2A) with most scales removed and the tegulae in place over the attachment of the forewings. Behind them are two elliptical domes about 10 µm x 70 µm wide (arrows) that could be tymbals. The anterior and underside of a tegula (Figure 4.2B) have no striations. The tympanum was open (Figure 4.2C), revealing the central connections of the scolopidium to the tympanal membrane and the tympanal cavity.

4.5.2 **Sound and ultrasound produced by IMM**

Males and females had similar wingbeat frequencies: 51 ± 2.1 Hz and 50 ± 10.2 Hz, respectively (n=6 males, n=6 females, t=-0.06, *p*=0.95) (Figure 4.3). The waveform of the sound is complex and varies with the orientation of the moth with respect to the microphone. Recordings often showed two peaks of positive pressure per complete wingbeat, corresponding with the maximum velocity of the wings as they pass the microphone going up and down. The sound pressure level of the wingbeats at ~1.5 cm was 70 ± 4.4 dB and 73 ± 1.3 dB (n=6 males, n=5 females, t=-2.09 *p*=0.08). Ultrasound from the clicks was broadband, spanning 25 to 80 kHz; the most intense components were between 40 – 50 kHz (Figure 4.4). There was typically one click per wingbeat, emitted 2 – 2.5 ms after the peak pressure change, but sometimes a second, smaller click was seen after the large click. The interval between large clicks was 17 ± 0.001 ms (n=6 males), corresponding to the duration of their complete wingbeat (Figure 4.4). The peak sound pressure level of the clicks was 53 ± 2.9 dB and 55 ± 3.1 dB for males and females respectively (n=4 for males, n=4 for females, t= 0.70, *p*=0.51).

4.5.3 **How IMM produce ultrasound**

None of the alterations to potential ultrasound producing structures resulted in extinction of the ultrasound. Clicks had the same interclick interval and interclick frequency after tegulae were removed. Glueing both dorsal thoracic ‘tymbals’ did not remove ultrasound from recordings, and shortening the wings neither abolished the ultrasonic clicks from recordings, nor affected the interclick interval or interclick frequency (Table 4.4).

4.5.4 **Behavioural experiments**

More females were caught in treatment traps with sound than the silent traps ($F_{(2,27)}$ = 2.22, *p*=0.03). Although slightly more females were caught when wingbeat was used as a treatment than when clicks or wingbeat + clicks were

used, there was no significant difference between the treatments ($F_{(2,27)}$ = 1.26, p=0.3).(Table 4.5).

4.5.5 **Tympanal vibration**

Tympanal organs did not vibrate with stimuli below 1 kHz, but did in response to ultrasound from 20-100 kHz, with the proportion of tympana responding increasing with increasing frequency. Male tympana vibrated maximally at 90 kHz with a smaller peak at 70 kHz and female tympana had a peak response at 70 kHz with a smaller one at 100 kHz (Figure 4.6, n=6 for each sex). The strength of the tympanal response to sound stimuli that varied in frequency varied with frequency ($F_{(8,74)}$=17.7, p<0.0001) but did not differ between the sexes ($F_{(1,74)}$=2.45, p=0.15).

4.5.6 **Antennal vibration**

No vibration matching the frequency of the stimulus was seen from the antennae of either sex in response to ultrasound, but all (z=2.45, p<0.05, n=6: for both sexes) vibrated in response to conspecific sound. A response was considered to occur when the frequency of the antennal vibration was the same as the frequency of the stimulus (representative female antennal response shown in Figure 4.7).

Both male and female antennae vibrated maximally at a single frequency in response to sine wave stimuli swept from 30 Hz to 1 kHz (Figure 4.8). To see if this was due to mechanical resonance, as it is in the antennae of Diptera, I compared the phase of the response and stimulus over the sweep and found a change of approximately 180° with the steepest part of the change at the frequency of maximal vibration (Figure 4.8). This shows that the antennae are tuned mechanical systems. Male antennae were tuned to a slightly higher frequency than female antennae (157 ±6 and 149 ±14 Hz respectively, n=6 for, each sex, t=1.18, p=0.27); the third harmonic of the female and male wingbeat frequency. The amplitude of vibration of male antennae at their best frequency

was 135 nm, slightly but not significantly, more than that of females at 120 nm, with a sound pressure level of 65-82 dB increasing with the sweep (Table 4.6). Q values were not significantly different between the sexes, with females at 2.05 $\pm$ 0.48 and males at 2.55 $\pm$ 0.44 (n=5 for each sex, t=1.68, p=0.131).

4.5.7 **Electrophysiology of antennae**

The background noise in the laboratory made it difficult to obtain consistent responses from the antennae. Nerve impulses 1 ms in duration and negative-going up to 15 mV in amplitude could be seen and heard in response to the sweep from 100 to 1000 Hz and the 150 Hz sine wave in both sexes (Figure 4.9), (n=7 of each sex). The nerve impulses did not follow the frequency of the stimulus closely except for a few seconds in one recording where a man was speaking during the 150 Hz sine wave stimulus (Figure 4.10); the antennae responded at a mean frequency of 66 $\pm$ 18 Hz, n=11 (spikes in the recording). Subsequent analysis of the voice showed that the impulses occurred when the dominant frequency of the voice was 150 Hz, the best frequency of the antenna (Figure 4.11).

4.6 Discussion

This research shows that IMM produce and can detect sound and ultrasound. The tympana detect ultrasonic frequencies as produced by bats and moths. How the moths generate ultrasound remains unknown. The tympana do not vibrate in response to the wingbeat frequency or its harmonics at natural levels. However, the antennae do vibrate in response to the wingbeat, and may be used to communicate with others of their species.

4.6.1 **Morphology**

While the flagella of females are significantly longer than those of males, the difference is proportional to their size. Because their pedicels, which contain the vibration-sensitive scolopidia, are the same size, it seems unlikely that hearing is more important for one sex. The ring of neurons in the distal pedicel (Johnston's

organ) is present in all Insecta (Regier *et al*., 2010), but has so far only been shown to detect sounds in two of the 29 orders of insects, the Diptera and Hymenoptera (Hoy & Yack, 2008). The antennae of IMM and many other moths are similar in structure to those of bees that are known to communicate with sound from their wings (Nadrowski *et al*., 2011). The JOs of much larger sphingid moths have several hundred scolopidia, but from his preliminary electrophysiological investigation, Vande Berg (1971) thought they had no auditory function.

The tympana of IMM have the same basic structure as those of other tympanate moths (Surlykke *et al*., 2003; Windmill *et al*., 2007), and have been described by Mullen & Tsao (1971). However, their examination focused on the external morphology of the tympana, the image here shows the internal morphology. The scolopidium is believed homologous with a proprioceptive chordotonal organ, while the tympanum itself is believed homologous with an apodeme (ingrowth of the exoskeleton serving as attachment points) on the ancestral ditrysian sternum (ventral side) of the second abdominal segment (Minet & Surlykke, 2003).

4.6.2 **Sound and ultrasound produced by IMM**

During flight, IMM produce wingbeat and ultrasonic sounds of similar frequencies. Belton (1986) showed a distinct relationship between wingbeat and frequency for Lepidoptera, with the frequency increasing as size decreases. Although this relationship could lead a person to believe that any significant difference in size, as seen in IMM, equals a frequency difference, the actual size difference between the sexes is so small that the frequency is the same between males and females. However, with the same wingbeat and associated click frequencies, IMM communicating with sounds would not be able to discriminate between sexes as mosquitoes do. This similarity in frequencies fits with the lack of sexual dimorphism in the antennae. The similar pedicel size and overall antennal morphology of IMM suggest both sexes hear equally.

The mechanism of click production remains unknown. Ultrasonic clicks, in moth species that emit them, are commonly produced by tymbals on the thorax (Arctiidae) (Fullard & Heller, 1990; Conner, 1999; Corcoran *et al.*, 2010), or abdomen (Noctuoidea) (Skals & Surlykke, 1999; Conner, 1999). The pyralids *Symmoracma minoralis* and *Syntonarcha iriastis* possess tymbals on the last abdominal segment (Gwynne & Edwards, 1986; Heller & Krahe, 1994). Pyralids in the galleriine group generate clicks with their tegulae (Spangler, 1988). Examination of the IMM body did not yield a structure that resembled any previously described tymbal, in any of the aforementioned locations, or anywhere else on the body. Removal of the tegulae did not eliminate the ultrasonic clicks, contrary to the results of Trematerra and Pavan (1995) who recorded clicks from IMM and two related *Ephestia* species and found that in the three IMM from which they removed tegulae, clicks ceased.

Shortening the wings is known to increase wingbeat frequency in insects (Sotavalta, 1952), but it did not eliminate the clicks. With shortened wings it is impossible for contact between them to create the clicks generated by wing contact in some Lepidoptera (Bailey, 1978). Although the exact mechanism of click generation by IMM remains unclear, I believe it must be associated with movement of the wings or thorax, because the clicks are locked to the wingbeat, as shown by the interclick frequency equalling the wingbeat frequency. If the wings are the source, then the clicking mechanism may have been on the remaining half of the wings. However, insects would not move when I cut their wings to the base so I could not record them; another method of immobolizing their wings would show if they do produce the clicks. The crambid species *Ostrinia furnacalis* possesses specialized scales that rub against the wings to produce ultrasound (Nakano *et al.,* 2008 & 2009). Scales and other structures, such as the patagia, that might be disturbed by the wingbeat could be examined and removed to test this.

4.6.3 **Behavioural experiments**

Although there was a trend toward the wingbeat being more attractive than the other treatments, no significant difference between treatments implies that no particular frequency is favoured over another. Trematerra and Pavan (1995) ran preliminary tests that showed cessation of movement by males when exposed to bursts of ultrasound, mimicking bat echolocation. If ultrasound was the only sound detectable by IMM, they would not have responded to all the treatments; thus, that the treatment traps in the behavioural experiment overall caught more females than the control traps supports the idea that IMM detect sounds. More replicates may make a difference between treatments apparent.

4.6.4 **Tympanal responses**

The sensitivity and frequency range of the tympana fit well with behavioural research showing that males detected 20 kHz, but only at a sound pressure of 95 dB, and 30 kHz just above 80 dB; their best hearing range fell between 40 to 70 kHz (Svensson *et al.*, 2007). The insect's ability to detect ultrasound allows it to hear the echolocating calls of foraging bats, fitting with the theory of tympanal evolution (Conner, 1999; Miller & Surlykke, 2001; Waters, 2003). Both sexes of IMM can detect their ultrasonic clicks, as shown by the laser vibrometry. The graphs of the frequency response of the tympana place the best frequency at 70 kHz for females and 90 kHz for males, with the overall trend of response increasing with increasing frequency. The clicks of IMM are broadband, with the upper limit of 80 kHz falling within the range of hearing for both sexes. The second, smaller peak in sensitivity at 100 kHz for females and 70 kHz for males may be a common feature of pyralid tympana because peaks at 25 and 70 kHz are present in *Ostrinia nubilalis* (Agee, 1969), at 20 and 60 kHz in *Anagasta kuehniella* (Pérez & Zhantiev, 1976) and 33 and 100 kHz in *Achroia grisella* (Spangler & Takessian, 1983).

That the tympanum did not respond to the low frequencies, including the wingbeat frequency and some of its harmonics, is unsurprising. Bennet-Clark

notes that insect tympana can respond from a few hundred Hz to frequencies above 60 kHz (1971). However, because a 20 kHz signal required a 95 dB sound pressure level to elicit a response in males (Svensson *et al.*, 2007), the 50 Hz signal I tested at 65 dB (a biologically relevant level) would need to be at a sound pressure level much greater than that which the insect would experience in nature. Rowland, working with *Lymantria* species, was able to elicit a tympanal vibration with sounds at 56 dB (Rowland *et al*., 2011), 8 cm from the insect (Rowland, personal communication). As this would make the sound at the insect 38 dB in a room where ambient noise is ~50 dB (my measurement), the tympanum in *Lymantria* must be exceptionally sensitive. Spangler elicited a response from *Galleria mellonella* at a sound pressure level just below 60 dB with a frequency of ~12 kHz (1984). Electrophysiological recordings are needed to resolve this question.

Ultrasound detection by IMM is accepted as occurring with tympanate ears; however, the mechanism by which the moths detect sound had not been determined. In his PhD dissertation on the almond moth, *Cadra cautella*, and IMM, Lollis suggests that the lack of sensitivity of pyralids to low frequencies may indicate that the moths have no means of receiving airborne sound, or that they do not display an observable behaviour to these lower frequencies (1971). Studies on *Galleria mellonella* (GWM) (Spangler *et al.*, 1985) demonstrate that the low frequencies of wingfanning are important to that species, even though there is no described method by which the moths detect them. Spangler has suggested that GWM hear with their wings outspread (Spangler, 1985), but this has not been substantiated. This proposed method of hearing is unlikely in IMM because the moths do not rest with their wings spread like GWM. Orci and Szocs (2009) suggest that hair sensilla could be receptors in the Crambidae, a closely-related family of moths previously placed in the Pyralidae. Hair sensilla have been shown to detect sound up to 300 Hz in other insects (Bennet-Clark, 1971); however, because adult pyralids have few long setae compared with the sound detecting cerci of grasshoppers or cockroaches, I believe that the

antennae, with their Johnston's organs, are the most likely candidates for hearing.

4.6.5 **Antennal responses**

The laser vibrometry of antennae exposed to frequency sweeps and playback of male recordings show they respond to sound, but not ultrasound; therefore, they could be used as ears for low frequencies. The mechanical resonance of the antennae is almost identical to that of mosquitoes (Clements, 1999) and brachyceran flies (Robert & Göpfert, 2002) known to use their antennae as wingbeat receptors. The velocity (from 112 to 136 µm/s) and displacement (120 to 135 nm) values are similar to those measured from *Ae. togoi* in Chapter 3 (velocity from 71.3 to 116.4 µm/s, displacement from 34.65 to 78.30 nm). However, the Q value is smaller than that seen for female *Ae. togoi*, and more similar to the value for males, suggesting the antennae are not highly tuned. The small number of scolopidia in JO of Lepidoptera may allow the pedicel-flagellar joint to flex more than in mosquitoes where thousands must be stretched. The main difference between the mosquito and the moth responses is that the antennae of mosquitoes have a best frequency close to the female wingbeat frequency, whereas the IMM respond best to its third harmonic. However, as seen in the spectrum of the male wingbeat recording, the third harmonic can be louder than the fundamental frequency.

4.6.6 **Electrophysiology of antennae**

Although marred by loud background noise, the electrophysiological recordings add support for the antennae as hearing organs. If you listen to the recordings, the nerve responses from the antenna in response to sound are audible; unfortunately, their visual depiction is difficult against the background. In the most clear example of the spikes, the responses are seen as 1 ms in duration, which demonstrates that they are from nerves firing; muscle responses would last between 5 to 10 ms. Moreover, the spikes from the antenna responding to a man's spoken voice show a response at 150 Hz, the best frequency of the

antennae. Although the recordings need to be improved, they do still show that the antennae are capable of detecting sound. More electrophysiological recordings to support the laser findings should be done to demonstrate more clearly that the observed response is an example of hearing.

4.7 Conclusion

I demonstrate for the first time, that the antennae of Lepidoptera vibrate as mechanical systems like those of Diptera. Laser vibrometry shows that at biologically relevant levels, the tympanum does not detect low frequencies in the wingbeat, but the antennae do, and have a best frequency around the third harmonic of the wingbeat. The tympana can detect ultrasound in the 40 – 50 kHz range, frequencies generated by clicks during the wingbeat of both females and males. The source of the clicks does not seem to be the tegulae, or contact between the wings. Because background noise in the electrophysiological recordings made detection of the nerve responses difficult, more experiments are needed to convince researchers that the moths use their antennae to detect sound. However, based on my results, I believe IMM have developed two sets of hearing organs specialized for detecting different frequencies: one for the ultrasound of echolocating bats and the clicks of either sex, the other for the wingbeats of conspecifics. It seems unlikely that the moths can use sound to discriminate between the sexes because their wingbeats and associated clicks are not different by sex. It is more likely that pheromones serve to distinguish the sexes. Preliminary tests show that females are attracted to sound in combination with blue-violet light, but observations in nature may be needed to understand the role of sound and ultrasound in the biology of these economically important moths. This is the first evidence that moths can use their antennae to detect sound, and thus possess two pairs of hearing organs.

4.8 Figures and Tables

Table 4.3. Comparison of female and male IMM antennal measurements

Part measured	Male Mean	SD	Female Mean	SD	t statistic	*p*-value
Scape length (mm)	0.33	0.035	0.34	0.008	-0.34	0.75
Scape width (mm)	0.16	0.012	0.17	0.010	-0.52	0.61
Pedicel length (mm)	0.10	0.009	0.11	0.009	0.60	0.56
Pedicel width (mm)	0.13	0.013	0.13	0.009	0.25	0.80
Flagellum length (mm)	4.17	0.19	4.56	0.230	-2.03	0.01*
Last flagellomere length (mm)	0.06	0.012	0.07	0.015	-0.42	0.68
Last flagellomere width (mm)	0.04	0.005	0.04	0.005	0.54	0.60

.

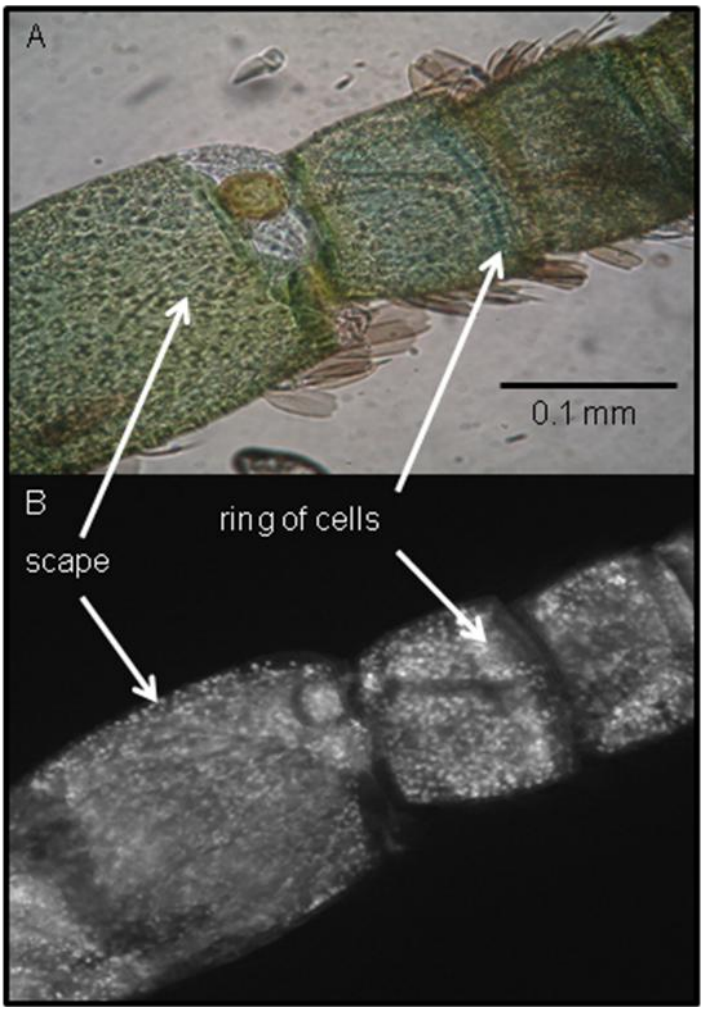


Figure 4.1. Photograph of a male IMM antenna stained with A) methylene blue to show structure; and, B) Neurotrace to highlight nerve cells, showing the location of the Johnston's organ (ring of cells) in the second antennal segment.

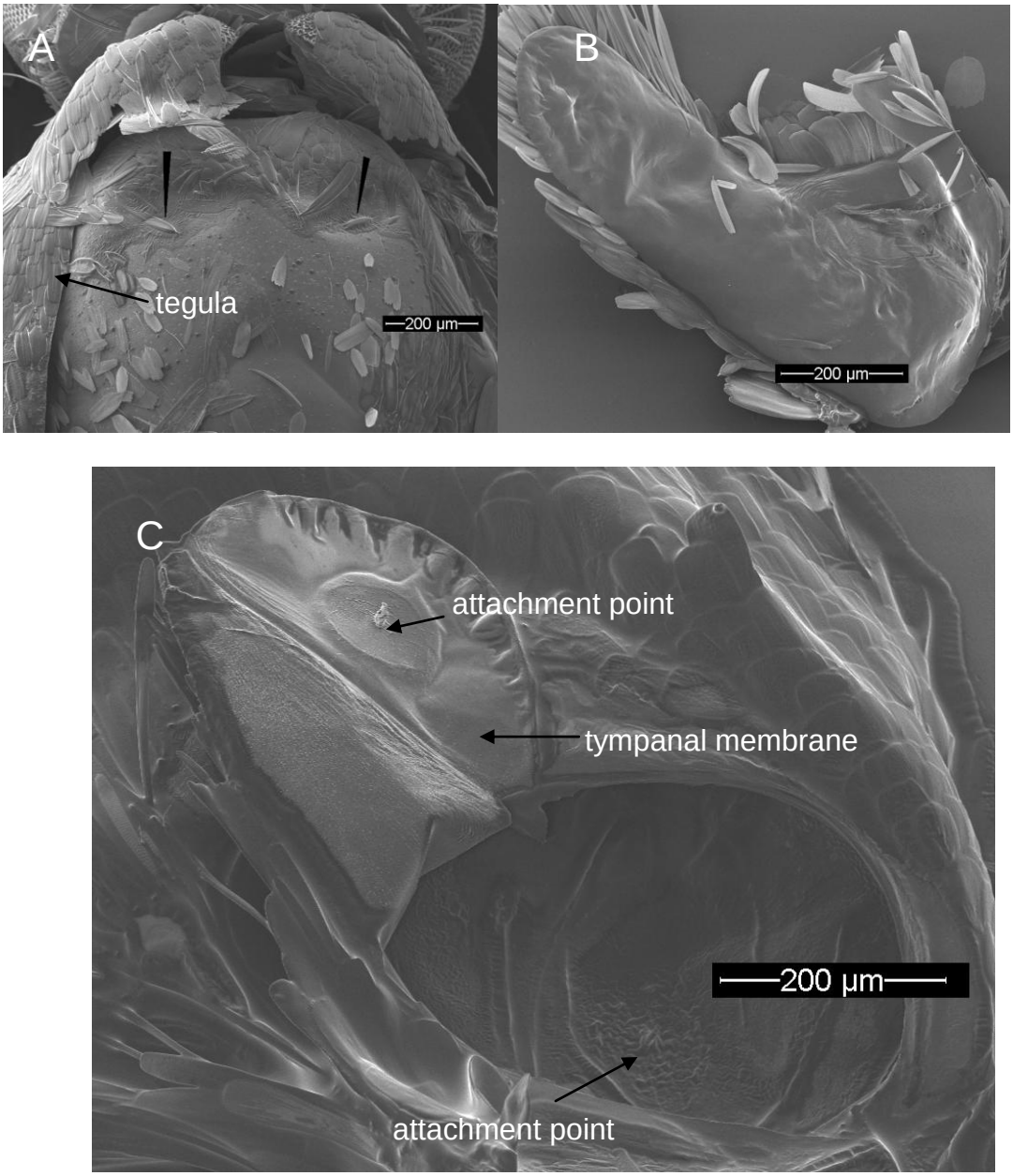


Figure 4.2. Scanning electron micrographs of: A) tymbal-like indentations (arrows) on the thorax, with the tymbal-like structure on the left covered by a scale; B) a left tegula, ventral side up, in the position it would be as taken directly off the moth (the left edge being the right most edge of the tegula shown in A); and, C) tympanum with the membrane folded back revealing the interior of the hearing organ and attachment points of the scolopale.

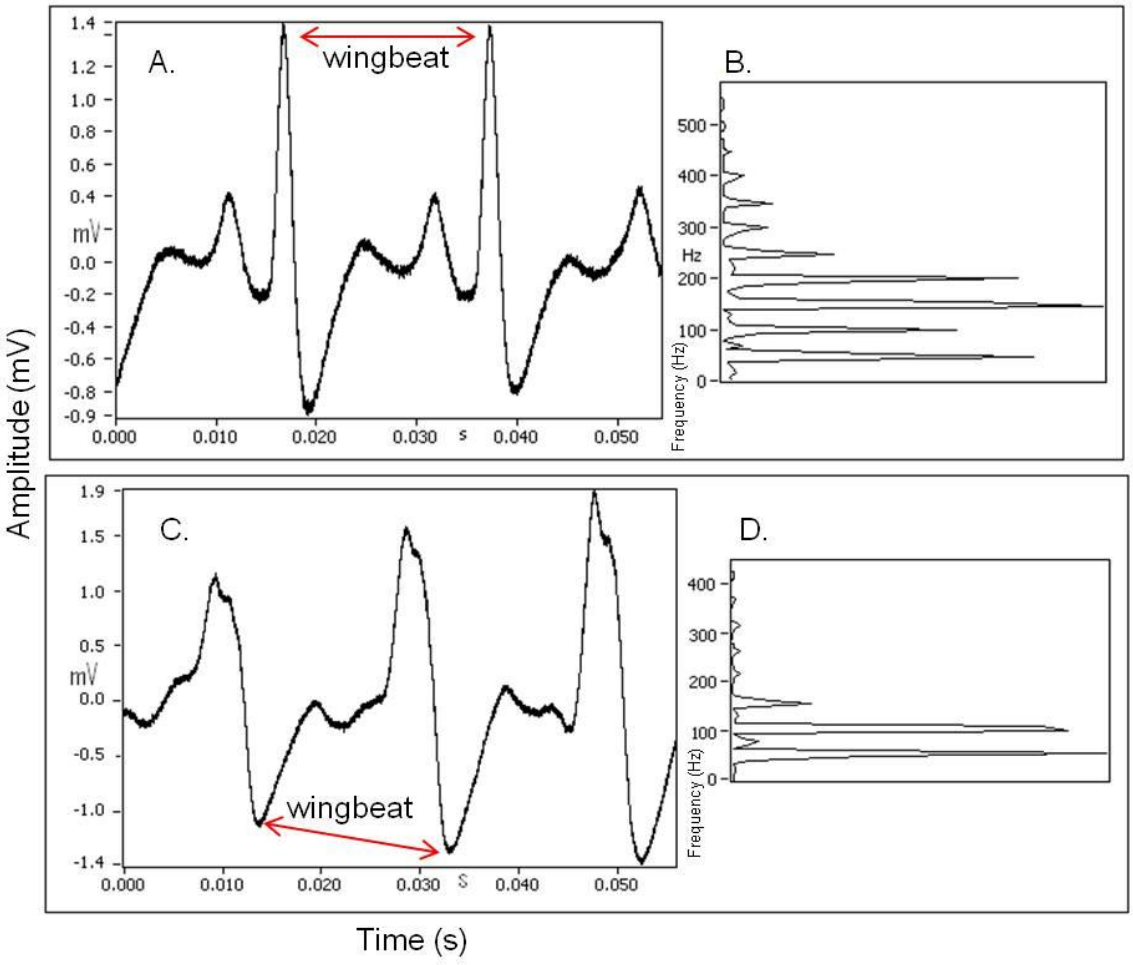


Figure 4.3. Wingbeats of male and female IMM. A) Waveform from a representative male; B) the frequency spectrum of the wingbeat shown in A (wingbeat frequency = 49 Hz); C) waveform from a representative female IMM; and D) the frequency spectrum of the wingbeat shown in C (wingbeat frequency = 51 Hz).

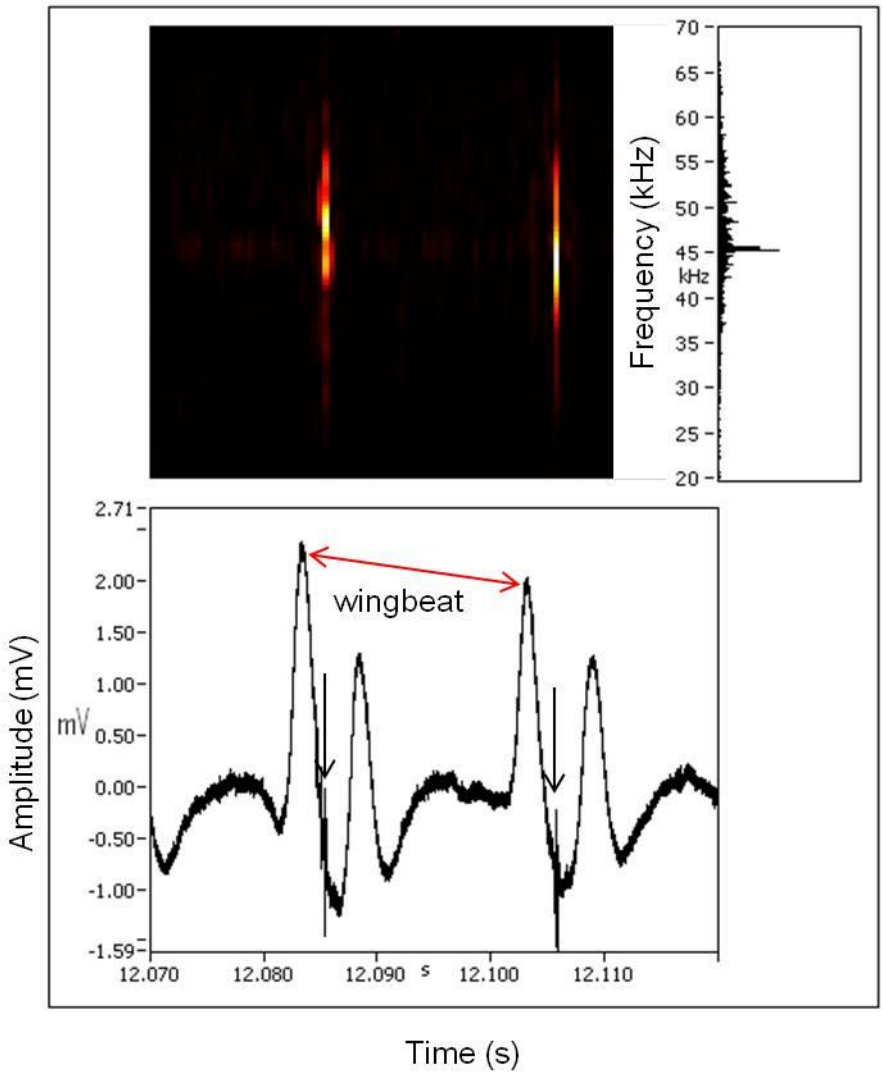


Figure 4.4. Representative ultrasonic clicks from a female IMM recorded with a Knowles ultrasonic microphone. A) power spectrum of the clicks where the whiter the colour, the more intense the frequency; B) the corresponding frequency distribution of the power spectrum; and, C) the position of the clicks (single arrows) on the waveform of the wingbeat (46.1 Hz).

Table 4.4. Intervals between ultrasonic clicks and wingbeat frequency before and after alteration of body parts on male IMM.

Body part altered		Before alteration		After alteration		n	t statistic	*p* value
		Mean	SD	Mean	SD			
Tegulae	Interval between clicks (s)	0.015	0.002	0.02	0.002	5	1.20	0.30
	Interclick frequency (Hz)	67.8	9.2	63.5	8.9	5	1.46	0.22
thoracic "tymbals"	Interval between clicks (s)	0.018	0.002	0.02	0.003	2	*	*
	Interclick frequency (Hz)	57.6	7.0	63.5	11.2	2	*	*
Wings	Interval between clicks (s)	0.016	0.001	0.01	0.001	4	2.83	0.07
	Interclick frequency (Hz)	61.6	1.8	70.4	4.9	4	2.70	0.07

- n precludes comparison of the sound produced before and after glueing of thoracic "tymbals"

Table 4.5. Mean numbers of virgin female IMM caught in 10 comparisons of 'noisy' and silent light-traps + light (10 females released in each trial, 10 replicates per treatment).

Treatment	Wingbeat		Wingbeat + click		Click	
Stimulus	**Treatment**	**Control**	**Treatment**	**Control**	**Treatment**	**Control**
Number of females caught (mean)	3.2	1.4	1.8	1.3	2.2	2.0
SD	1.2	1.3	1.2	0.9	1.8	0.6

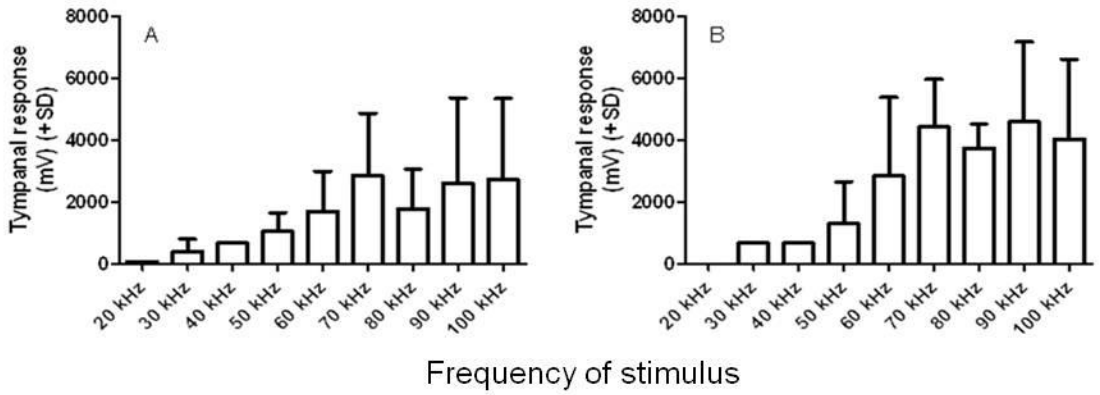


Figure 4.5. Amplitude of vibration of female (A) and male (B) IMM tympana, measured with maser vibrometry, in response to ultrasound above 20 kHz at 80 dB SPL at the insect (n=6, for each sex).

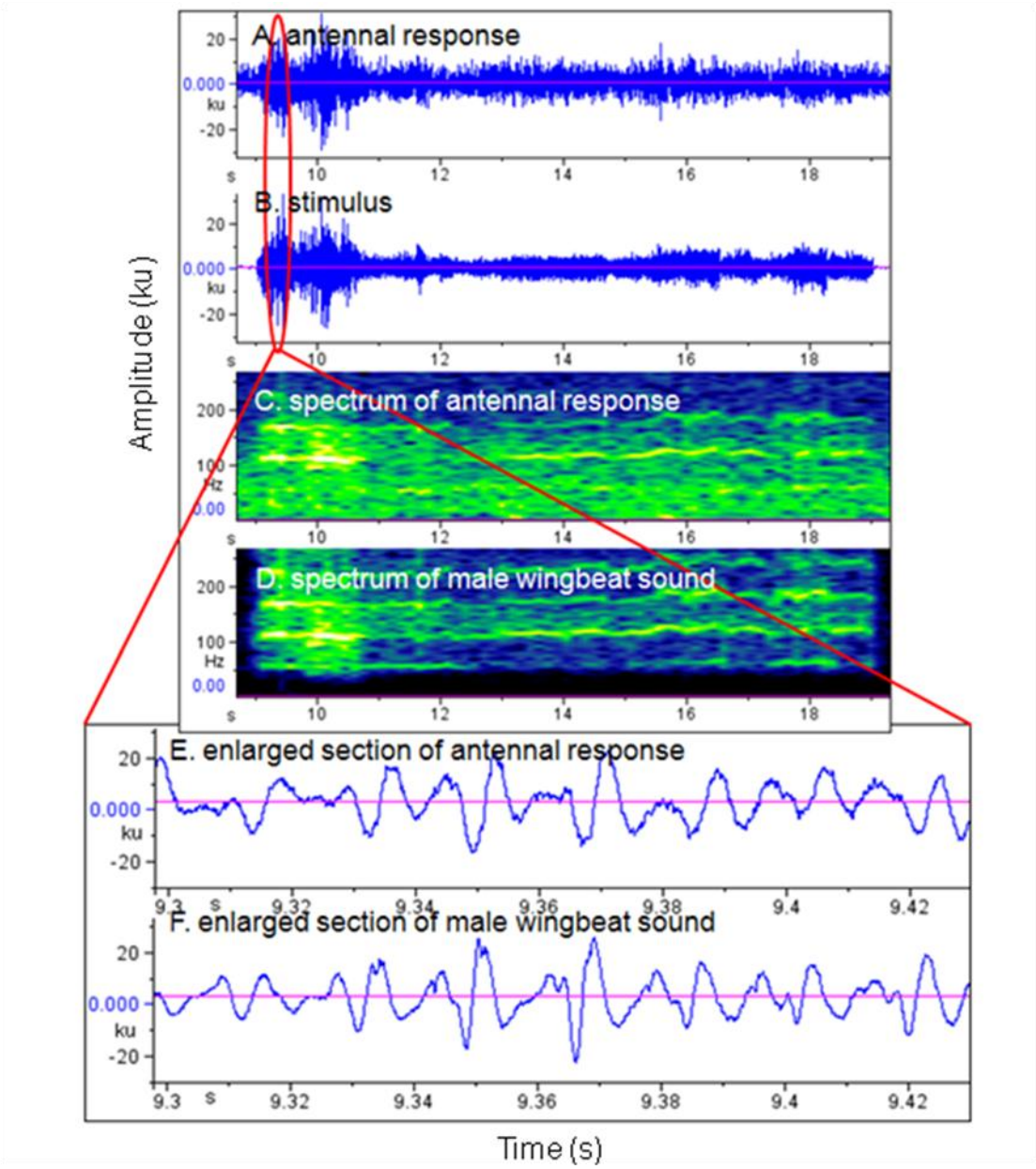


Figure 4.6. Laser vibrometry of a female IMM antenna. A) Waveform of an antennal response; B) to male wingbeat sound replayed at 65 dB SPL at the insect; C) spectrogram of antennal response; and, D) spectrogram of male wingbeat sound. The enlarged section shows the antennal vibration (E), matching the conspecific sound (F).

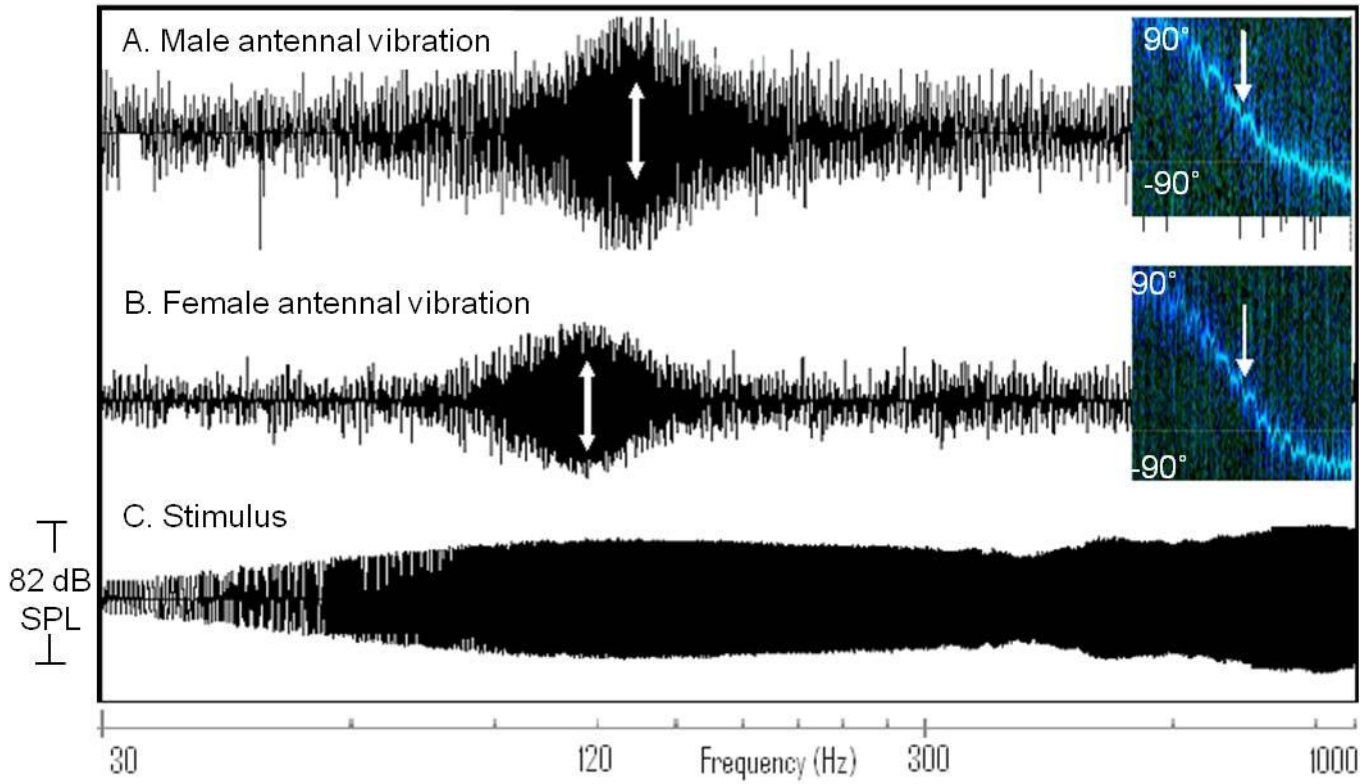


Figure 4.7. Vibration of female and male IMM antennae in response to a sine wave swept from 30-1000 Hz. A). a representative male response ~ 150 Hz; B) a representative female response ~115 Hz; and C) stimulus sine wave swept from 30-1000 Hz, increasing with frequency from 65-82 dB SPL at the insect. The insets associated with A and B show the phase change (single arrow) that occurs at the peak of the responses from the antennae (double arrows indicate best frequency response, where the phase change occurs).

Table 4.6. Vibration of male and female IMM antennae measured at the base of the flagellum to a sine wave swept from 30 to 1000 Hz (n=6 for each sex).

Parameter	Female	Female SD	Male	Male SD	t statistic	*p*-value
Best frequency (Hz)	149	14	157	8	1.18	0.27
Displacement at resonance (nm)	120	26.55	135	56.52	0.62	0.55
Velocity at resonance (μm/s)	112	23.51	136	66.78	0.84	0.43

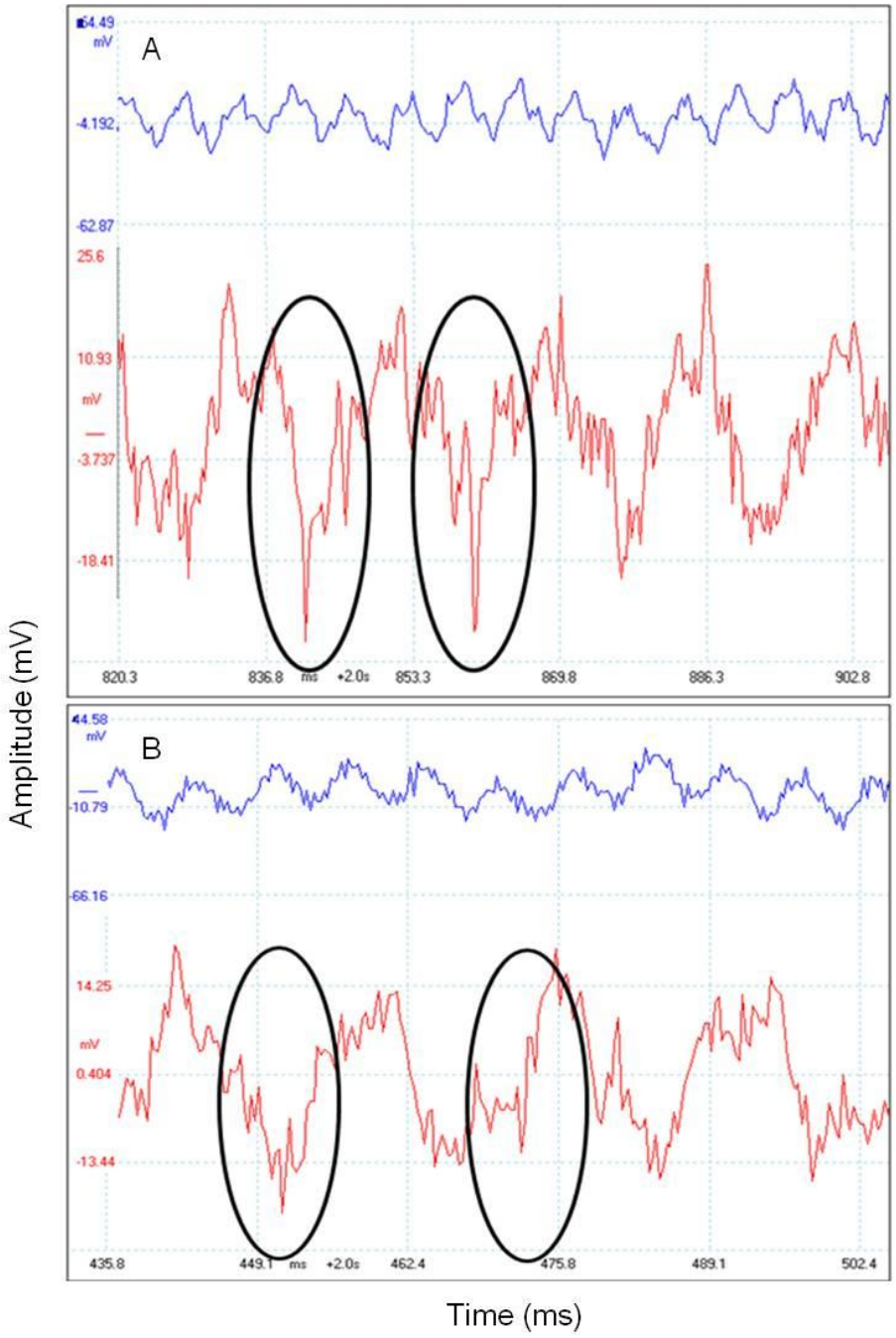


Figure 4.8. Electrical activity recorded from the scape of a male IMM (lower traces of A & B) in response to a 150 Hz sine wave at 65 dB at antenna (upper traces of A & B). Negative-going spikes occur clearly in A, but are masked by background noise in B. Circles contain examples of spikes recorded from the antennae.

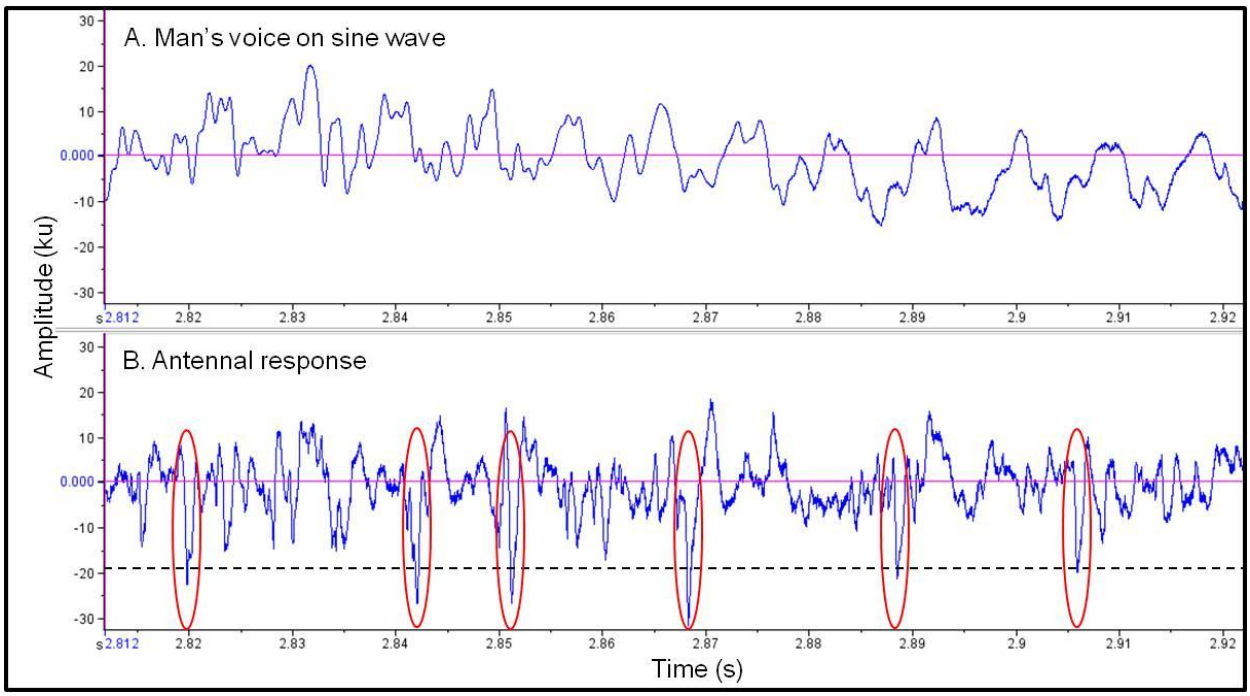


Figure 4.9. A) A 150 Hz sine wave stimulus with a man's voice superimposed; and, B) an electrophysiological recording of an IMM antenna. Red circles highlight six nerve impulses that met the requirements of being considered an impulse (1 ms in duration and below -20 ku). The x-axis is in seconds; the y-axis is in ku (thousands of digital samples). The dashed line indicates the line below which a signal was considered a response.

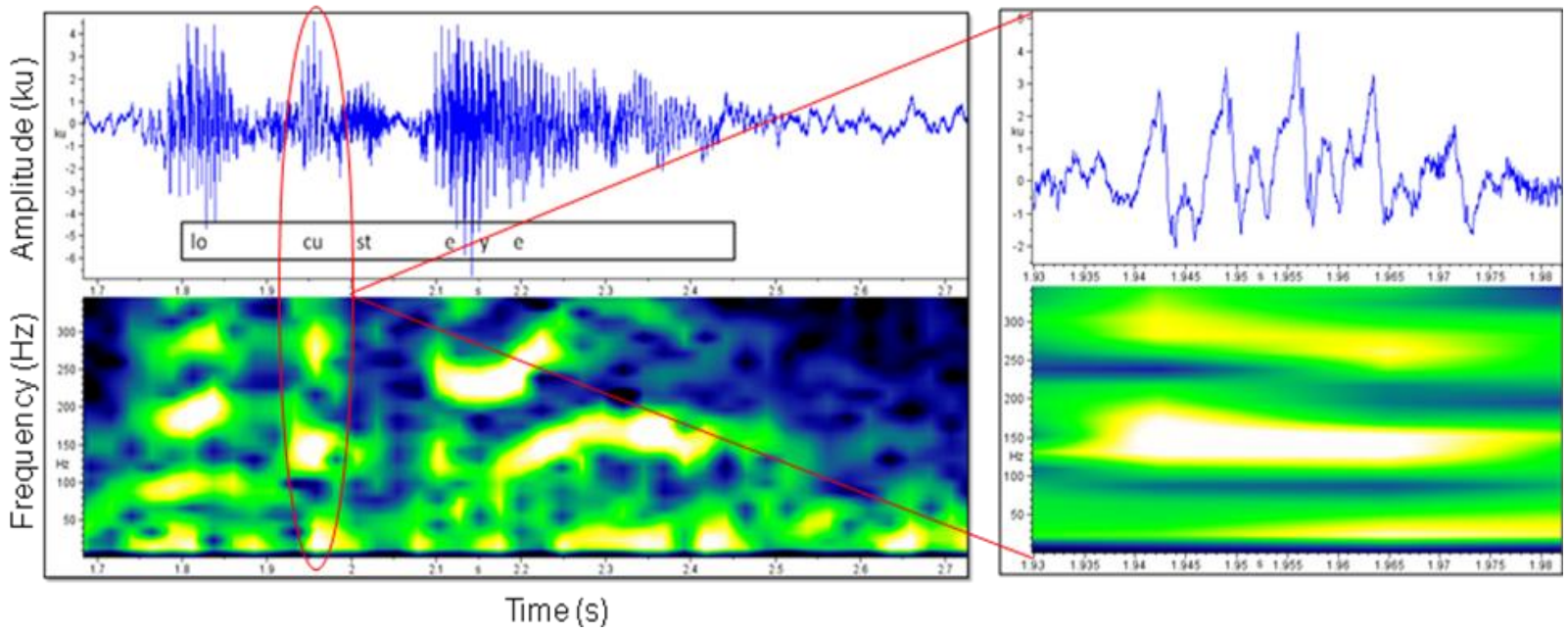


Figure 4.10. Man's speaking voice that elicited the antennal response from a male IMM (Figure 4.9). The top trace shows the waveform of his words "locust" and "eye" while the bottom trace represents the spectrogram of the waveform. The circled portion, enlarged on the right, shows the "kuh" phoneme of locust, with a frequency around 150 Hz.

LINKING STATEMENT

In chapter 4, I described the use and detection of sound and ultrasound by *Plodia interpunctella* (IMM). I demonstrated that the tympana of both sexes can detect ultrasound, but not sound at biologically relevant levels. From the results of laser vibrometry and electrophysiology of antennae, I believe that these organs allow IMM to detect the low frequency components of wingbeats. This is the first evidence for a moth having two pairs of hearing organs tuned to different frequencies.

In chapter 5, I study the peach twig borer, *Anarsia lineatella,* and the webbing clothes moth, *Tineola bisselliella*, two atympanate pest species reported to communicate with sound, to determine if they use their antennae to detect sound. The results of sound and ultrasound recordings, laser vibrometry, and morphological examination support the hypothesis that these moths, like IMM, can detect conspecific sound with their antennae, and for *Anarsia lineatella*, perhaps ultrasound with a tympanum-like structure.

5 HOW PEACH TWIG BORER AND WEBBING CLOTHES MOTHS COMMUNICATE WITH SOUND

5.1 Abstract

The peach twig borer, *Anarsia lineatella*, an orchard pest, and the webbing clothes moth, *Tineola bisselliella,* a stored products pest, have been reported to use sound in their communication system, but the extent of its use, and how they detect it is unknown. I used morphological studies, sound and ultrasound recordings, behavioural experiments and laser vibrometry to investigate how these two moth species differed in their production, detection and use of sound and ultrasound. *A. lineatella* produces sound (males at a fundamental frequency of 56 Hz, females at 57 Hz) and broadband ultrasonic clicks (from 20 to 80 kHz in both sexes) when they flew or fluttered their wings. In contrast, *T. bisselliella* only produced sound (males with a fundamental frequency of 58 Hz, females did not wingfan or fly in captivity). In the field, significantly more *A. lineatella* males were caught in pheromone traps playing the sound of a female than in identical silent traps. I found that the filiform antennae of the two moth species are not sexually dimorphic and the pedicels of both sexes contained a Johnston's organ. The antennae of both species vibrated to played back recordings of the wingbeats of conspecifics. *A. lineatella* antennae had best frequencies ranging from 130 to 500 Hz, whereas *T. bisselliella* had a best frequency near 100 Hz, making the second harmonic of the wingbeat frequency potentially audible. Although the family is thought to be atympanate, I discovered a circular region on the metepisternite of *A. lineatella* that vibrated with ultrasound (best frequencies at 90 kHz for each sex) and thus may be used to receive ultrasound. Possible reasons that *A. lineatella* but not *T. bisselliella* generate ultrasound are briefly discussed.

5.2 Introduction

Numerous species of moths use a combination of pheromones and sounds for communication (Rowland *et al*., 2011; Scoble, 1992). Insects may use two modalities because pheromones, good for long distance communication, may not be effective in close settings for moths seeking a mate (Willis & Baker, 1984). Thus, a second, more directional modality, such as sound or ultrasound would allow moths to find one another in a pheromonal miasma at night.

Most of the moth species studied that communicate with acoustic signals emit ultrasound that can be detected by tympana (Scoble, 1992; Conner, 1999). Tympana are the only hearing organs identified thus far in Lepidoptera and only the tympanate pyralids *Achroia grisella* and *Galleria mellonella*, the crambid *Ostrinia nubilalis* (Orci & Szocs, 2009), and species of *Lymantria* (Rowland *et al*., 2011) have been shown to use low frequency wingbeats in addition to pheromones and ultrasound. However, only in *Lymantria*, where flying males and females hear both ultrasound and sound has it been claimed that the moths use their tympana to detect wingbeat frequencies (Rowland *et al*., 2011). The sensitivity of the tympana of *A. grisella*, *G. mellonella* and *O. nubilalis* to sound has not been tested. In contrast, the atympanate moths *Tineola bisselliella* and *Anarsia lineatella* have been described as using wingbeat sounds to communicate (Takács *et al*., 2003; Hart, 2006), but with no tympana they must have other hearing organs.

Ultrasonic signals make sense in terms of evolution and effectiveness when they are used in open spaces. Tympana, believed to have evolved to detect the ultrasound of echolocating bats, pre-adapted moths to use ultrasound for intraspecific communication (Conner, 1999). Ultrasound is transmitted directionally, and although the shorter wavelengths of ultrasound are more readily absorbed by surfaces than the longer wavelengths of sound, an animal sending it from an elevated perch diminishes the effects of absorption on the signal and can be located readily (Forrest, 1994). There is ample evidence that

many species of moths can detect the ultrasound of predators (Jacobs *et al*., 2008, Svensson *et al*., 2007, Hristov & Conner, 2005) or conspecifics (Rodriguez & Greenfield, 2004, Yang & Greenfield, 1996) with tympana, or labial palps in the case of sphingids (Göpfert *et al*., 2002, Göpfert & Wasserthal, 1999). Even though an early study showed that significantly fewer atympanate microlepidoptera were caught in light-traps "protected" by ultrasound than in silent traps (Treat, 1962), the sense organs they use to detect sound or ultrasound remain unknown.

In this study, I investigated how these two species, *Anarsia lineatella*, the peach twig borer (PTB), and *Tineola bisselliella*, the webbing clothes moth (WCM), might use sound and ultrasound. Specifically, with PTB: a) I investigated the internal morphology of the antennae and an area of the metepisternite with light and scanning electron microscopy; b) I recorded the acoustic signals produced by females and males; c) I investigated the behaviour of the sexes to acoustic signals, testing male response to female sounds with experiments in the field and female responses to male sounds in laboratory; and, d) I measured the best frequency and tuning of antennae, and the best frequency of the central area of the metepisternite (a potential ultrasound-receiver) with laser vibrometry. With WCM, I investigated a) the internal morphology of the antennae with light microscopy; b) the sounds produced by males; c) the behaviour of the sexes in response to wingbeat sound and white noise in the laboratory; and, d) I measured the best frequency and tuning of antennae with laser vibrometry. I compare the physical properties of the moth antennae with Diptera antennae, known to be among the most sensitive ears in the animal kingdom (Gibson *et al*., 2011).

5.3 Insect natural histories

5.3.1 Peach twig borer

PTB is a small moth that is a pest of fruits of *Prunus* species. It was first noted in 1839 in Germany (Duruz, 1923) and is thought to have originated in Asia or Western Europe (Marlatt, 1898, Duruz, 1923). First found in Canada in 1902 (Belton, 1988), it was the main lepidopteran pest of nectarines and peaches in Canada until the accidental introduction of the oriental fruit moth, *Grapholita molesta* (Daane *et al*., 1993).

The life cycle from egg to adult lasts between 31 to 44 days, but the last summer generation overwinters as a larva and continues development in the spring (Bailey, 1948). Adults live up to two weeks, feed on juice from damaged fruit and available water sources, and can mate multiple times (Bailey, 1948). Females emerge first, have a preoviposition period between 1-4 days (Bailey, 1948) and call with a pheromone around dawn (Schlamp *et al*., 2006). Males use the pheromone signals to orient toward the female, producing sound as they fly (Hart, 2006). When exposed to the male sound, the female lowers pheromone emission and begins fluttering and jumping in flight, emitting sound of her own that the male may use to pinpoint her location (Hart, 2006). Once the male has alighted near a female, he begins wingfanning and attempts to mate with her (Hart, unpublished observations). Once mated, females typically lay their eggs the next night, but will oviposit during the day as well (Bailey, 1948). Oviposition can last 2 weeks (Ponomarenko, 1990) with a female producing up to 100 eggs (Bailey, 1948). She lays these in clusters of 2-5 in leaf axils, or on the bark, terminal shoots, fruit, and bud bases (Bailey, 1948; Sarai, 1966; Ponomarenko, 1990). After hatching, larvae develop through four to five instars (Bailey, 1948).

When ready to pupate, fifth instar larvae find a location inside folded leaves, fruit, or cracks in the bark (Marlatt, 1898; Treherne, 1923; Ponomarenko, 1990). When pupating in bark, the larvae create hibernacula (chimneys), small reddish-

brown tubes of bark pieces fastened with silk that protrude from the bark surface and serve an unknown purpose (Duruz, 1923; Treherne, 1923). Larvae emerging early in the summer may construct a hibernaculum which they leave to continue foraging before constructing another hibernaculum in which to pupate (Marlatt, 1898; Price & Summers, 1961; Ponomarenko, 1990), but not all larvae do this. Larvae that emerge later in the summer pupate and develop into adults if they feed on fruit, whereas those that feed on bark overwinter in their larval state (Sarai, 1966).

Adults of both sexes are positively phototactic, negatively geotactic, and move by hopping and flying. They do not fly great distances unless foliage is present; however, their flight range has not been determined (Sziraki, 1984; Ahmad, 1989). The greatest damage to fruit and new growth and highest trap captures of moths was in the upper levels of trees (Rice & Jones, 1975; Ahmad & Khadhum, 1986; Weakley *et al*., 1990).

Three to four generations per year have been observed in California (Price & Summers, 1961): two to three in British Columbia (Sarai, 1966). The actual number may be underestimated because it can be difficult to identify overlapping generations (Bailey, 1948; Brunner & Rice, 1984).

5.3.2 **Webbing clothes moth**

WCM was first described, albeit as *Tinea bisselliella*, in 1823 (Cox & Pinniger, 2007). Of course, people of the 19th century were not the first to observe these pests. In his thoughts on nature, Aristotle documents clothes moth in dirty woolens (Wentworth-Thompson, 1994). The poet Horace, writing a little later in the first century BC, acknowledges their destructive nature: “who has bed-clothes rotting in his chest, the food of worms and moths” (Smart, 1863).

The geographical origin of WCM is uncertain, but it probably started out as a pest of animal lairs and bird nests before infesting human dwellings (Cox & Pinniger,

2007). Wherever their origin, these small moths have successfully spread throughout the world, most likely with humans (Cox & Pinniger, 2007).

After eclosion, adult WCM live for up to 38 days depending on temperature and humidity (Cox & Pinniger, 2007). They have fused mouthparts, so do not feed or drink as adults (Cox & Pinniger, 2007), making their longevity remarkable. Although reported to fly as far as 90 m (Herrich, 1933), females typically run in a seemingly haphazard manner (Key & Common, 1959). When dispersing, the moths rely on a series of chemical signals. Males arrive at a food source earlier than females and begin wingfanning and emitting an aggregation pheromone that attracts males and females and when a female arrives, she emits a sex pheromone that attracts males (Takács *et al*., 2002). Males walk towards the females in a rapid, erratic manner, wingfanning continuously (Cox & Pinniger, 2007). The sound associated with the wingfanning may attract males and females to a suitable larval habitat (Takács *et al*., 2003).

WCM adults can mate within hours of emergence and females will oviposit in the following 24 hours (Griswold, 1944). The eggs are laid in groups or singly (Griswold, 1944) with females selecting soiled, yeast-scented or fleecy sites on fabric (Kan & Waku, 1985; Traynier *et al.*, 1994). Females have about 100 eggs (Cox & Pinniger, 2007) which they lay over 11-17 days, depending on temperature (Griswold, 1944). In particularly heavy infestations, larvae pupate at the edges of the infested material, most likely to facilitate dispersal of the adults (Cox & Pinniger, 2007).

Adults of both sexes are negatively phototactic (Griswold, 1944), and are most active three hours after sunset (Key & Common, 1959). Because WCMs are stored products pests, their habitat has few seasonal changes so they develop continuously and, under ideal indoor conditions, have up to six generations a year (Ebeling, 1978; Cox & Pinniger, 2007). Outdoors, WCM is subject to lack of food, and temperature fluctuations that limit the number of generations per year;

consequently, the larvae have more than the five to six instars that they typically undergo in optimal conditions (Hinton, 1956). Although it is still debated, the larvae might enter diapause to survive exceptionally adverse times (Pelham-Clinton, 1985).

5.4 Materials and methods

5.4.1 **Rearing: PTB**

PTB collected as larvae from a fruit orchard in Cawston BC, were kept at 24˚C, on a 16:8 hour light:dark cycle. Larvae were reared on diet as described by Hart (2006). Pupae were separated from the diet and kept in Petri dishes until they emerged, after which they were transferred to glass jars, given access to food (~15 mL of a 10% sucrose solution) wicked through dental cotton, and felt on which to lay eggs.

5.4.2 **Morphology: PTB**

I examined the morphology of two possible sound receptors, the antennae and metepisternite. To examine the antennae, I used the Nikon digital fluorescence imaging system described in Chapter 4. Tissue from five freshly killed moths in saline, and five moths kept in Bouin's fixative were stained with methylene blue to show overall form and structure and with Neurotrace to distinguish nerve cells (Invitrogen, USA). For visualizing the Neurotrace, fluorescence images used a 490nm excitation filter, a 536nm emission filter and a "C-Y-R" dichroic mirror (Chroma Technology).

I examined the metepisternite by putting freshly dissected legs onto slides, holding them in place with plasticine, and clearing any scales obscuring the sclerite using a paintbrush. Photographs of the dissections were taken with a Canon Powershot SD1000 through the lens of a Carl Zeiss Technival 2 dissecting microscope.

5.4.3 **Sound recordings: PTB**

To investigate the frequencies and sound pressure level (SPL) of wingbeat sounds, I recorded them with an AKG condenser microphone (sensitivity 20 mV/Pa; flat frequency response: 20 Hz to 20 kHz $\pm$ 1 dB; AKG Acoustics Nashville Tennessee, USA), and a Knowles SPM0404UD5 ultrasonic microphone (specified frequency response: 1-60 kHz $\pm$ 5 dB – own measurement: 10 Hz- 100 kHz $\pm$ 12 dB; Knowles Electronics, Itasca IL, USA; Appendix B). The sensitivity of the microphone was confirmed by comparison with a Brüel and Kjær 2204 sound level meter with a 2203 ½" microphone. Signals were amplified x 800 with a National Instruments (NI) SC 2040 differential amplifier (National Instruments Corporation, Austin, TX, USA). I tethered six males and seven females PTB behind the head with a human hair and flew them ~1.5 cm in front of the microphone; concurrent digital video recordings confirmed that tethering did not impede flight or cause unwanted sounds from wings hitting the tether. Recordings up to two minutes of individual moths were saved to play in later experiments.

Spectra of the wingbeat and clicks were displayed using LabView with the procedure described in Chapter 4.

5.4.4 **Behaviour: PTB**

5.4.4.1 Field Experiments

I ran experiments in the field to see if the males were attracted to a recording of the female wingbeat including ultrasound up to 24 kHz. Experiments were done at an organic orchard in Cawston, BC during the summers of 2006 (n=31 paired traps) and 2007 (n=17 paired traps). Pairs of Delta traps (PheroTech, Delta, BC, CAN), each with a Panasonic WM-R57A speaker facing inward at one entrance of the trap, were baited with synthetic pheromone (100ng of a 10:1 mixture by weight of (E)-5-decen-1-yl acetate and (E)-5-decen-1-ol) on a rubber septum. Female wingbeat sounds were played from one of the pair of traps (chosen at

random) from dusk to dawn using Sony D-EJ120 portable CD players. I set the SPL to 65 dB ~1.5 cm from the speaker as in previous field experiments (Hart, 2006). Traps were placed, a pair in each tree, ~5' above the ground.

5.4.4.2 Laboratory experiments

Experiments in the laboratory tested the response of the female to male wingbeat sounds at different intensities and to the different frequency components of the male sound. Concurrent videotaping with a Sony digital handicam (Model DCR-VX1000) and audio recordings with the AKG condenser microphone as described earlier (Chapter 4) were used to quantify the female responses.

5.4.4.2.1 **Distance**

I tested the sensitivity of the hearing organs by examining the distance at which females responded to male sounds, with five or six females at each distance. Each female, leashed with a ~15 cm human hair, was placed on a 5 cm diameter round filter paper, with the end of the hair taped to the paper, and the paper taped to the benchtop. The wingbeat sounds were played first at 2.5 cm (hereafter 0 m) from a Sennheiser 70 headphone speaker (Sennheiser Electronic Corporation, Old Lyme, CT, USA; flat frequency response: 10 to 39 500 Hz, 0.05% THD) where the sound level of the male recording was 65 dB. If the female showed a response (flight, wing fluttering, or other movement) to the male signal, she was placed at a randomly assigned distance (0, 0.5, 1.0, or 1.5 m) and observed over two minutes for a response. The SPL at the speaker was kept constant, so it decreased with distance: 0 m=65 dB, 0.5 m=39 dB, 1 m=33 dB and 1.5 m=29 dB.

5.4.4.2.2 **Sound and ultrasound**

To examine female responses to the different frequencies in the male wingbeat, virgin females were leashed with a ~15 cm human hair and the hair taped to a 5 cm filter paper taped to the benchtop ~5 cm in front of the Sennheiser speaker. The female was then played one of three male sounds, wingbeat, wingbeat + click (the complete signal), or click, at 65 db at the moth for 48 seconds. The

components of the male sound were bandstop filtered above 1000 Hz (wingbeat), unfiltered (wingbeat + click), or bandstop filtered below 20 kHz (click). Female wing fluttering sounds were recorded with the AKG condenser microphone and evaluated using LabView, as time to first emitted sound, the number of clicks, and the SPL of her wingbeats. I determined the SPL from the peak to peak amplitude of the microphone output in mV, calculating its root mean square (RMS) and reading SPL in dB from a nomogram (Appendix B).

Equation 5.1. Calculating RMS

$$\text{RMS} = ½ \text{ (peak to peak amplitude)} \times 0.707$$

5.4.5 **Laser vibrometry of receptors: PTB**

A 2-channel OFV 2500 Polytec laser vibrometer (Polytec Inc., Irvine, CA, USA) was used. The output from the laser, focused on the base of the antennal flagellum or the metepisternite, went to one channel of the vibrometer, and the output of the AKG microphone for sonic recordings, adjacent to the moth, to the other. Because the Polytec software cannot store ultrasound, the output of a Brüel and Kjær 2204 sound level meter with a linear response, using a 4133 ½" microphone adjacent to the receptor was sent, with that of the laser, to a 2-channel digital Picoscope 2203 oscilloscope (Pico Technology, Cambridgeshire, UK) with a sampling rate >200 kHz to avoid aliasing. Acoustic stimuli were: a) a sine wave swept from 30 Hz to 1000 Hz, increasing in amplitude from 65 to 82 dB SPL at the moth, covering the range of wingbeat and harmonics; b) pure tones every 10 kHz from 20 kHz to 100 kHz at 85 dB at the moth to test the mepisternal sclerite's response to ultrasound; c) recordings of the male and female wingbeat played at 65 dB. I determined mechanical resonance of the antennae in Adobe Audition 3.0 (San Jose, CA, USA) by noting the time of steepest portion of the phase change and determining the frequency at that time using Raven (Cornell Laboratory of Ornithology, Ithaca, NY, USA). The frequency response of the sclerite to pure ultrasonic tones was plotted from the

mean of three measurements at each frequency for each insect, converting the amplitude of the mean responses at each frequency to a proportion of the greatest response, and taking the mean of those proportions for the six males and females. A metepisternite vibration was considered to be vibrating in response to the stimulus when the sine waves of each were at the same frequency, in phase with each other, and at a measureable level (> 0.2 mV). I calculated quality factor (Q) values from the antennal responses to the swept sine waves, using the equation and method described in Chapter 4. Details of the sound and ultrasound-producing equipment are also the same as those used for IMM (Chapter 4).

5.4.6 **Rearing: WCM**

I purchased WCM from Central Science Laboratory (York, UK) and fed larvae on untanned beaver, squirrel and raccoon pelts purchased from local trappers. The insects were maintained at 24˚C, on a 8:16 hour light: dark light cycle.

5.4.7 **Morphology: WCM**

Examination of the antennae used the Nikon digital fluorescence imaging system described for PTB. Antennae from five freshly killed male and female moths kept in saline, and five preserved moths kept in Bouin's were stained with methylene blue and Neurotrace following the methods described for PTB.

5.4.8 **Sound recordings: WCM**

I recorded wingbeat sounds with the Knowles microphone and equipment described for PTB. Recording from males or females suspended from a human hair was not feasible because they would not fly or flutter, so I recorded from six individual males that wingfanned for up to three minutes, approximately 0.5 cm from the microphone. Spectra of the sounds were displayed and analyzed with a program written with LabVIEW as described in Chapter 4.

5.4.9 **Behaviour: WCM**

I ran the following behavioural experiments in the laboratory to determine the moths' response to sound.

5.4.9.1 Laboratory experiments.

5.4.9.1.1 **Antennal ablation**

I ablated one or both male antennae at the base of the scape, or left both intact, to investigate whether or not they receive sound. Four hours before the experiment, males of unknown mating status were treated as follows: antennae left intact, one antenna cut at the scape, or both antennae cut at the scape. In 20 replicates, I placed groups of nine males, three with each treatment, in a petri dish and put it in a 1 m^3 mesh cage an hour before the experiment. Traps were 10 cm diameter circles cut from milk cartons and coated with Tanglefoot®, above which sat the Sennheiser headphone on top of a glass cylinder (Figure 5.1). Gaussian white noise with uniform output from 0-20 kHz (Takács *et al.* 2003) generated through a program in LabView and playback of the male wingbeat sound from the computers was set at 65 dB ~ 1.5 cm from the speaker. The male wingbeat was a 17 second recording of one male, with a wingbeat frequency of 49 Hz, looped with one second of silence between repetitions. At the start of the scotophase I opened the petri dish. Each of the 20 replicates ran for 16 hours, when the number and category of moths stuck on each trap was recorded, as were the number of moths remaining in the dish or in the cage.

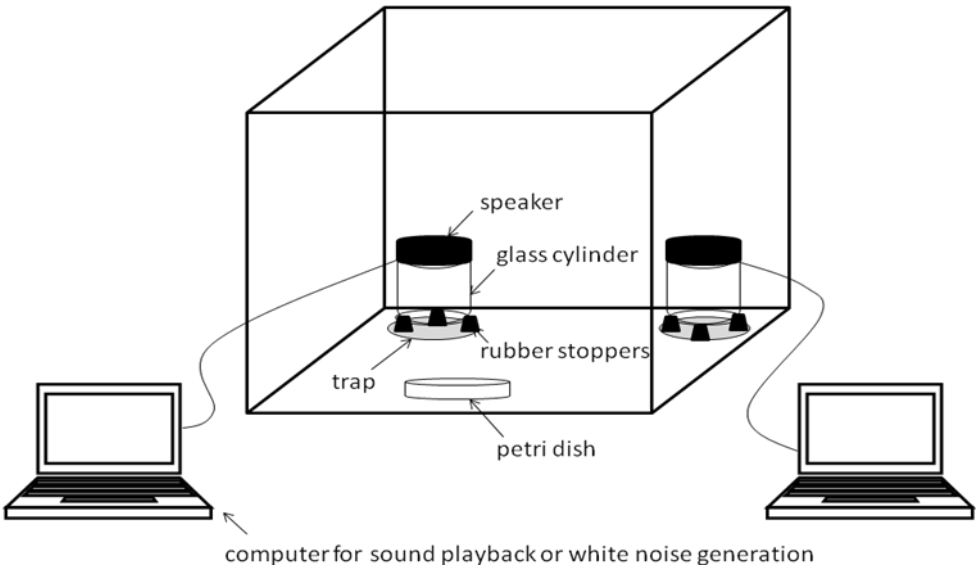


Figure 5.1. Experimental set-up for the WCM antennal ablation experiment.

5.4.9.1.2 **Intermittent sound**

Because some moths of both sexes moved when male wingbeat sounds were played, I ran the following experiment to quantify their response. Twenty virgin moths of each sex were leashed with a ~15 cm human hair that was taped to a 5 cm filter paper which was taped to the benchtop, ~ 5 cm in front of the Sennheiser speaker. After a moth was stationary for more than 10 seconds, it was left in silence for a further minute, then played a wingbeat recording of a single male (65 dB at insect) for 2 minutes, followed by another minute of silence. Video footage was examined for: a) the duration of antennal movement (waving or twitching); and, b) the duration of any other movements, including wing fluttering, walking, movement of legs.

5.4.10 **Laser vibrometry of receptors: WCM**

I used the laser set-up described for PTB to investigate the vibration of the antennae in response to sound. Stimuli were: a) a sine wave swept from 30 Hz to 1000 Hz, increasing in amplitude from 65 to 82 dB SPL at the moth to cover the range of wingbeat frequency and its lower harmonics; and, b) recordings of the conspecific male wingbeat at an SPL of 65dB at the antenna. I replayed

sounds recorded from the moths in the laboratory using computers with software programs developed in LabVIEW (NI) through a Sennheiser 70 headphone speaker. I determined mechanical resonance of the antennae using Adobe Audition 3.0 (San Jose, CA, USA) and the methods described for PTB. I calculated quality factor (Q) values from the antennal responses to the swept sine waves, using the equation and method described in Chapter 4. Details of the sound and ultrasound-producing equipment are the same as those used for IMM (Chapter 4).

5.5 Statistics

Statistics used JMP software (SAS®, Cary, NC, USA). Throughout, data are presented as the mean $\pm$ standard deviation. I analyzed differences in antennal size between the sexes, overall moth size between the sexes and differences between the frequency and intensity of the sounds produced by both sexes with t-tests. In the PTB field tests, I used an ANOVA, with year as a random effect to see if more males were attracted to the pheromone traps playing female wingbeat sounds than to identical silent traps. In the lab, I compared the proportion of female PTB fluttering in response to the male wingbeat sound at distances up to 1.5 m. I used a chi-square analysis to see if there was an optimal distance at which females would respond. To determine if females preferred one part of the played back male sound over another, I examined female time to respond to the sound, intensity of response to the sound and number of sounds produced in response to male sound with an ANOVA. I evaluated whether male and female PTB differed in their amplitude of vibration in response to ultrasound stimuli that varied in frequency every 10 kHz from 20 kHz to 100 kHz using a mixed model. Individual was included as a random term. Stimuli that did not elicit a measureable vibration from the metepisternite at the same frequency as the stimuli were excluded from analyses. To see if male and female antennae both responded to conspecific sound, I used a contingency table to analyze the number of moths whose antennae vibrated in response to the stimulus in PTB and WCM. In the ablation experiment, I calculated the difference in the number

of WCM with zero, one and two antennae attracted to the playback of male wingbeat sound or white noise after each trial. I used a repeated measures ANOVA to see if the number of antennae influenced the catch. I also used a repeated measures ANOVA to compare movements of female and male WCM in response to the male sound. For laser vibrometry comparing the best frequency, tuning (Q) and displacement between female and male WCM antennae in response to sweeps, I used t-tests.

5.6 Results: PTB

5.6.1 Morphology

Basing body size on winglength, females 5.18 $\pm$ 0.14 mm, males 5.52 $\pm$ 0.09 mm, there is no difference between the sexes (t=2.21, p=0.13, n= 3 for each sex). Antennae were also the same between the sexes, with female antennae 3.91 $\pm$ 0.19 mm long and males 3.82 $\pm$ 0.20 mm (t=0.11, p=0.92, n=3 for each sex). Antennae were kept flat against the back when the moths were at rest, and upright when mate seeking occurred. In flight, the antennae were angled into the incoming air. Microscopy of the antennae of both sexes showed a ring of cells in the distal pedicel, where I expected to find Johnston's organ. Staining with Neurotrace confirmed that they were neurons (Figure 5.2). A search of the thorax and abdomen where tympana are found in other moth families revealed no similar structures; however, when the scales were removed, the metepisternite was different from other sclerites. It had a circular area ~ 0.13 mm in diameter that was a translucent white colour (Figure 5.3). Although I saw an air space below these spots when I removed the sclerite, I found no scolopidium.

5.6.2 Production of sound and ultrasound

The seven female PTB had a mean ($\pm$SD) wingbeat frequency of 57 $\pm$ 1.6 Hz, and the six males a wingbeat frequency of 56 $\pm$ 2.6 Hz (t=-0.36, p=0.73) (Figure 5.4). The SPL of the wingbeat was 64 $\pm$ 5.6 dB for females and 61 $\pm$ 3.3 dB for males (t=-0.99, p=0.34) at ~1 cm from the insect. Ultrasonic clicks were

broadband, spanning 25 to 80 kHz; the most intense components (the brightest area on the click spectra) were at 42.7 ± 1.4 kHz for females, 43.7 ± 2.6 kHz for males, (t=0.34, *p*=0.74, n=8 for females, n= 6 for males) (Figure 5.5). Sound pressure levels of the clicks were 64 ± 4.4 dB for females and 63 ± 1.8 dB for males (t=-0.44, *p*=0.67, n=7 for each sex). Ultrasonic clicks were present in all recordings of tethered male and female moths. They occurred consistently at a particular phase of each wingbeat, but the number of clicks per wingbeat varied from moth to moth (Figure 5.5). One two or three clicks were seen in one wingbeat cycle in both sexes, but the number of clicks was not studied further. In Figure 5.5B, a female, a single click is generated as the wing is at its highest velocity during an up or downstroke. In Figure 5.5D, a male, two clicks are generated at opposite phases of the wingbeat, one is probably near the top and the other near the bottom of the stroke. In Figure 5.5F, a female also generated clicks at opposite phases of the wingbeat, but the larger is a closely-spaced pair of clicks. All the clicks had similar frequency components in their ultrasonic spectra (Figure 5.5 A, C and E).

Video recordings of tethered male moths show clearly that the forewings clap together above the thorax at the highest point of each wingbeat but do not touch at the lowest point (Fig 5.6).

5.6.3 **Behavioural experiments**

5.6.3.1 Field experiments

Males are attracted to the playback of recordings of female wingbeat sound and ultrasound in the field. Treatment traps in both 2006 and 2007 caught more moths than the control traps ($F_{(1,45)}$=2.48, *p*=0.02; treatment*year $F_{(1,45)}$=0.29, *p*=0.59). However, there was a difference between the total numbers of moths caught in each year ($F_{(1,45)}$=11.15, *p*=0.002) (Figure 5.7).

5.6.3.2 Laboratory experiments

Females have not been tested in the field for their attraction to male sounds, so experiments were done in the laboratory to record their fluttering or flight in response to different intensities and frequency components of the male flight sound.

5.6.3.2.1 **Distance**

More than half the tethered females fluttered or flew during a two minute exposure to the complete male sound (wingbeat + click). None flew or fluttered during the two minutes of silence preceding their exposure to male sound. More than half the females fluttered, producing acoustic signals to the male sound at 1.5 m (29 dB) (Figure 5.8), the farthest distance tested in the experiment. All females tested at 1.0 m (33 dB) fluttered to the male wingbeat, but no other distance elicited this level of response. However, the difference between the number of moths responding at each distance was not significant (chi-square = 4.46, df=3, p=0.216).

5.6.3.2.2 **Component frequencies**

Playback of the complete signal and playback of the click alone elicited significantly louder and quicker responses compared with playback of the wingbeat alone. The number of times females fluttered in response to either the complete signal, the wingbeat or the click alone, was not significantly different (Table 5.1).

5.6.4 **Laser vibrometry of antennae and metepisternites**

The antennae of six specimens of each sex showed mechanical resonance in response to the swept sine wave (representative response shown in Figure 5.9). Unexpectedly, the best frequencies varied widely from individual to individual, from ~130 to 500 Hz (males: 204 $\pm$ 80 Hz females 299 $\pm$ 157 Hz, t=-1.33, p=0.22, n=6 for each sex). The antennae of both sexes vibrated at the replayed fundamental wingbeat frequency of either sex (Figure 5.10). The metipisternite of six specimens of each sex vibrated measureably between 40 and 100 kHz in

response to ultrasound played at 10 kHz intervals from 20 to 100 kHz at 85 dB, with a peak amplitude at 90 kHz (Figure 5.11). Analysis of the fixed effects of the proportional data showed a significant difference between the sexes, with females vibrating at a greater amplitude than males at all frequencies ($F_{(1,56)}$=18.47, p<0.01). There was also a significant difference between the tested frequencies ($F_{(6,56)}$=7.63, p<0.01). The Q values of the antennae were not significantly different between the sexes, with females at 3.98 $\pm$ 1.3 and males at 3.25 $\pm$ 1.47 (t=-0.83, p=0.43, n=5 for each sex). Displacement of the antennae at the best frequency was also not significantly different between the sexes, with females at 67 $\pm$ 40 nm and males at 67 $\pm$ 36 nm (t=1.36, p=0.20, n=6 for each sex) at an SPL of 70 dB at the antenna.

5.7 Results: WCM

5.8 Morphology

The antennae of females and males were the same length, 3.55 $\pm$ 0.70 and 3.93 $\pm$ 0.50 mm, respectively (t=1.07, p=0.31, n=6 for both sexes). Antennae were flat across the back when the moths were at rest, and upright during mate seeking times. Microscopy of WCM antennae showed a ring of cells at the apex of the pedicel, the location of the Johnston's organ. They were brightly stained with Neurotrace (Figure 5.12). Basing body size on winglength measurements, females 4.88 $\pm$ 0.32 mm, males 4.16 $\pm$ 0.54 mm, females were larger than females (t=-2.83, p=0.02, n=6 for both sexes).

5.8.1 Production of sound

Male WCM had a wingfanning frequency of 58 $\pm$ 3.22 Hz (n=6) (Figure 5.13) with a sound pressure ~0.5 cm from the insect of 71 $\pm$ 2.8 dB (n=7). No ultrasonic clicks were detected and because females were never seen flying or wingfanning, no recordings of them could be made.

5.8.2 **Behavioural experiments**

5.8.2.1 Antennal ablation

The number of antennae ablated did not influence whether male moths were more likely to be attracted to the sound of conspecifics than to white noise (Table 5.2) ($F_{(2,57)=}$1.22, p=0.30). The total number of moths trapped over the 20 16 hour replicates was 35. Counter to expectations, even moths with no antennae removed did not show a preference for playbacks of conspecifics compared to white noise (single sample t-test mean difference = -0.1 ± 0.79, n=20, *p* = 0.58).

5.8.2.2 Intermittent sound

In response to playback of the male wingbeat sound, females moved their legs and wings significantly more than they did during the preceding and following minutes of silence (Figure 5.14A). The amount of antennal movement before, during and after playback of conspecific sound was not significantly different between treatments (Figure 5.14B).

5.8.3 **Laser vibrometry of antennae**

Laser vibrometry of WCM antennae showed a mechanical resonance with best frequencies at 106 + 21.2 Hz for females (n=11) and 111 $\pm$ 33.9 Hz for males (n=8) (t=0.37, *p*=0.72,) (Figure 5.15). Vibration of the female and male antennae closely followed the amplitude and frequency changes in the male wingbeat sound (n=6 for each sex) (Figure 5.16). The Q values were low, with females at 1.97 $\pm$ 0.54 and males at 1.85 $\pm$ 0.51 (t=-0.35, *p*=0.73, n=5 for each sex). Displacement of the antennae at best frequency was 217 $\pm$ 129 nm for females and 116 $\pm$ 45 nm for males (t=-2.29, *p*=0.04, n=10, females, n=7, males) with a SPL of 75 dB.

5.9 Discussion

These small moths can evidently detect the wingbeat sounds of their own species, but the sense organs they use have not been identified. I examined the

antennae as potential sound receivers. I show that their filiform antennae are similar in both sexes of the moths and have a Johnston's organ in their second segment, similar in structure to that of the IMM. The antennae behave as simple resonant physical systems and are sensitive enough to vibrate in response to sound frequencies generated by the wingbeat of nearby conspecifics of either sex. They could clearly function as directional sound receptors.

I discovered that PTB generate ultrasonic clicks with each wingbeat and that pheromone traps emitting wingbeat sounds attract more males in the field than traps without sound. Their antennae do not vibrate in response to ultrasound but both sexes of PTB have an area of cuticle above the hind leg that does vibrate with ultrasound and may be an ultrasound receptor. To compare the species I will discuss the results from both in the following sections.

5.10 Morphology

The antennae of PTB and WCM are long, filiform structures similar between the sexes and to those of IMM (Chapter 4), but without the swollen pedicels found in mosquitoes (eg. *Ae. togoi* (Chapter 3)). All have similar innervation of the antennae, with a ring of neurons in their pedicel in the expected position of Johnston's organ. The upright position in which the moths hold their antennae during mate location would be ideal for the reception of sound waves. However, electrophysiological recordings from the antennae are needed to show conclusively that they are hearing organs. Their morphology is very similar to that of antennae with known auditory function in other insects (Dreller & Kirchner, 1993; Todi *et al*., 2004).

The metepisternite in PTB resembles the tympana of other moths (eg. IMM Chapter 4). In some atympanate moths the metathoracic wing-hinge chordotonal organ is attached to the metepimeron, and is homologous with the scoloparium of noctuids (Minet & Surlykke, 2003). The metepimeron touches the metepisternite, so it is possible that the metathoracic wing-hinge chordotonal organ attached to

the metepisternite instead of the metepimeron. Tympana are believed to have evolved from sclerites (Minet & Surlykke, 2003). The chordotonal proprioceptors present in numerous locations throughout the insect body are believed to require few modifications to become tympana. Tympana have already evolved independently at least three times in the Lepidoptera (Yack & Fullard, 1993). The vibration of the sclerite in response to ultrasound, the air space I saw below it, and the proximity of the wing-hinge chordotonal organ fits well with its possible function as an ear. Finding a scolopidium and taking electrophysiological recordings are needed to confirm its function as an ear.

5.10.1 **Sound and ultrasound recordings**

The wingbeat of all flying insects generates a complex sound containing many harmonics. Even with two wings, each wingbeat creates two peaks of pressure, generated during the upstroke and downstroke at the point when the wing reaches its highest velocity (Bennet-Clark & Ewing, 1968). Because wingbeat frequency is associated with wing size (Belton, 1986), I expected that male and female PTB and WCM, similar in size, would have similar wingbeat frequencies and that their sound pressure levels would not differ greatly between the sexes. Although I was unable to obtain wingbeat recordings from female WCM, female and male PTB had similar wingbeat frequencies and SPL.

PTB's ultrasonic clicks are in the same frequency range (25-80 kHz) as those of IMM (Trematerra & Pavan, 1995; Chapter 4). The ultrasound produced by PTB has not been described previously. The clicks are evidently associated with the wingbeat because they occur consistently at a particular phase. Many noctuid moths, larger species thought to have evolved after the two clicking species I studied (Maddison & Schulz, 2007), generate ultrasonic clicks similar to those of PTB as they fly. No obvious tymbals or other sound producing organs have been found in four species of *Lymantria* (Rowland, 2009), *Prodenia eridania* (Roeder & Treat, 1957), *Heliothis zea* (Kay, 1969) or *Noctua pronuba* and *Agrotis segetum* (Waters & Jones, 1994) all of which generate clicks at particular phases of each

wingbeat in tethered flight. Waters and Jones show that *Noctua pronuba* clap their hind wings together at the top of the wingbeat in free flight and propose that this is the source of the ultrasonic clicks (1994). This could not explain my recording of two similar clicks produced at different phases of one wingbeat (Figure 5.3D), and I recorded ultrasonic clicks from females fluttering in response to nearby males when there was no possibility of their wings touching above the body. Because all the clicks generated by male and female PTB had similar ultrasonic spectra, it seems most likely that they share the same generating mechanism. High speed video and concurrent audio recordings may help elucidate how PTB produces ultrasonic clicks. The single frames from the video of a flying male (Figure 5.6) show the wings bending and straightening, and this could possibly generate clicks.

In contrast to PTB, male WCM do not click or produce ultrasound. Recordings of them fluttering are very similar to previous recordings of males in the presence of conspecifics (Takács *et al*., 2003). The wingbeats I recorded had a sound pressure level of 71 dB at 0.5 cm, corresponding with the 55 dB reported by Takács *et al*. (2003) at 2.5 cm where the pressure would be less. The frequency of their fluttering (58 Hz) is in the middle of the range reported by Takács *et al*. (2003) of 49-63 Hz. Females were never observed to wingfan, but because of the correlation between size and frequency (Belton, 1986), I believe females would have sounds similar to those of males. Because they are known to fly, females would produce a similar sound to that of males although Takács *et al*. (2003) found no evidence for it. Sounds are probably less important than the pheromones females produce attract males.

5.10.2 **Behavioural experiments**

5.10.2.1 Field experiments: PTB

Because significantly more male moths were caught in pheromone traps playing female sound than in silent traps, I conclude that the males must detect female sounds. Although there was an effect of year, treatment traps in both years

caught more moths than the control traps, so the effect may be due to differences in population numbers between the years. PTB produces sound and ultrasound; therefore, to determine if the males prefer one set of frequencies over the other, field tests of the components should be run.

5.10.2.2 Laboratory experiments: PTB

The significant difference between the acoustic replies of female PTB to the wingbeat alone, compared with those in response to the wingbeat + click, and click is evidence that females detect ultrasound. Female PTB responded more rapidly, and produced louder acoustic signals, in response to playbacks of conspecifics that included the ultrasonic click component than playbacks that only included the sound produced by wingbeats indicating that females hear the clicks and respond to them acoustically. However, females did show some response to playbacks incorporating only the wingbeat indicating that the wingbeat component may be detected, but be less important than the click.

The distance trials indicate that there may be an optimal distance for females to respond to male sound. Given that animals communicating with sound must evolve strategies to overcome environmental constraints on sound transmission (Römer & Lewald, 1992), having an optimal calling distance might be another adaptive strategy to use sound communication in a complex environment. At 1 m where all females fluttered in response to playback of the male sound, the SPL would be 33 dB, whereas at 1.5 m away, where some females still fluttered their wings upon exposure to playback of the male sound, the wingbeat would be 29 dB. Twenty-nine dB is close to the lower limit of sensitivity for an insect ear (Robert & Göpfert, 2002), so it is unlikely that this distance would be optimal for accurate sound transmission. Although in nature, 33 dB might also be too quiet when background noise is taken into consideration, the response of all females at 1 m is promising evidence for an optimal calling distance, despite the low number of replicates. To discern the sensitivity of PTB hearing in nature, field tests using different sound pressure levels are needed.

5.10.2.3 Antennal ablation: WCM

The conclusion that WCM use their antennae as sound receptors was not supported by the antennal ablation experiment. Whereas I had expected to show that the absence of antennae altered the ability of WCM to discriminate between playback and white noise, there was no effect of antennae on stimulus choice. However, few moths were attracted to and caught in the traps placed in front of the speakers, and even moths with both antennae showed no preference for playback over white noise. Therefore, the results of this experiment cannot be interpreted as evidence for or against the hypothesis that antennae are sound receptors. This is surprising, given that Takács *et al*. found that more WCM were attracted to wingfanning than to white noise (2003), and found this difference by catching fewer moths than I did.

Whereas the ablation experiment did not support the hypothesis that the antennae detect sound, the increase in female activity and antennal movement with sound compared to before and after sound suggests the moths hear. If they were unable to detect sound, the moths should not have increased their movement when sound was present. This idea that WCM hear is in agreement with Takács *et al*. (2003) who believe the moths use sound to communicate. However, my findings run contrary to ther belief that males are more responsive to sound than females (Takács *et al*., 2003), as the females in my experiment were more active than the males.

Although the laboratory tests do not support the hypothesis that the antennae detect sound, they should not be dismissed as sound receptors. Because the laser vibrometry shows that WCM antennae do vibrate at harmonics of the wingbeat frequency, I believe that the antennae do detect sound, but that it may not be as important in the WCM communication system as are pheromones and host volatiles. Clearly, more work is needed to discern the exact role and level of importance of sound in WCM communication.

5.10.2.4 Laser vibrometry of antennae and metepisternites

Both PTB and WCM antennae are clearly mechanically resonant systems. The antennal vibration of PTB to sound but not to ultrasound shows that they are potential receivers for low frequencies as suggested by Busnel (1963) and Field and Matheson (1998). It is curious that the antennae of both sexes of PTB varied so much from individual to individual because IMM and WCM both have best frequencies consistently close to a harmonic of the wingbeat. The range of best frequencies could indicate that they might detect more harmonics than IMM (Chapter 4) or WCM (below). Their Q values (in the range of 3 to 4), almost the same as those from female *Ae. togoi* (Chapter 3), yet higher than IMM (in the range of 2 to 3: Chapter 4) indicate a tuned system. Their sensitivity, measured as displacement in response to a known sound pressure is the closest to sensitivity of all the moths examined to *Ae. togoi*.

Antennae of male and female WCM had a best frequency at the second harmonic of the wingbeat frequency, and vibrated in response to natural sound levels as recorded from the moths. Thus, the laser vibrometry shows that the antennae can detect the wingbeat. That the Q value of the antennae was relatively low (compared to PTB and *Ae. togoi*) implies that the antennae are not highly tuned; however, their displacement values, similar to those of IMM and *Ae. togoi*, and their mechanical resonance provide evidence for their use in hearing. A small degree of tuning may be all that is required for the moth to identify another as conspecific, or it may be that WCM use sound, but not to the same extent as PTB, IMM, or *Ae. togoi*. Nonetheless, their antennal responses are in line with those from the Johnston's organ of Diptera such as mosquitoes, well documented as highly sensitive to airborne sound (Clements, 1999, Chapter 2), and with those of Gyrinid beetles, used to detect vibrations on the surface of water (Kolmes, 1983). Tympanate ears have been described in the *Micrerethista* and *Harmaclona* genera of the subfamily Harmacloninae (Davis, 1998), but WCM is in the Tineinae subfamily for which tympana have not been described. Davis and Heppner (1987) reported that another tineid, *Ischnuridia virginella* had

tympana-like structures on its second abdominal sternite, but I did not find these structures on WCM. Tympanate ears so far studied in moths would not be suited to detect the wingbeat sound of WCM. However a tarso-pretarsal chordotonal organ of the non-connective type has been described for WCM (Faucheux, 1985). Perhaps this chordotonal organ, like the subgenual organ, might also detect sound through vibration of the substrate. However, this would be unlikely in their environment because the low frequency, low amplitude sounds would not transmit well through fur, bird's nests, and the other sound absorbing materials of their typical habitat.

The PTB metepisternite did not vibrate with low frequencies; but did respond to ultrasound at levels similar to those played to the IMM tympanum (Chapter 4). In their 1993 paper, Yack and Fullard propose three criteria for an insect ear: a morphologically specialized region for detection, an adaptive behavioural response to the sound, and neuronal responses of the area to sound. The spot on the metepisternite meets two of these criteria, the morphologically specialized region, and the behavioural response; unfortunately, not enough specimens were available to attempt histological or electrophysiological studies to support or refute this. However, the results of laser vibrometry are evidence for its functioning as a hearing organ. Whereas Yack and Fullard suggest that many proposed ears are simply body parts resonating to sounds in the 1-5 kHz range that vibrate the exoskeleton (1993), the responses of the metepisternite were best to signals above 70 kHz. The argument might be made that the behavioural responses are due to the subgenual organ. However, subgenual organs are for detecting substrate vibrations, most sensitive to frequencies up to ~5 kHz (Yack, 2004), and in the Lepidoptera (*Manduca sexta*) have only been reported in the pro and mesothoracic legs (Kent & Griffin, 1990), not the metathoracic leg where the PTB proposed ultrasound-detecting structure is.

Electrophysiology on the antennae of both species and the metepisternite of PTB are needed to confirm the laser recordings. To check that chordotonal organs in

the legs are not responding to airborne sound, researchers may also want to run electrophysiology of these structures.

5.11 Conclusion

PTB and WCM share many similarities; both are major pests, evolving early in the history of Lepidoptera, live as adults for a relatively long time, are small in size, and lack tympana, but produce and respond to sound as part of their communication system. Both species have simple filiform antennae about as long as their forewing and because they are similar in size, have similar wingbeat frequencies. However, the similarities end there. Their environments provide different challenges for the use of sounds. In the open, it is advantageous for PTB to use ultrasound, and at close range for WCM to use lower frequencies. The orchard environment at dawn when PTB call, at the tops of trees, would allow more effective transmission of ultrasound than lower in the orchard canopy at a different time of day (Bennet-Clark, 1971; 1998; Forrest, 1994; Sanborn, 2005). In contrast, the animal lairs, bird's nests and closets in which WCM live would readily absorb ultrasound (Bennet-Clark, 1971; Forrest, 1994).

With the incorporation of ultrasound in their communication system, PTB would need to detect those signals, whereas WCM, not utilizing ultrasound, would not. All the previously studied moths that click have tympana that could detect them. Perhaps PTB, like tympanate moths, developed a detector system against foraging bats and turned that to the purpose of communication. If so, the circular area on the metepisternite would be a new type of ear for Lepidoptera, and perhaps represents an evolutionary transition between stretch receptor and tympanum.

5.12 Figures and Tables

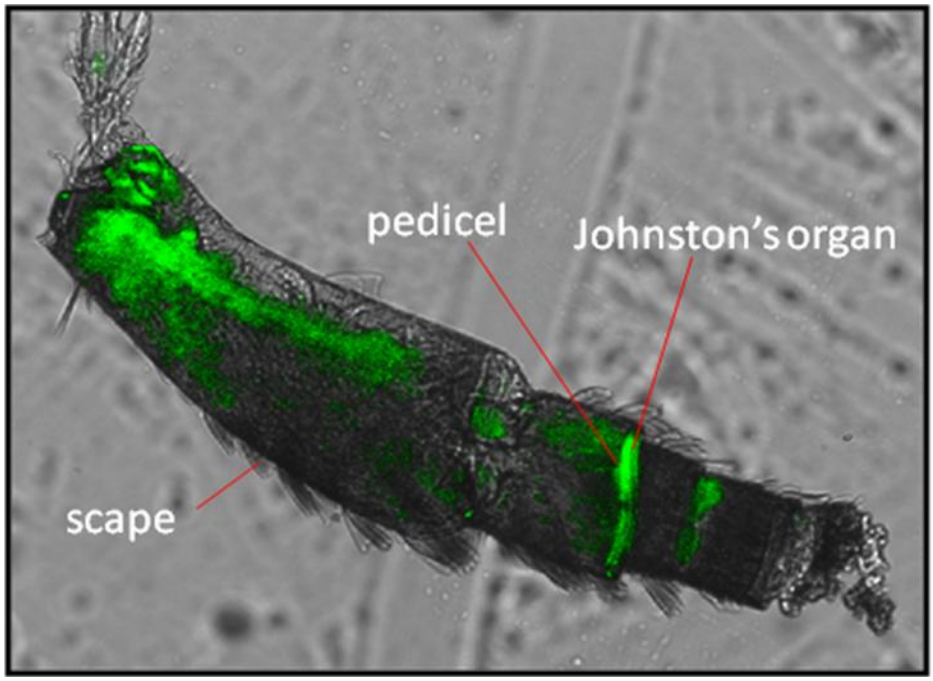


Figure 5.2. The scape, pedicel and first three flagellomeres of a male PTB antenna stained with Neurotrace, showing the location of the Johnston's organ (ring of neurons) in the pedicel. The female pedicel had a similar structure.

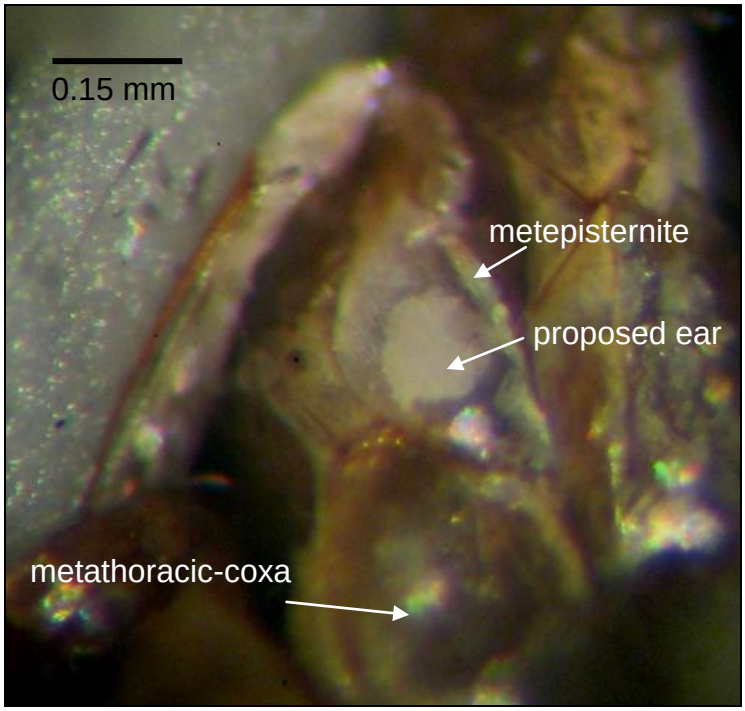


Figure 5.3. Part of the metathorax of a female PTB showing pale circular cuticle (proposed ear) on the metepisternite. The male had an identical area on the metepisternite.

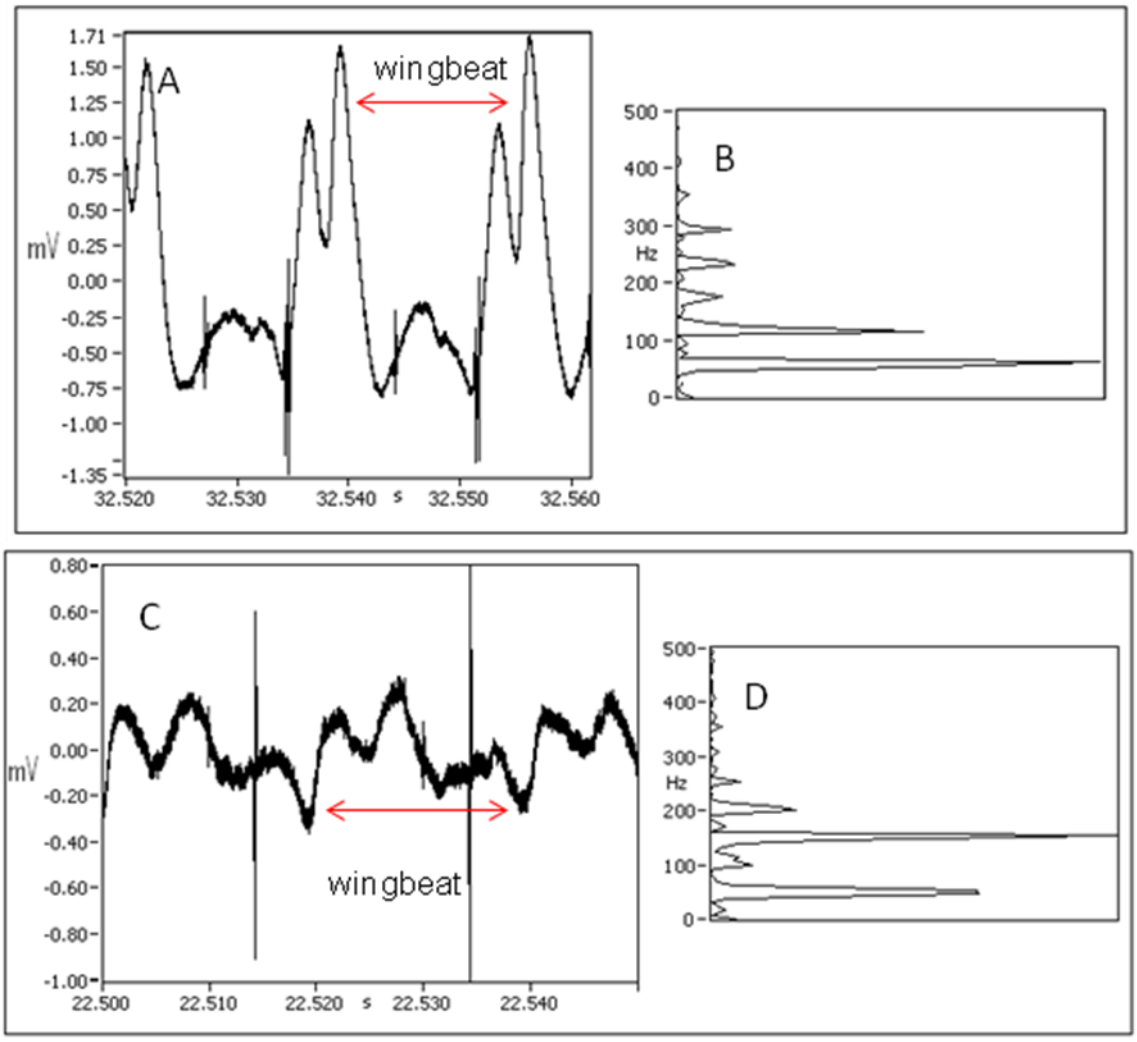


Figure 5.4. Wingbeat frequency of PTBs. A) waveform of male wingbeat (61 Hz); B) its low frequency spectrum C) waveform of a female wingbeat (46 Hz); and D) its low frequency spectrum. In these recordings, both wingbeats generate two clicks.

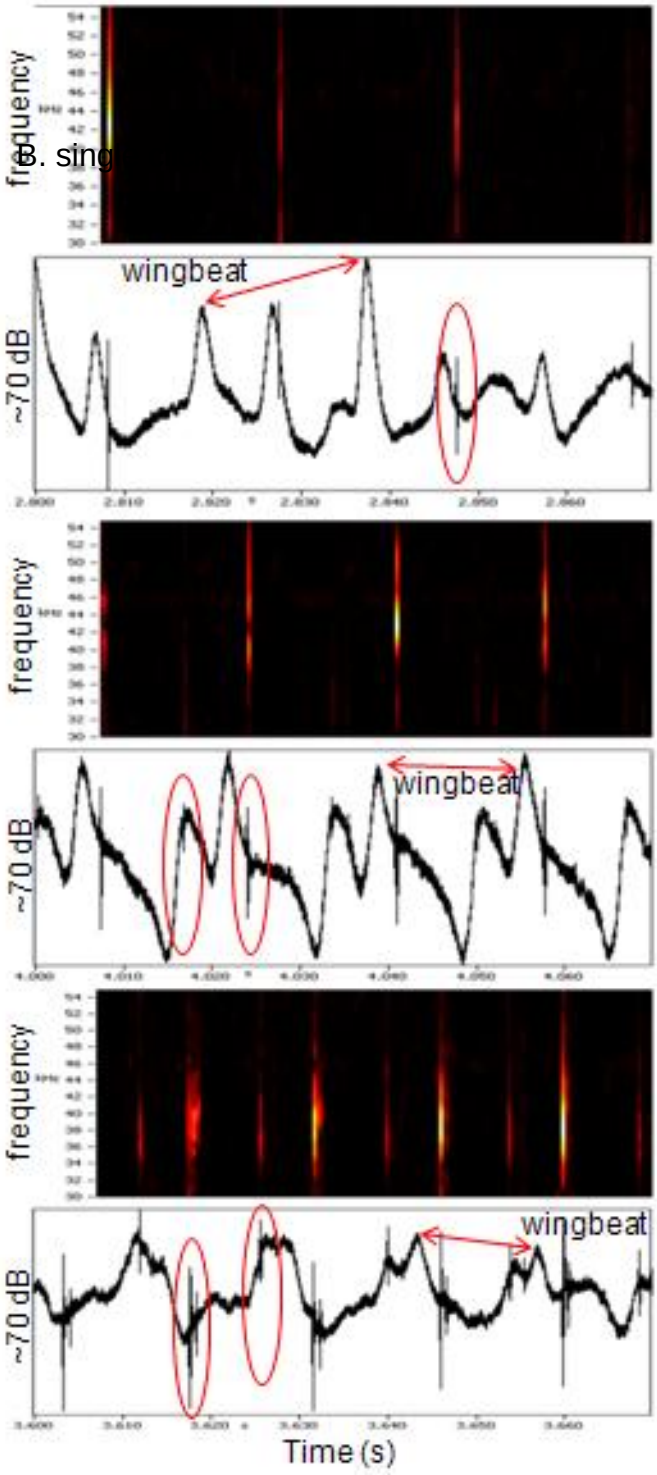


Figure 5.5. Ultrasonic clicks of PTB. A) high frequency spectrum (spectrogram) of the B) showing one click per wingbeat, a female; C) spectrum of D) two clicks per wingbeat, a male; and E) spectrum of F) three clicks per wingbeat, a female. The waveform of the wingbeat varies greatly with the position of the moth and examples of clicks are circled in red. In F) three clicks per wingbeat, the first red circle contains a double click.

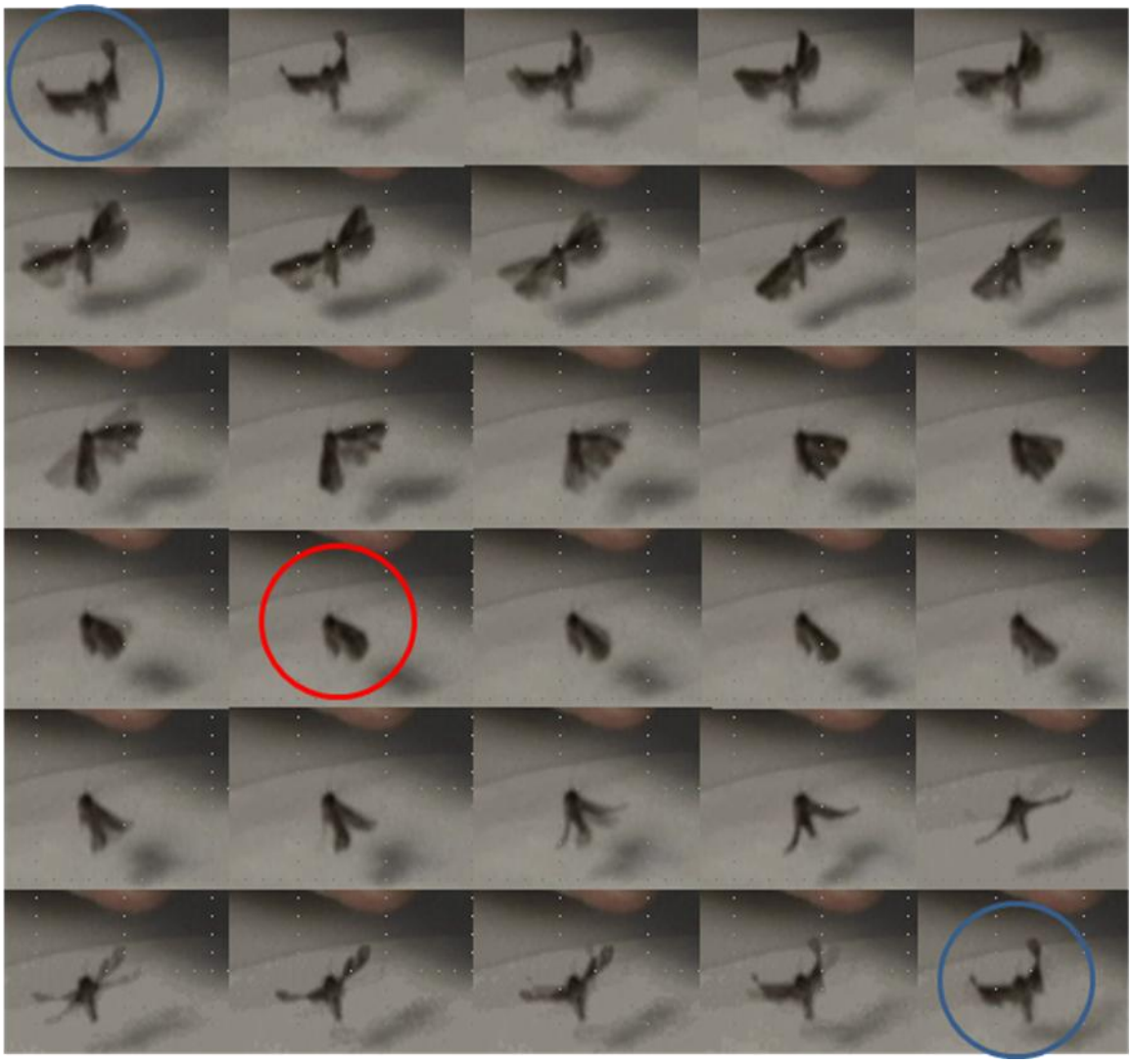


Figure 5.6. Consecutive frames from a video recording showing one complete wingbeat of a tethered PTB male, wingspan ~1.9 cm. The wings touch at the highest (red circle), but not at the lowest point of the wingbeat (blue circles).

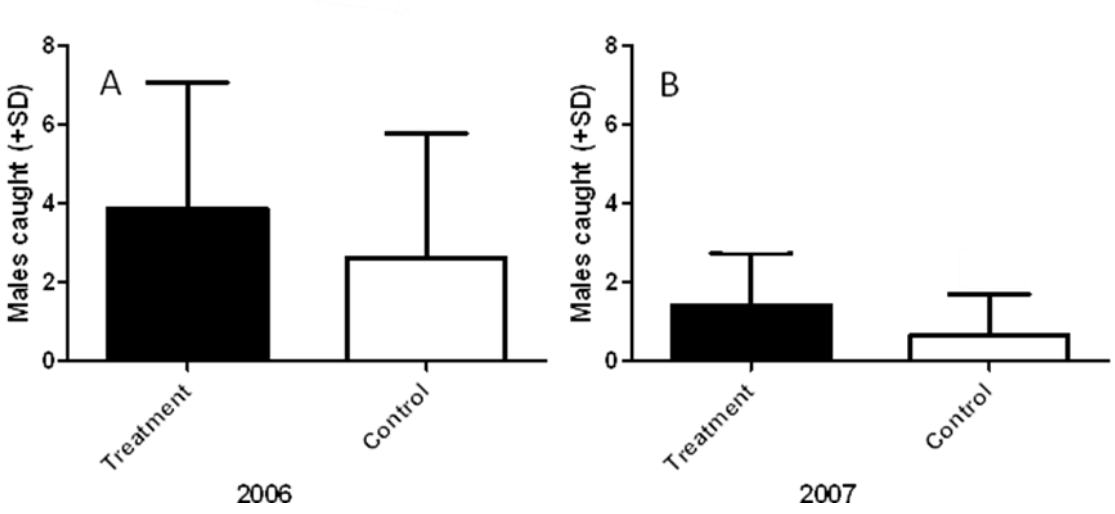


Figure 5.7. Comparisons of the mean catches of 31 trap pairs in 2006 (A) and 17 trap pairs in 2007 (B) of pheromone traps. Traps played female PTB wingbeat sounds (treatment) or were silent (contol).

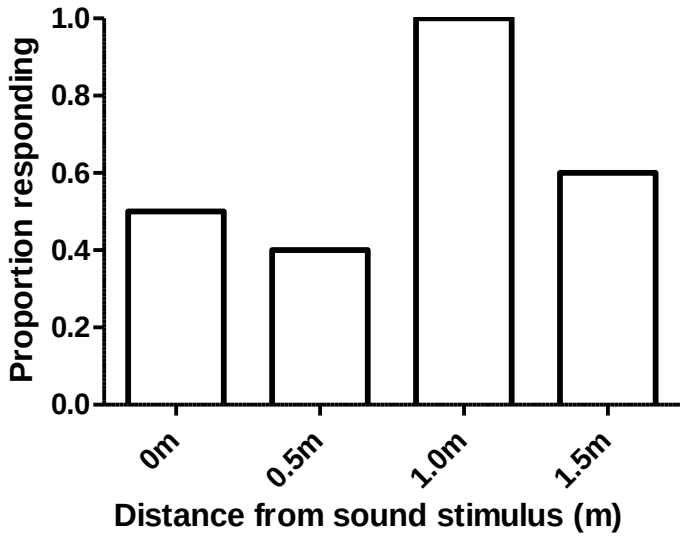


Figure 5.8. Proportion of female PTB fluttering during playback of the complete male sound at different differences (n=6 for 0m, 65 dB; n=5 for 0.5m, 39 dB, 1.0m, 33 dB, and 1.5m, 29 dB).

Table 5.1. Fluttering of female PTB to playback of male sound that includes the wingbeat alone, the wingbeat and ultrasonic clicks, and the ultrasonic clicks alone. The overall results of the ANOVA are presented with different letters that indicate means that differ based on post-hoc Student's t-tests.

Measurement	Wingbeat			Wingbeat + click			Click			F-ratio	*p*-value
	Mean	SD	n	Mean	SD	n	Mean	SD	n		
Amplitude of response (dB)	28.2^{A}	30.1	10	57.6^{B}	2.1	10	49.8^{B}	17.8	10	5.67	<0.01*
Time to respond (s)	69.2^{A}	60.3	9	8.1^{B}	6.8	9	21.3^{B}	37.3	9	5.49	0.01*
Number of times fluttering	27.8^{A}	54.3	10	77.1^{A}	61.8	10	51.1^{A}	88.1	10	1.26	0.30

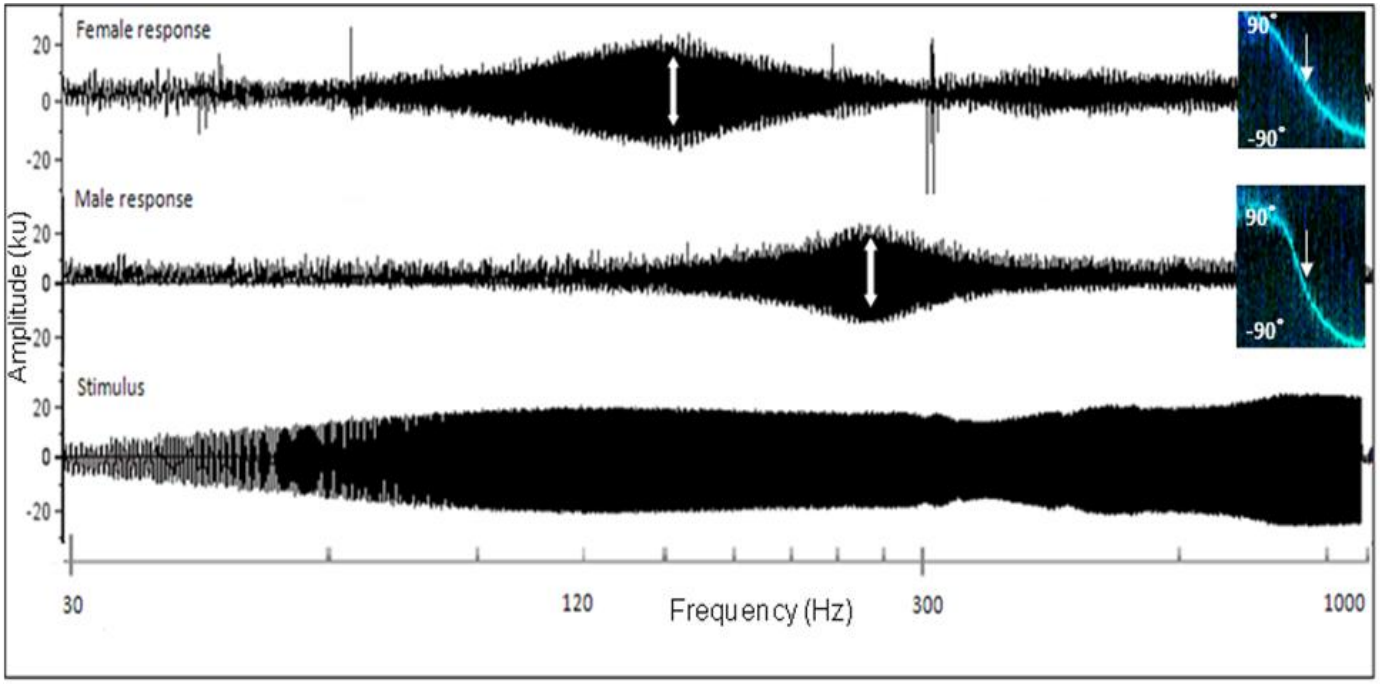


Figure 5.9. Vibration of representative female and male PTB antennae in response to a swept sine wave from 30-1000 Hz. Insets show the phase changes (single arrows) at the best frequency (double arrows).

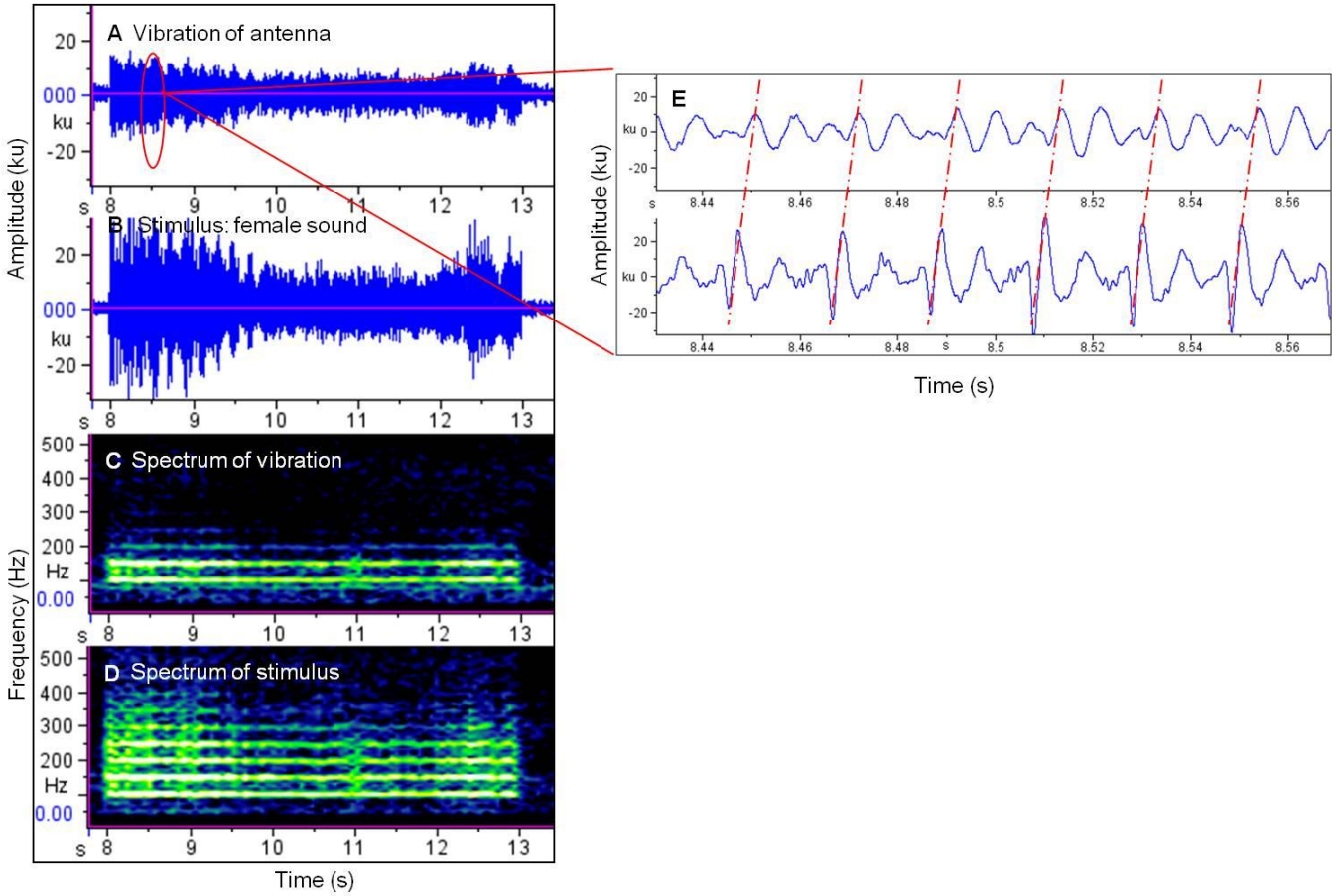


Figure 5.10. Vibration of a male PTB antenna in response to playback of female sound. A) Vibration of the antenna; B) Female wingbeat sound; C) spectrogram of A, with the second harmonic dominant; D) spectrogram of B; and E) enlarged portion of A & B. Dotted lines show correlation between the vibrating antenna and the stimulus.

.

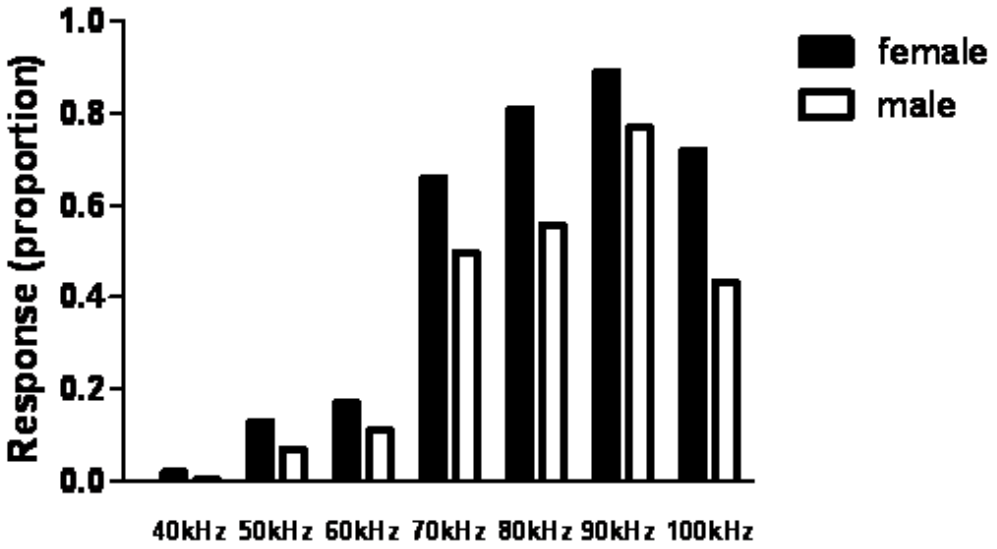


Figure 5.11. Relative amplitude of vibration of the metepisternite of male and female PTB. Stimuli were pure tones at 85 dB. Responses are the mean of three measurements from six moths of each sex, expressed as a proportion of the greatest response from each insect.

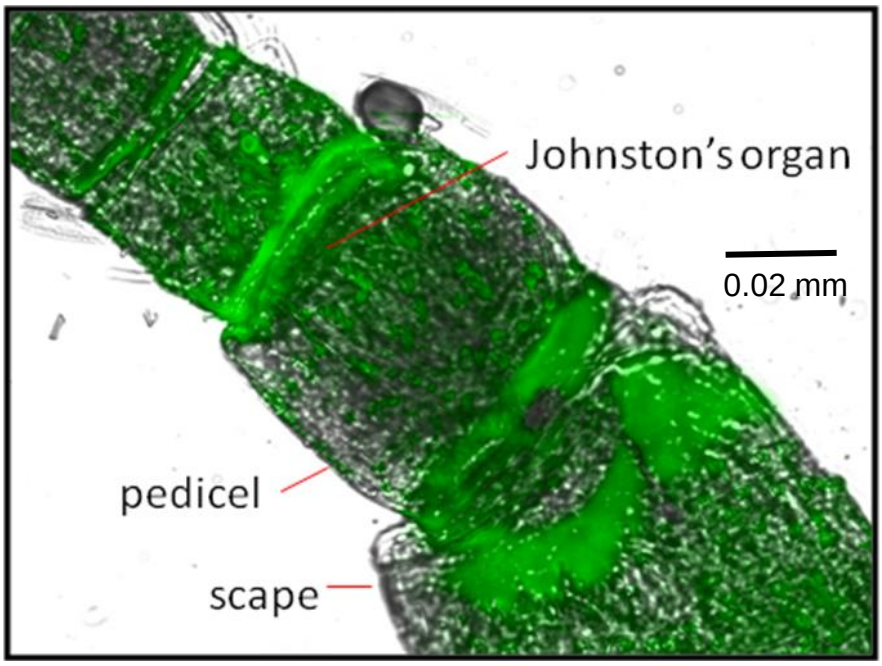


Figure 5.12. Base of an antenna from a male WCM stained with Neurotrace showing a ring of neurons in the distal pedicel.

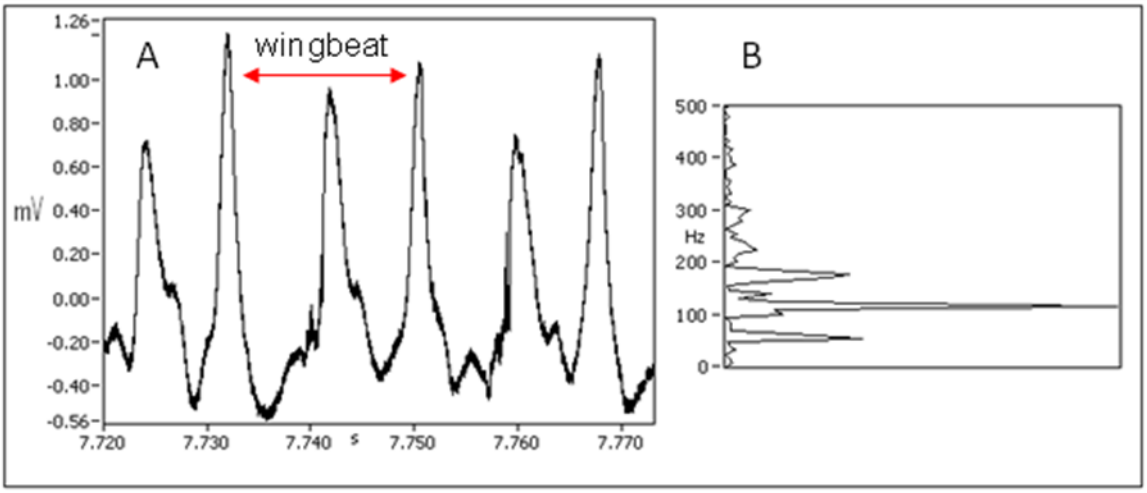


Figure 5.13. Wingbeat of wingfanning male WCM (females were never seen wingfanning). A) waveform of male wingbeat (53.9 Hz), one cycle between arrows; B) frequency spectrogram of waveform shown in A: the second harmonic predominates. The mean sound pressure level at 0.5 cm was 71 dB.

Table 5.2. Mean catch of male WCM with 0, 1 or 2 antennae to playback of male wingbeat (wingfanning) or white noise in 20 trials with three males of each antennal type.

Number of antennae	Wingfanning		White noise	
	Moths caught (mean)	SD	Moths caught (mean)	SD
0 antennae	0.10	0.31	0.25	0.44
1 antenna	0.45	0.60	0.25	0.44
2 antennae	0.30	0.47	0.40	0.60

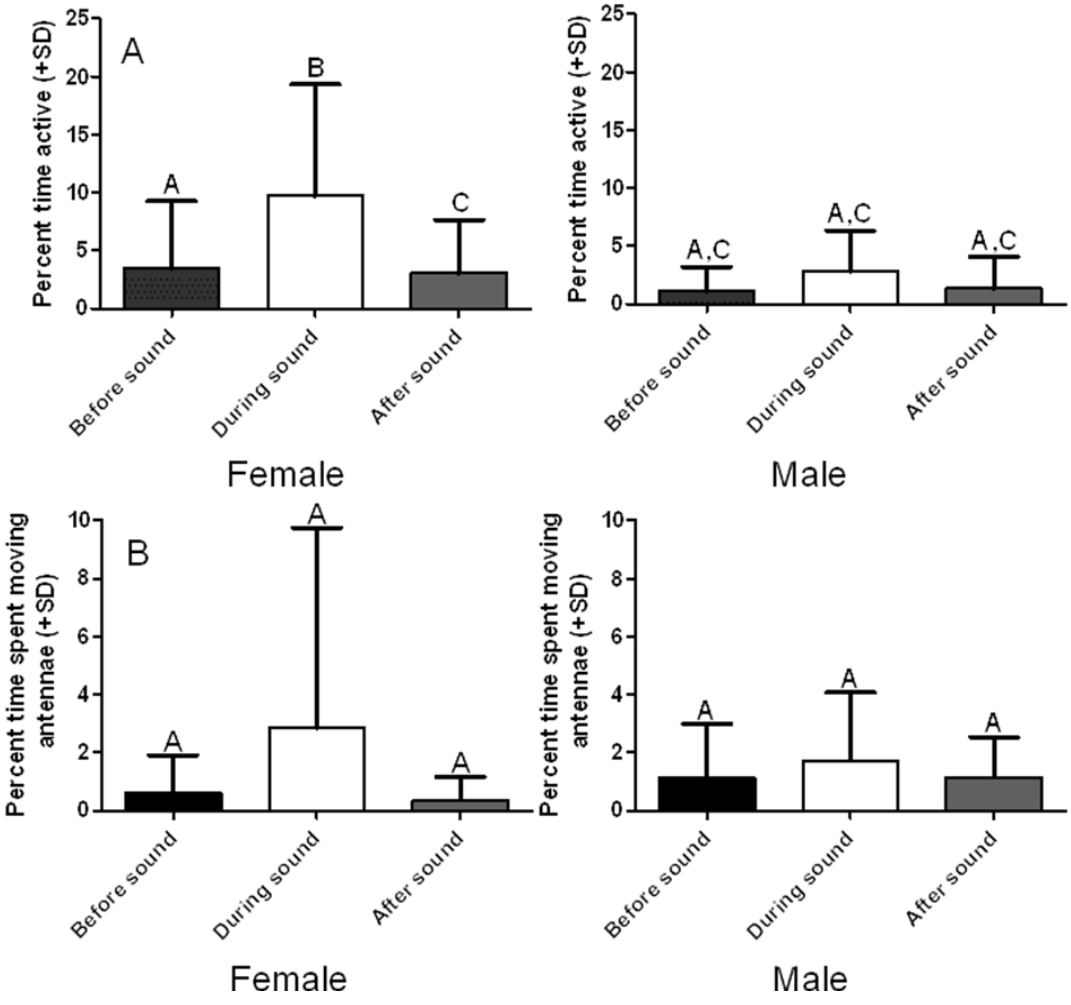


Figure 5.14. Movement of 20 female and 20 male WCM to playback of male wingbeat (before sound = 1 minute silence, during sound = 2 minutes sound, after sound = 1 minute silence). A) mean duration of antennal movement in response to sound; B) mean duration of activity (other than antennal movement) in response to sound. Times between the sexes with different letters are significantly different from each other.

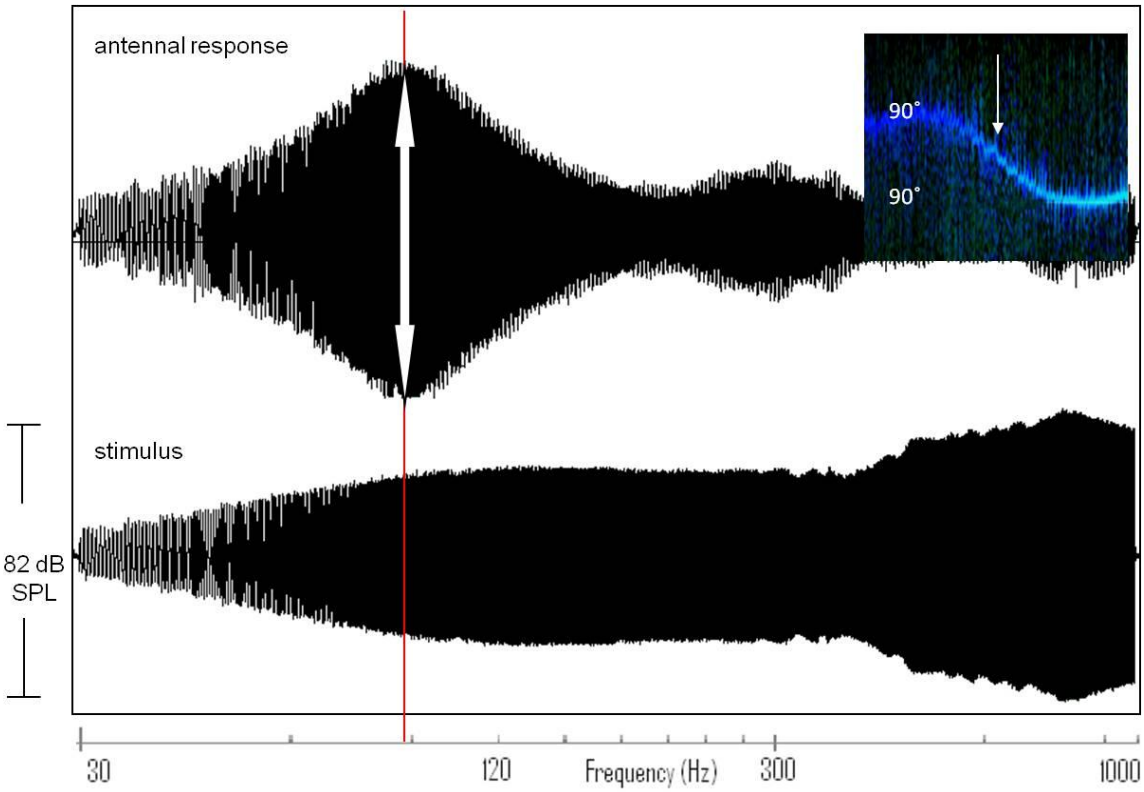


Figure 5.15. Vibration of a typical female WCM antenna in response to a swept sine wave from 30-1000 Hz. Inset, upper right, shows the phase change (single arrow) at best frequency of 90 Hz (double arrows).

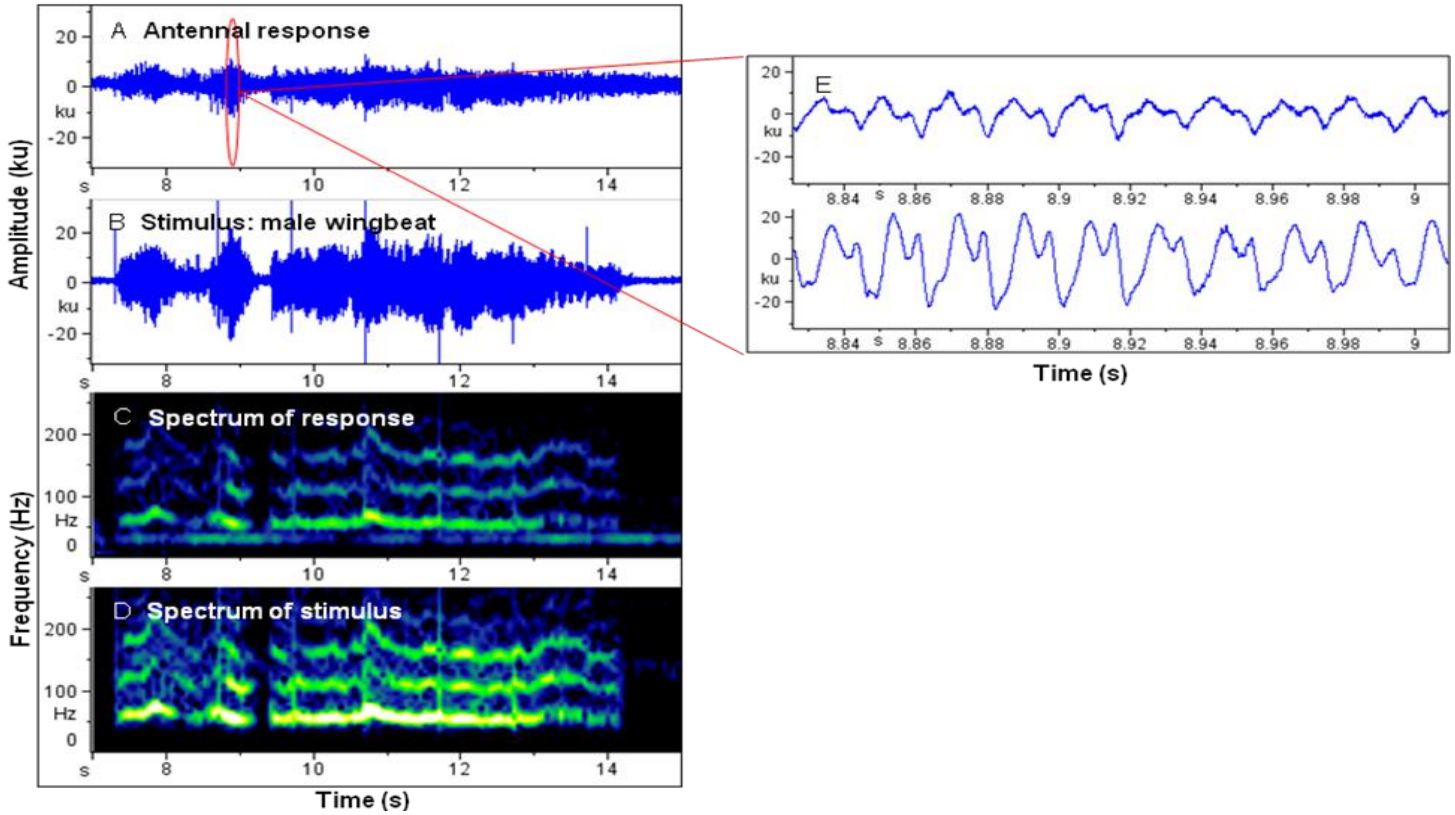


Figure 5.16. Vibration of WCM female antennae to male wingbeat; A) waveform of female response; B) waveform of male sound; C) spectrogram of A; D) spectrogram of B; and E) enlarged section of A & B.

6 CONCLUSION

My main goal was to investigate the structure and function of the antennae of three economically important species of moths. I compared them with the antennae of males and females of a species of mosquito, *Aedes togoi.* Mosquitoes have among the most sensitive ears known in the animal kingdom in the Johnston's organs of their antennae. My results show for the first time that the antennae of both sexes of all three species of moth can potentially detect sound. However, the results also provide ideas for future research and raise questions about the application of sound for pest management.

Both sexes of *Aedes togoi* produce sounds with their wings as they fly. Whereas males detect the fundamental frequency of the female wingbeat, the relevance of the frequency to which females are tuned remains unknown. The best frequency of the female antennae fits well with recent studies showing that female mosquitoes hear the difference between their wingbeat frequency and that of males or their harmonics (Warren *et al*. 2010, Pennetier *et al*., 2010). If females hear difference tones, research into why and how this happens is needed. Although female mosquitoes have not been trapped using male wingbeat sounds, those that feed on frog blood have been attracted to traps playing frog sounds. The possibility that female *Ae. togoi* are attracted to the sound of their hosts cannot be ruled out. Because mosquitoes vector numerous viruses and diseases, better understanding of their use of sound might facilitate more effective control methods.

Both sexes of Indianmeal moths produce wingbeat sounds and ultrasonic clicks. As a stored products pest, IMMs are a prime target of control efforts. Previous research showed that infrasound accelerated larval development (Mullen, 1973), whereas extended exposure to sound from 120 Hz to 2 kHz at

amplified levels led to 75% fewer larvae surviving (Kirkpatrick & Harein, 1965). This is the extent of research into lower frequencies on IMM. Sounds of all frequencies may show promise for control of the IMM, although the moths may adapt to the signals, just as tympanate moths have evolved to counter echolocating techniques of bats (Conner & Corcoran, 2012).

Both sexes of peach twig borers generate low frequency sounds and ultrasonic clicks as they fly, with the wingbeat vibrating the antennae and the ultrasound vibrating a circular area on the metepisternite. Although pheromones are used to trap PTB, using pheromones to disrupt mating has not always been successful (Bentley, 2012). The moth's reported use of sound could be the reason for this inconsistency; if they use pheromones for long distance communication and sound for pinpointing a mate, saturating an orchard with pheromone will not affect the close range sound communication. Even worse, the high pheromone concentration could draw moths into an area where they then find a mate through acoustic signalling. Understanding the role of sound in PTB biology could allow for better, environmentally friendly control of the moths in the field. Their use and detection of ultrasound also has intriguing implications for evolutionary biologists. The PTB is an evolutionarily primitive atympanate moth (Maddison & Schulz, 2007). If the metepisternite can detect ultrasound, then a new hearing organ, different from tympana, exists in the Lepidoptera. Electrophysiology must be done to confirm the structure's ability to function as an ultrasound detector. Other atympanate moths in the same and related families should be examined for similar structures, and the evolution of these ears in relation to the appearance of bats examined.

Male Webbing clothes moths only generate low frequency sounds when they fan their wings or fly. In this and a previous study (Takács *et al*., 2003), the females were never seen to wingfan or fly. Female antennae vibrated best at a harmonic of the male wingbeat frequency; however, behavioural experiments showed no significant effects of sound on the moths. WCM lives in habitats

where wingbeat sounds would be readily absorbed. Their antennal vibrations may indicate that WCMs can hear low frequency sounds, whereas their behaviour may indicate that other cues, such as olfactory signals, may be more important. The wingfanning could then play a more important role in disseminating chemical cues. If chemoreception is more important than sound reception in the moths' communication, perhaps research into completely disrupting the scents through the use of fans (possibly running at the same frequency as the best frequency response of the antennae to confuse any sound stimuli) would be a more effective approach to controlling WCM.

Overall, the work presented in this thesis has shown the moths, like mosquitoes, can use their their antennae to detect sound. The extent to which each species incorporates sound in its communication needs further exploration. The use of antennal sound reception should also be examined in other tympanate and atympanate moths to see how widespread it is in Lepidoptera. Management of these economically important moths using sounds, either by disrupting their sound communication, or using acoustics to attract them or alter their behaviour, might be feasible. However, problems with annoying people with the use of sound in control efforts, effective deployment of sound signals in the environment, effects of the signals on other organisms and the time before moths adapt to the sounds all need to be investigated before acoustics are used in pest control. Knowing that moths can use their antennae to detect sound and that some could possess non-tympanate ultrasound-detecting ears are exciting discoveries; the research possibilities that extend from these finding are limitless.

APPENDICES

6.1 Appendix A

I used a B&K microphone to test the full range of a Knowles SPM0404UD5 microphone, the specifications of which did not describe the microphone's sensitivity to the frequency range I was interested in recording. The results fit well with a report that has since been published, characterizing the frequency response of the SPM0404UD5 (http://www.digikey.ca/product-detail/en/SPM0404UD5/423-1086-1-ND/1587388).

6.2

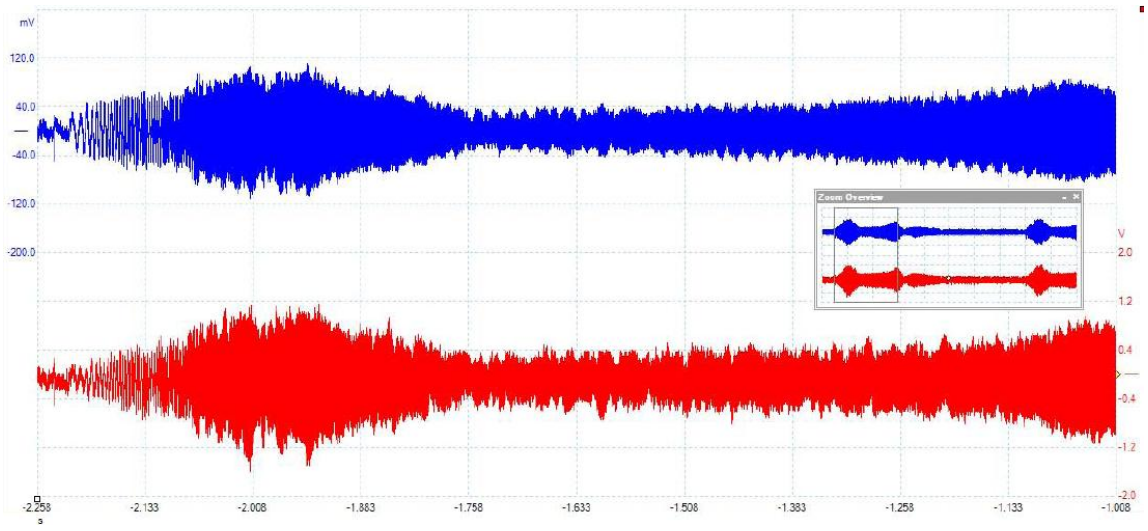


Figure B. 1. Testing the frequency response of the Knowles SPM0404UD5, upper blue trace, against a 1/4" B & K condenser microphone (C weighted). Both detect 15 Hz to 15 kHz (small inset). Zoomed in portion, shown surrounded by a box in the inset, shows the low-frequency responses of each microphone.

6.3 Appendix B

I used a nomogram to calculate the SPL from the output voltage of the various microphones used.

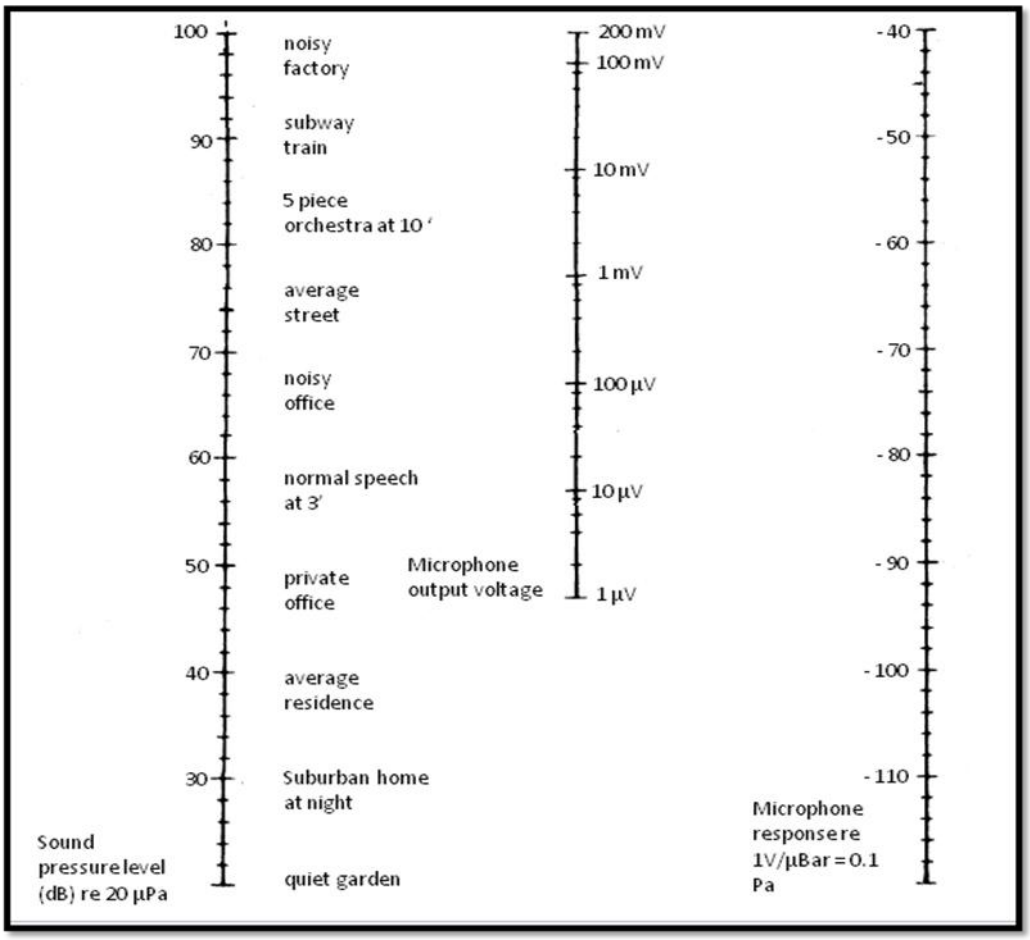


Figure C. 1. Nomogram used to calculate sound pressure level dB from root mean square values of mV responses from sound recordings. The nomogram is modified from Conover (1956).

REFERENCES

Agee HR (1969) Acoustic sensitivity of the European corn borer moth, *Ostrinia nubilalis*. Annals of the Entomological Society of America. 62(6): 1364-1367.

Ahmad TR (1989) Pheromone concentration and plant cover types on movement of *Anarsia lineatella* (Zeller) (Lepidoptera, Gelechiidae). Insect Science and its Application. 10: 43-48.

Ahmad TR & Khadhum, AA (1986) Influence of pheromone trap design and placement on capture of peach twig borer, *Anarsia lineatella* (Zeller) (Lepidoptera: Gelechiidae). Insect Science and its Application. 7: 637-640.

Anonymous (2008) *Aedes* (Finlaya) *togoi* (Theobald) pest information sheet. N.Z. BioSecure. http://www.smsl.co.nz/site/southernmonitoring/files/NZB/Aedes%20togoi%20-%20profile%20Feb%2008.pdf

Arthur BJ, Wyttenbach RA, Harrington LC & Hoy RR (2010) Responses to one and two tone stimuli in the hearing organ of the dengue vector mosquito. Journal of Experimental Biology. 213: 1376-1385.

Autrum H (1963) Anatomy and physiology of sound receptors in invertebrates. In Acoustic Behaviour of Animals. Ed. RG Busnel pp 412-433 Elsevier, Amsterdam.

Avitabile D, Homer M, Champneys AR, Jackson JC & Robert D (2010) Mathematical modelling of the active hearing process in mosquitoes. Journal of the Royal Society Interface. 7**:** 105-122.

Bailey SF (1948) The peach twig borer. California Agricultural Experiment Station Bulletin no. 708. University of California, Berkeley, CA, USA.

Bailey WJ (1978) Resonant wing systems in the Australian whistling moth *Hecatesia* (Agarasidae, Lepidoptera). Nature*.* 272: 444–446.

Bartlett-Healy K, Crans W & Gaugler R (2008) Phonotaxis to amphibian vocalizations in *Culex territans* (Diptera: Culicidae). Annals of the Entomological Society of America. 101(1): 95-103.

Belton EM (1988) Lepidoptera on fruit crops in Canada. Pest Management Papers no. 30, pp.18-19.

Belton P (1962) Responses to sound in pyralid moths. Nature*.* 196: 1188–1189.

Belton P (1986) In: Insect Flight: Dispersal and Migration. Ed. W. Danthanarayana.Springer Verlag, Berlin.

Belton P & Belton OC (1990) *Aedes togoi* comes aboard. Journal of the American Mosquito Control Association. 6(2): 328-329.

Belton P & Costello RA (1979) Flight sounds of the females of some mosquitoes of Western Canada. Entomologia Experimentalis et Applicata 26: 105-114.

Bennet-Clark HC (1971) Acoustics of insect song. Nature. 234: 255–259.

Bennet-Clark HC (1998) Size and Scale Effects as Constraints in Insect Sound Communication. Philosophical Transactions: Biological Sciences. 353(1367): 407-419.

Bennet-Clark HC (1999) Which Q's to choose: questions of quality in bioacoustics? Bioacoustics Journal. 9(4): 351-359.

Bennet-Clark HC & Ewing AW (1968) The wing mechanism involved in the courtship of *Drosophila*. Journal of Experimental Biology. 49: 117-128.

Bennett AL (1989) On the mechanism of ultrasound production in *Galleria mellonella* (L) (Lepidoptera, Pyralidae). Journal of the Entomological Society of Southern Africa. 52(2): 517-523.

Bennett AL, Atkinson PR & Lacroix NJS (1991) On communication in the African sugaracane borer, *Eldana saccharina* Walker (Lepidoptera, Pyralidae). Journal of the Entomological Society of Southern Africa. 54(2): 243-259.

Bentley WJ (2012) Peach Twig Borer. UC IPM Pest Management Guidelines: Peach. UC ANR Publication 3454.

Birch MC & Keenlyside JJ (1991) Tapping behavior is a rhythmic communication in the death-watch beetle, *Xestobium rufovillosum* (Coleoptera: Anobiidae). Journal of Insect Behavior. 4: 257-263.

Blest AD, Collett TS & Pye JD (1963) The generation of ultrasonic signals by a New World arctiid moth. Proceedings of the Royal Society of London Series B. 158:196-207.

Boo KS & Richards AG (1975) Fine structure of the scolopidia in the Johnston's organ of male *Aedes aegypti* (L.) (Diptera:Culicidae). International Journal of Insect Morphology and Embryology 4: 549-566.

Borkent A (2008) The Frog-Biting Midges of the World (Corethrellidae: Diptera). Zootaxa. 1804:1-456.

Borkent A & Belton P (2006). Attraction of female *Uranotaenia lowii* (Diptera: Culicidae) to frog calls in Costa Rica. The Canadian Entomologist. 138: 91-94.

Bosworth W & Ewert A (1971) Infectivity of *Brugia malayi* larvae recovered from *Aedes togoi* mosquitoes at various times after a single blood meal. Journal of Medical Entomology. 6(30):717-719.

Brady UE, Jay EG, Redlinger LM & Pearman G (1975) Mating activity of *Plodia interpunctella* and *Cadra cautella* during exposure to synthetic sex pheromone in the field. Environmental Entomology. 4: 441-444.

Brunel-Pons O, Alem S & Greenfield MD (2011) The complex auditory scene at leks: balancing antipredator behaviour and competitive signalling in an acoustic moth. Animal Behaviour 81(1): 231–239.

Brunner JF & Rice RE (1984) Peach twig borer *Anarsia lineatella* Zeller (Lepidoptera: Gelechiidae), development in Washington and California. Environmental Entomology 13: 607-610.

Bura VL, Rohwer VG, Martin PR & Yack JE (2011) Whistling in caterpillars (*Amorpha juglandis*, Bombycoidea): sound-producing mechanism and function. The Journal of Experimental Biology. 214: 30-37.

Busnel RG (1963) Acoustic Behaviour of Animals. Elsevier, Amsterdam. 933 pp.

Campbell JF, Mullen, MA & Dowdy AK (2002) Monitoring stored-product pests in food processing plants with pheromone trapping, contour mapping and mark-recapture. Journal of Economic Entomology. 95:1089-1101.

Christensen-Dalsgaard J & Manley GA (2005) Directionality of the lizard ear. Journal of Experimental Biology. 208: 1209-1217.

Christophers SR (1960) *Aedes aegypti* (L.), the yellow fever mosquito; its life history, bionomics, and structure. University Press, Cambridge, England. 738 pp.

Clements AN (1999) The biology of mosquitoes volume 2: Sensory reception and behavior. CABI Publishing. New York, NY. p. 55-87.

Cokl A (1983) Functional properties of vibroreceptors in the legs of *Nezara viridula* (L.) (Heteroptera: Pentatomidae). Journal of Comparative Physiology. 150: 261-269.

Cokl A, Virant-Doberlet M & McDowell A (1999) Vibrational directionality in the Southern green stink bug, *Nezara viridula* (L.), is mediated by female song. Animal Behavior. 58:1277-1283.

Conner WE (1999) 'Un chant d'appel amoureux': Acoustic communication in moths. Journal of Experimental Biology 202: 1711-1723.

Conner WE & Corcoran AJ (2012) Sound strategies: the 65-million-year-old battle between bats and insects. Annual Review of Entomology. 57:21-39.

Connétable S, Robert A, Bouffault F & Bordereau C (1999) Vibratory alarm signals in two sympatric higher termite species: *Pseudacanthotermes spiniger* and *P. militaris* (Termitidae, Macrotermitinae). Journal of Insect Behavior. 12: 329-342.

Conover W (1956) Micophone nomograph. Electronics. Vol. 29(6): r24.

Corcoran AJ, Conner WE & Barber JR (2010) Anti-bat tiger moth sounds: form and function. Current Zoology. 56(3): 358-369.

Costello RA (1974) Effects of environmental and physiological factors on the acoustic behavior of *Aedes aegypti* (L.) (Diptera: Culicidae). PhD Thesis. SFU, Burnaby.

Cowan T & Gries G (2009) Ultraviolet and violet light: attractive orientation cues for the Indian meal moth, *Plodia interpunctella*. Entomologia Experimentalis et Applicata. 131(2): 148-158.

Cox PD & Pinniger DB (2007) Biology, behaviour and environmentally sustainable control of *Tineola bisselliella* (Hummel) (Lepidoptera: Tineidae). Journal of Stored Products Research. 43:2-32.

Daane KM, Yokota GY & Dlott JW (1993) Dormant-season sprays affect the mortality of peach twig borer (Lepidoptera: Gelechiidae) and its parasitoids. Journal of Economic Entomology 86: 1679-1685.

Davis DR (1998) A world classification of the *Harmacloninae*, a new subfamily of *Tineidae* (Lepidoptera: Tineoidea). Smithsonian Contributions to Zoology. 597: 1-81.

Davis DR & Heppner JB (1987) New discoveries concerning *Ischnuridia* Sauber, A Remarkable Genus of Indo-Australian Tineidae (Lepidoptera). *Tinea*. 12(suppl): 145-150.

Desutter-Grandcolas L (1995) Functional forewing morphology and stridulation in crickets (Orthoptera, Grylloidea). Journal of Zoology, London. 236: 243-252.

Downes JA (1969) The swarming and mating flight of Diptera. Annual Review of Entomology. 14:271–298.

Dreller C & Kirchner WH (1993) Localization of the auditory sense organ. Journal of Comparative Physiology A. 173(3): 275-279.

Drosopoulos S & Claridge MF (eds) 2005: Insect sounds and communication: physiology, behaviour, ecology and evolution. CRC Press, Taylor & Francis Group, New York. 532 pp.

Dunning DC & Krüger M (1996) Predation upon Moths by Free-Foraging *Hipposideros caffer*. Journal of Mammalogy. 77(3): 708-715.

Dunning DC & Roeder KD (1965) Moth sounds and the insect-catching behavior of bats. Science. 147: 173-174.

Duruz WP (1923) The peach twig-borer (*Anarsia lineatella* Zeller). Agricultural Experimental Station Bulletin Berkley California 355: 421-463.

Ebeling W (1978) Urban Entomology. University of California Press. Berkeley. 695 pp.

Edgecomb RS, Robert D, Read MP & Hoy RR (1995) The tympanal hearing organ of a fly: phylogenetic analysis of its morphological origins. Cell & Tissue Research. 282:251-268.

Faucheux MJ (1985) Structure of the tarso-pretarsal chordotonal organ in the imago of *Tineola bisselliella* Humm (Lepidoptera, Tineidae). International Journal of Insect Morphology & Embryology. 14(3): 147-154.

Field LH (2004) The Chordotonal Organ: A Uniquely Invertebrate Mechanoreceptor. In: Methods in Insect Sensory Neuroscience. Ed. TA Christensen. CRC Press, Florida. 61-106.

Field LH & Matheson T (1998) Chordotonal organs of insects. Advances in Insect Physiology. 27: 1-228.

Fletcher NH (1992) Acoustic Systems in Biology. Oxford University Press, New York. 333 pp.

Forrest TG (1994) Sender to receiver: propagation and environmental effects on acoustic signals. American Zoologist. 34(6): 644-654.

Fullard JH & Heller B (1990) Functional organization of the arctiid moth tymbal (Insecta, Lepidoptera). Journal of Morphology. 204 (1): 57-65.

Gerhardt HC & Huber F (2002) Acoustic Communication in Insects and Anurans: Common Problems and Diverse Solutions. University Of Chicago Press. Chicago. pp. 542

Gerstner AT, Poulsen M & Currie CR (2011) Recruitment of minor workers for defense against a specialized parasite of *Atta* leaf-cutting ant fungus gardens. Ethology Ecology & Evolution. 23(1): 61-75.

Gibson G & Russell IJ (2006) Flying in tune: Sexual recognition in mosquitoes. Current Biology. 16: 1311-1316.

Gibson G, Warren B & Russell IJ (2010) Humming in tune: sex and species recognition by mosquitoes. Journal of the Association for Research in Otolaryngology. 11: 527-540.

Göpfert MC, Briegel H & Robert D (1999) Mosquito-hearing: sound-induced antennal vibrations in male and female *Aedes aegypti*. The Journal of Experimental Biology. 202: 2727-2738.

Göpfert MC & Robert D (2000) Nanometre-range acoustic sensitivity in male and female mosquitoes. Proceedings: Biological Sciences. 267: 453-457.

Göpfert MC & Robert D (2001) Active auditory mechanics in mosquitoes. Proceedings: Biological Sciences. 268: 333-339.

Göpfert MC & Robert D (2002) The mechanical basis of *Drosophila* audition. The Journal of Experimental Biology. 205:1199-1208

Göpfert MC, Surlykke AM & Wasserthal LT (2002) Tympanal and atympanal 'mouth-ears' in hawkmoths (Sphingidae). Proceedings of the Royal Society of London Series B. 269(1486): 89-95.

Göpfert MC & Wasserthal LT (1999) Auditory sensory cells in hawkmoths: identification, physiology and structure. Journal of Experimental Biology. 202(12): 1579-1587.

Grant GG (1974) Male sex pheromone from the wing glands of the Indian meal moth, *Plodia interpunctella* (Hbn.) (Lepidoptera: Phycitidae). Experientia. 30: 917-918.

Grant GG & Brady UE (1975) Courtship behavior of phycitid moths. I. Comparison of *Plodia interpunctella* and *Cadra cautella* and role of male scent glands. Canadian Journal of Zoology. 53: 813-826.

Greenfield MD & Coffelt JA (1983) Reproductive behaviour of the lesser waxmoth, *Achroia grisella* (Pyralidae, Galleriinae) Signalling, pair formation, male interactions, and mate guarding. Behaviour. 84: 287-314.

Grimaldi D & Engel MS (2005) Evolution of the Insects. Cambridge University Press. New York. 755 pp.

Griswold GH (1944) Studies on the biology of the webbing clothes moth (*Tineola bisselliella*). Memoirs of Cornell University Agricultural Experimental Station. 262: 1–59.

Gwynne DT & Edwards ED (1986) Ultrasound production by genital stridulation in *Syntonarcha iriastis* (Lepidoptera: Pyralidae): long distance signalling by male moths? Zoological Journal of the Linnean Society. 88: 363–376.

Hart M (2006) The role of sonic signals in the sexual communication of peach twig borers, *Anarsia lineatella* (Zeller) (Lepidoptera: Gelechiidae). MSc thesis, Simon Fraser University, Burnaby.

Hart M, Belton P & Gries G (2010) The effect of fibril erection on hearing in male *Aedes togoi*: an open and shut case. Canadian Acoustics. 38(3): 36-37.

Hart M, Belton P & Kuhn R (2011) The Risler Manuscript. European Mosquito Bulletin. 29:103-113.

Haskell PT (1961) Insect Sounds. Quadrangle Books, Chicago. xviii + 189 pp

Haskell PT & Belton P (1956) Electrical responses of certain Lepidopterous tympana. Science. 177:139-140.

Heller KG & Achmann R (1995) Ultrasound communication in the pyralid moth species *Symmoracma minoralis* (Lepidoptera: Pyralidae: Nymphulinae). Entomologia Generalis. 20, 1–9.

Heller KG & Krahe R (1994) Sound production and hearing in the pyralid moth *Symmoracma minoralis*. Journal of Experimental Biology. 187: 101-111.

Herrich GW (1933) An unusual invasion of the clothes moth, *Tineola bisselliella*. Entomological News 44: 99–101.

Hinton HE (1956) The larvae of the species of Tineidae of economic importance. Bulletin of Entomological Research. 47: 251–346.

Hoy RR (1998) Acute as a bug's ear: an informal discussion of hearing in insects. In: Comparative hearing: insects. Ed. RR Hoy & AN Popper. New York: Springer. 1–17.

Hoy RR & Yack J (2008) Hearing. In: The Encyclopedia of Entomology. Ed. JL Capinera. Springer, The Netherlands. 440-446.

Hristov NI & Conner WE (2005) Sound strategy: acoustic aposematism in the bat–tiger moth arms race. Naturwissenschaften. 92: 164–169.

Huang F & Subramanyam BH (2003) Effects of delayed mating on reproductive performance. of *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae). Journal of Stored Products Research 39: 53–63.

Huang F & Subramanyam BH (2004) Behavioral and reproductive effects of ultrasound on the Indian meal moth, *Plodia interpunctella.* Entomologia Experimentalis et Applicata. 113: 157-164.

Huang F, Subramanyam BH & Taylor R (2003) Ultrasound affects spermatophore transfer, larval numbers, and larval weight of *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae). Journal of Stored Products Research 39: 413–422.

Insley SJ (1992) Mother-Offspring Separation and Acoustic Stereotypy: A Comparison of Call Morphology in Two Species of Pinnipeds. Behaviour. 120(1/2): 103-122.

Jacobs DS, Ratcliffe JM & Fullard JH (2008) Beware of bats, beware of birds: the auditory responses of eared moths to bat and bird predation. Bevavioral Ecology. 10(6): 1333-1342.

Kan SP & Ho BC (1973) Development of *Brugia pahangi* in the flight muscles of *Aedes togoi*: Ultrastructural changes in the infected muscle fibers and the infecting filarial larvae. American Journal of Tropical Medicine and Hygiene. 22(2): 179-188.

Kan E & Waku Y (1985) Analysis of oviposition preference in the webbing clothes moth, *Tineola bisselliella* (Lepidoptera: Tineidae). Applied Entomology and Zoology. 20: 322–330.

Kasper J & Hirschberger P (2005) Stridulation in *Aphodius* dung beetles: songs and morphology of stridulatory organs in North American *Aphodius* species (Scarabaeidae). Journal of Theoretical Biology. 39(1):91-99.

Kay RE (1969) Acoustic signaling and its possible relationship to assembling and navigation in the moth, *Heliothis zea*. Journal of Insect Physiology. 15: 989-1001.

Keating P & Buhr R (1978) Fundamental frequency in the speech of infants and children. Journal of the Acoustical Society of America. 63(2): 567-571.

Kent KS & Griffin LM (1990) Sensory organs of the thoracic legs of the moth *Manduca sexta.* Cell Tissue Research. 259:209-223.

Key KHL & Common IFB (1959) Observations on the ecology of the clothes moths *Tineola bisselliella* and *Tinea pellionella* in a bulk wool store. Australian Journal of Zoology. 7: 39–77.

Kindl J, Kalinová B, Červenka M, Jílek M & Valterová I (2011) Male moth songs tempt females to accept mating: the role of acoustic and pheromonal communication in the reproductive behaviour of *Aphomia sociella.* Plos ONE. 6(10): e26476.

Kolmes SA (1983) Ecological and sensory aspects of prey capture by the whirligig beetle *Dineutes discolor* (Coleoptera: Gyrinidae). Journal of the New York Entomological Society. 91:405–412.

Kirkpatrick RL & Harein PK (1965) Inhibition of Reproduction of Indian-Meal Moths, *Plodia interpunctella*, by Exposure to Amplified Sound. Journal of Economic Entomology. 58(5): 920-921.

Kuhl W, Schodder GR & Schroeder FK (1954) Condenser transmitters and microphones with solid dielectric for airborne ultrasonics. Acoustica. 4: 519-532.

Kuhn R (2002) Colonisation of the flood-water mosquito *Aedes vexans* (Meigen) (Diptera:Culicidae). European Mosquito Bulletin 12: 7-16.

Lafaille M, Bimbard G & Greenfield MD (2010) Risk trading in mating behavior: forgoing anti-predator responses reduces the likelihood of missing terminal mating opportunities. Behavioral Ecology and Sociobiology. 64: 1485-1494.

Lapshin DN & Voronstov DD (2007) Acoustic Irradiation Produced by Flying Moths (Lepidoptera, Noctuidae). Entomological Review. 87(9): 1115–1125.

Lee DK (2001) Field evaluation of an insect growth regulator, pyriproxyfen, against *Aedes togoi* larvae in brackish water in South Korea. Journal of Vector Ecology. 26(10: 39-42.

Lollis DW (1971) The auditory (ultrasonic) reception and behaviour of two stored products pests: the almond moth *Cadra cautella* and the Indianmeal moth *Plodia interpunctella*. PhD thesis. University of Oklahoma.

Lum PTM (1974) Effect of carbon dioxide anesthesia at eclosion upon mating efficiency of male *Plodia interpunctella* (Lepidoptera: Pyralidae). Journal of Stored Products Research. 10: 69–71.

Maddison DR & Schulz KS (2007) The Tree of Life Web Project. Internet address: http://tolweb.org

Mankin RW, Arbogast RT, Kendra PE & Weaver DK (1999) Active spaces of pheromone traps for *Plodia interpunctella* (Lepidoptera: Pyralidae) in enclosed environments. Environmental Entomology. 28(4): 557-565.

Marlatt CL (1898) The peach twig borer. US Department of Agriculture Farmers' Bulletin 80: 1-15.

Marshall JF (1938) The British Mosquitoes. British Museum (Natural History) London. 341 pp.

Masters WM, Tautz J, Fletcher NH & Markl H (1983) Body vibration and sound production in an insect (*Atta sexdens)* without specialized radiating structures. Journal of Comparative Physiology A. 150: 239–249.

Mayor HD, Hampton CJ& Rosario B (1961) A simple method for removing the resin from epoxy-embedded tissue. Journal of Biophysical Cytology 9: 909-910.

Mbata GN & Osuji FNC (1983) Some aspects of the biology of *Plodia interpunctella* (Hubner) Lepidoptera: Pyralidae) as a pest of stored groundnuts in Nigeria. Journal of Stored Products Research. 19: 141–151.

Menzel JG & Tautz J (1994) Functional morphology of the subgenual organ of the carpenter ant. Tissue Cell. 26(5):735-46.

Miller LA & Surlykke A (2001) How some insects detect and avoid being eaten by bats: Tactics and countertactics of prey and predator. BioScience. 51: 570–581.

Minet J & Surlykke A (2003) Auditory and sound producing organs. In: Handbook of Zoology, Vol. IV (Arthropoda: Insecta. Lepidoptera, Moths and Butterflies, Vol. 2) (ed. N. P. Kristensen). New York: W.G. de Gruyter. 289-323.

Miyagi I (1972) Feeding habits of some Japanese mosquitoes on cold-blooded animals in the laboratory. Tropical Medicine. 14:203-217.

Morse PM (1948) Vibration and Sound, 2nd edition. McGraw-Hill. New York. 468 pp.

Moulds MS (2009) Cicadas. In: Encyclopedia of Insects (Second Edition). Ed VH Resh & RT Cardé. Academic Press, San Diego. 163-164.

Mullen MA (1973) Low frequency sound affecting the development of the *Indian meal moth*. Journal of the Georgia Entomological Society. 8: 320–321.

Mullen MA & Tsao CH (1971) Tympanic organ of Indian-meal moth, *Plodia interpunctella* (Hübner), almond moth, *Cadra cautella* (Walker) and tobacco moth, *Ephestia elutella* (Hübner) (Lepidoptera: Pyralididae). International Journal of Insect Morphology and Embryology.1: 3-10.

Nadrowski B, Effertz T, Senthilan PR & Göpfert MC (2011) Antennal hearing in insects - New findings, new questions. Hearing Research. 273(1–2): 7–13.

Nakano R, Skals N, Takanashi T, Surlykke A, Koike T, Yoshida K, Maruyama H, Tatsuki S & Ishikawa Y (2008) Moths produce extremely quiet ultrasonic courtship songs by rubbing specialized scales. Proceedings of the National Academy of Sciences of the USA. 105: 11812–11817.

Nakano R, Takanashi T, Fujii T,Skals N, Surlykke A & Ishikawa Y (2009) Moths are not silent, but whisper ultrasonic courtship songs. Journal of Experimental Biology. 212(24): 4072-4078.

Nakano R, Takanashi T, Skals N, Surlykke A & Ishikawa Y (2010) Ultrasonic courtship songs of male Asian corn borer moths assist copulation attempts by making the females motionless. Physiological Entomology. 35: 76–81.

Nijhout HF (1977) Control of antennal hair erection in male mosquitoes. Biological Bulletin. 153: 591-603.

Nijhout HF & Martin SK (1978) Alpha-adrenergic activity of isoproterenol in mosquito antennae. Experientia. 36: 758-759.

Nijhout HF & Sheffield HG (1979) Hair erection in male mosquitoes: a new mechanical effector in insects. Science. 206(4418): 595-596.

Orci KM & Szocs G (2009) Ultrasounic and sonic emission during the courtship behaviour in males of Z- and E- pheromone strains of the European corn borer *Ostrinia nubilalis* Hubner (Lepidoptera, Pyraustidae). Bioacoustics: The International Journal of Animal Sound and its Recording. 19 (1-2): 93-107.

Pelham-Clinton EC (1985) Tineidae. In: The Moths and Butterflies of Great Britain and Ireland 2. Ed. J Heath, and AM Emmet. Harley Books, Colchester, UK. 152–207.

Pennetier C, Warren B, Roch-Dabire K, Russell IJ & Gibson G (2010) ‘‘Singing on the Wing’’ as a mechanism for species recognition in the malarial mosquito *Anopheles gambiae*. Current Biology. 20:131-136.

Pérez M & Zhantiev RD (1976). Functional organization of the tympanal organ of the flour moth, *Ephestia kuehniella*. Journal of Insect Physiology. 22: 1267–1273.

Pierce GW (1948) The Songs of Insects. Harvard University Press, Massachusetts. pp. 329.

Ponomarenko MG (1990) Review of the genus *Anarsia* Z. (Lepidoptera, Gelechiidae) of the USSR. Entomological Review 69: 102-116.

Price DW & Summers FM (1961) Cyclical changes in numbers of moths and larvae of the peach twig borer in California. Journal of Economic Entomology 54:933-936.

Pumphrey R & Rawdon-Smith AF (1936) Hearing in Insects: The Nature of the Response of Certain Receptors to Auditory Stimuli. Proceedings of the Royal Society of London. Series B, Biological Sciences. 121(820): 18-27.

Purnomo FP, Dennis DT & Atmosoedjono S (1976) Development of the Timor filaria in *Aedes togoi*: Preliminary Observations. Journal of Parasitology. 62(6): 881-885.

Regier JC, Shultz JW, Zwick A, Hussey A, Ball B, Wetzer R, Martin JW & Cunningham JW (2010) Arthropod relationships revealed by phylogenomic analysis of nuclear protein-coding sequences. Nature. 463: 1079-U98.

Rice RE & Jones RA (1975) Peach twig borer. Field use of a synthetic sex pheromone. Journal of Economic Entomology. 68:359-360.

Risler H (1953) Das Gehörorgan der Männchen von *Anopheles stephensi* Liston (Culicidae). Zoologische Jahrbucher (Anat. Ontog. Tiere) 73:165-176.

Risler H (1955) Das Gehörorgan der Männchen von *Culex pipiens* L., *Aedes aegypti* L. und *Anopheles stephensi* Liston (Culicidae), ein vergleichend morphologische Untersuchung. Zoologische Jahrbucher (Anat. Ontog. Tiere) 74: 478-490.

Risler H (1984) The auditory organ of male mosquitoes (Culicidae). (Studies on *Aedes vexans* Meigen 1830). Unpublished manuscript deposited in the Entomology Library of The Natural History Museum, London.

Risler H (1990) Strukturelle Grundlagen des Richtungshörens männlicher Stechmücken. Verhandlungen der Deutsche Zoologischer Gesellschaft. 83: 427.

Riyong D, Choochote W, Jitpakdi A, Suvannadabba S, Leemingswawat S & Shaithong U (2000) Autogenous *Aedes togoi* sub-colony (Shanthaburi, Thailand strain), and efficient laboratory vector in study of filariasis. Southeast Asian Journal of Tropical Medicine and Public Health. 31(2): 246-151.

Robert D & Göpfert MC (2002) Acoustic sensitivity of fly antennae. Journal of Insect Physiology. 48: 189-196.

Rodriguez RL & Greenfield MD (2004) Behavioural context regulates dual function of ultrasonic hearing in lesser waxmoths: bat avoidance and pair formation. Physiological Entomology. 29(2): 159-168.

Roeder KD & Treat AE (1957) Ultrasonic reception by the tympanic organ of noctuid moths. Journal of Experimental Zoology. 134: 127-58.

Roeder KD & Treat AE (1961) The reception of bat cries by the tympanic organ of noctuid moths. In: Sensory Communication. Ed. W. Rosenblith. Cambridge: M.I.T. Press. 545-60.

Roth L M (1948) A study of mosquito behavior. An experimental laboratory study of the sexual behavior of *Aedes aegypti* (Linnaeus). American Midland Naturalist. 40: 265-352.

Rowland E (2009) Morphological, physiological and behavioural evidence for sonic signals in the sexual communication system of Lymantriid moths. MSc Thesis, Simon Fraser University.

Rowland E, Schaefer PW, Belton P & Gries G (2011) Evidence for short-range sonic communication in Lymantriine moths. Journal of Insect Physiology. 57(2): 292-299.

Rydell J (1988) Bat defence in lekking ghost moths (*Hepialus humuli*), a moth without ultrasonic hearing. Proceedings of the Royal Society of London, Series B. (265): 1373-1376.

Rydell J, Skals N, Surlykke A & Svensson M (1997) Hearing and bat defence in geometrid winter moths. Proceedings of the Royal Society of London, Series B (264): 83-88.

Sambaraju KR, Phillips TW (2008) Effects of Physical and Chemical Factors on Oviposition by *Plodia interpunctella* (Lepidoptera: Pyralidae). Annals of the Entomological Society of America. 101(5): 955-963.

Sanborn AF (2005) Acoustic signals and temperature. In Insect Sounds and Communication (eds. S Drosopoulos and MF Claridge). CRC Taylor & Francis, Florida. p. 111–125.

Sane SP, Dieudonné A, Willis WA & Daniel TL (2007) Antennal mechanosensors mediate flight control in moths. Science. 315(5813): 863-866.

Sarai DS (1966) The peach twig borer, *Anarsia lineatella* Z. (Lepidoptera: Gelechiidae) in the Okanagan and Similkameen valleys of British Columbia. Journal of the Entomological Society of British Columbia. 63: 19-22.

Schlamp KK, Brown K, Gries R, Hart M, Gries G & Judd GGR (2006) Diel periodicity of sexual communication in *Anarsia lineatella* (Lepidoptera : Gelechiidae). Canadian Entomologist. 138(3): 384-389.

Schütze H & Elsner N (2001) Stridulatory pattern generation in acridid grasshoppers: metathoracic interneurons in *Stenobothrus rubicundus* (Germar 1817). Journal of Comparative Physiology A. 187(7): 529-540.

Scoble M (1992) The Lepidoptera: form, function and diversity. Oxford University Press, Oxford. 352 pp.

Shorey HH (1962) Nature of the sound produced by *Drosophila melanogaster* during courtship. Science. 137: 677-8.

Silhacek D, Murphy C & Arbogast RT (2003) Behavior and movements of Indian meal moths (*Plodia interpunctella* Hubner) during commodity infestation. Journal of Stored Products Research. 39(2): 171-184.

Simmons JA, Wever EG & Pylka JM (1971) Periodical cicada: Sound production and hearing. Science. 171: 212-213.

Skals N & Surlykke A (1999) Sound production by abdominal tymbal organs in two moth species: the green silver-line and the scarce silver-line (Noctuoidea: Nolidae: Chloephorinae). The Journal of Experimental Biology 202, 2937–2949.

Skals N & Surlykke A (2000) Hearing and evasive behaviour in the greater wax moth, *Galleria mellonella* (Pyralidae). Physiological Entomology. 25(4): 354-362.

Smart C (translator) (1863) The works of Horace. Ed. TA Buckley. American Book Company, New York. 232 pp.

Sotavalta O (1952) Flight-tone and wing-stroke frequency of insects and the dynamics of insect flight. Nature. 170: 1057–1058.

Sotavalta O (1963) The flight sounds of insects. In: Acoustic Behaviour of Animals. Ed. RG Busnel. Elsevier, Amsterdam. 374-390..

Spangler HG (1984) Responses of the Greater Wax Moth, *Galleria mellonella* L. (Lepidoptera: Pyralidae) to continuous high-frequency Sound. Journal of the Kansas Entomological Society. 57(1): 44-49.

Spangler HG (1985) Sound production and communication by the greater wax moth (Lepidoptera: Pyralidae). Annals of the Entomological Society of America. 78: 54-61.

Spangler HG (1987) Acoustically mediated pheromone release in *Galleria mellonella* (Lepidoptera, Pyralidae). Journal of Insect Physiology. 33(7): 465-468.

Spangler HG (1988) Moth hearing, defense and communication. Annual Review of Entomology. 33:59-81.

Spangler HG, Greenfield MD & Takessian A (1984) Ultrasonic mate calling in the lesser wax moth. Physiological Entomology. 9(1): 87-65.

Spangler HG & Takessian A (1983) Sound perception by 2 species of wax moths (Lepidoptera, Pyralidae). Annals of the Entomological Society of America. 76(1): 94-97.

Stout JF, Wilcox CR & Creitz LE (1969) Aggressive communication by *Larus glaucescens*. Part I. Sound communication. Behaviour 34: 30-41.

Strauss J & Lakes-Harlan R (2008) Neuroanatomy and physiology of the complex tibial organ of an atympanate ensiferan, *Ametrus tibialis* (Brunner von Wattenwyl, 1888) (Gryllacrididae, Orthoptera) and evolutionary implications. Brain, Behavior and Evolution. 71(3):167-80.

Sueur J & Aubin T (2006) When males whistle at females: complex FM acoustic signals in cockroaches. Naturwissenschaften. 93(10) 500-505.

Surlykke A, Yack JE, Spence AJ, & Hasenfuss I (2003) Hearing in hooktip moths (Drepanidae: Lepidoptera). The Journal of Experimental Biology. 206: 2653-2663.

Svensson GP, Skals N & Löfstedt C (2003) Disruption of the odour-mediated mating behaviour of *Plodia interpunctella* using high-frequency sound Entomologia Experimentalis et Applicata. 106: 187–192.

Svensson GP, Löfstedt C & Skals N (2004) The odour makes the difference: male moths attracted by sex pheromones ignore the threat by predatory bats. Oikos. 104: 91-97.

Svensson GP, Löfstedt C, Skals N (2007) Listening in pheromone plumes: Disruption of olfactory-guided mate attraction in a moth by a bat-like ultrasound. Journal of Insect Science. 7(59): 1-9.

Sziraki GY (1984) Dispersion and movement activity of some important moth pests living on stone fruits. Acta Phytopathologica Academiae Scientiarum Hungaricae. 19: 51-64.

Takács S, Gries G & Gries R (2002) Where to find a mate? Resource-based sexual communication of webbing clothes moth. Naturwissenschaften 89: 57-59.

Takács S, Mistal C & Gries G (2003) Communication ecology of webbing clothes moth: attractiveness and characterization of male-produced aggregation signals. Journal of Applied Entomology. 127: 127-133.

Tautz J & Markl H (1978) Caterpillars detect flying wasps by hairs sensitive to airborne vibration. Behavioral Ecology and Sociobiology. 4: 101-110.

Terman FE (1952) Electronic Measurements. McGraw-Hill, New York. pp. 707.

Tischner H (1953) Uber den Gehorsinn von Stechmiicken. Acustica 3:335-343.

Todi SV, Sharma Y, & Eberl DF (2004) Anatomical and molecular design of the *Drosophila* antenna as a flagellar auditory organ. Microscopy Research and Technique. 63: 388–399.

Toma T, Miyagi I, Higa Y, Okazawa T & Sasaki H (2005) Culicid and chaoborid flies (Diptera:Culicidae and Chaoboridae) attracted to CDC miniature frog call traps at Iriomote Island, the Ryuku Archipelago, Japan. Medical Entomology and Zoology 56: 65-7.

Traynier RMM, Schumacher RK, Lau DM, (1994) Oviposition site selection by *Tineola bisselliella*, *Tinea spp*. (Lepidoptera: Tineidae) and *Anthrenus flavipes* (Coleoptera: Dermestidae). Journal of Stored Products Research 30: 321–329.

Treat AE (1955) The response to sound in certain Lepidoptera. Annals of the Entomological Society of America. 48:272-284.

Treat AE (1962) Comparative moth captures by an ultrasonic and a silent light trap. Annals of the Entomological Society of America. 55: 716–720.

Treherne RC (1923) The peach twig borer, (*Anarsia lineatella*) in British Columbia. Proceedings of the British Columbia Entomological Society. 17&19: 176-183.

Trematerra P (1997) Some aspects of the sexual behaviour of the Lepidoptera Pyralidae infesting stored-products. Anzeiger fur Schadlingskunde Pflanzenschutz Umweltschutz. 70(5): 87-91.

Trematerra P & Pavan G (1995) Ultrasound production in the courtship behaviour of *Ephestia cautella* (Walk.), *E. kuehniella* Z. and *Plodia interpunctella* (Hb) (Lepidotpera: Pyralidae). Journal of Stored Products Research. 31: 43-48.

Vande Berg JS (1971) Fine structural studies of Johnston's organ in the tobacco hornworm moth, *Manduca sexta* (Johannson). Journal of Morphology. 133(4): 439–455.

Vogel S (1988) Life’s devices: the physical world of animals and plants. Princeton University Press. New Jersey. 367 pp.

Warren B, Gibson G & Russell IJ (2009) Sex recognition through midflight mating duets in *Culex* mosquitoes is mediated by acoustic distortion. Current Biology. 19: 485-491.

Warren B, Lukashkin AN & Russell IJ (2010) The dynein-tubulin motor powers active oscillations and amplification in the hearing organ of the mosquito. Proceedings of the Royal Society, London, Series B. 277: 1761- 1769.

Waters DA (2003) Bats and moths: What is there left to learn? Physiological Entomology. 28: 237–250.

Waters DA & Jones G (1994) Wingbeat-generated ultrasound in noctuid moths increases the discharge rate of the bat-detecting A1 cell. Proceedings of the Royal Society London, Series B. 258: 41-46.

Weakley CV, Kirsch PA & Zalom FG (1990) Within-orchard and within-tree distributions of peach twig borer (Lepidoptera: Gelechiidae) damage to peaches. Journal of Economic Entomology 83: 505-510.

Wentworth-Thompson DA (translator) (1994) Aristotle's The History of Animals. Web Atomics.

White FB (1877) Sense of hearing in insects and birds. Nature. 292-293.

Willis MA & Baker TC (1984) Effects of intermittent and continuous pheromone simulation on the flight behaviour of the Oriental fruit moth, *Grapholita molesta*. Physiological Entomology. 9:341-348.

Windmill JFC, Fullard JH & Robert D (2007) Mechanics of a 'simple' ear: tympanal vibrations in noctuid moths. The Journal of Experimental Biology. 210: 2637-2648.

Wishart G & Riordan CF (1959) Flight responses to various sounds by adult males of *Aedes aegypti*. Canadian Etnomologist. 23:181–191.

Wishart G, van Sickle GR & Riordan DF (1962) Orientation of the males of *Aedes aegypti* (L.) (Diptera:Culicidae) to sound. The Canadian Entomologist. 98: 613-626.

Yack JE (2004) The structure and function of auditory chordotonal organs in insects. Microscopy Research and Technique. 63:315-337.

Yack JE & Dawson J (2008) Insect Ears. In: The Senses: A Comprehensive Reference, Volume 3. (Eds. Hoy, RR, Shepherd GM, Basbaum AI, Kaneko A, & Westheimer G.) pp.35-54.

Yack JE & Fullard JH *(*1993*)* What is an insect ear? Annals of the Entomological Society of America 86: 677-682.

Yang YW & Greenfield MD (1996) Ultrasonic communication and sexual selection in wax moths: female choice based on energy and asynchrony of male signals. Animal Behaviour. 51: 1095-1106.

Yen PKF, Zaman V & Mak JW (1982) Identification of some common infective filarial larvae in Malaysia. Journal of Helminthology. 56: 69-80.

Yen S & Minet J (2007) Cimelioidea: A new superfamily name for the Gold Moths (Lepidoptera: Glossata). Zoological Studies. 46(3): 262-271.

Zagatti P (1981) Comportement sexual de la pyrale de la canne a sucre *Eldana saccharina* (Wlk.) lie a deux pheromones emises par le male. Behaviour. 78: 81–82.