MORPHOLOGICAL, PHYSIOLOGICAL AND BEHAVIOURAL EVIDENCE FOR SONIC SIGNALS IN THE SEXUAL COMMUNICATION SYSTEM OF LYMANTRIID MOTHS

by

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Abstract

Sexual communication of gypsy moth, *Lymantria dispar* (L.), nun moth, *L. monacha* (L.), pink gypsy moth, *L. mathura* (M.), and fumida tussock moth, *L. fumida* (B.) is known to be mediated by pheromones. Data presented here show that sonic and visual signals are also involved. Sound produced by flying male *L. dispar* induced wing fluttering and motion in conspecific females that may guide males to their micro-location. Analyses of sounds produced by wing fanning *L. monacha*, *L. mathura*, and *L. fumida* revealed species- and sex-specific wing beat and associated click frequencies that may contribute to reproductive isolation or close-range communication. Evidence for close-range communication in these lymnatriids includes (*i*) scanning electron micrographs of functional metathoracic tympanate ears, (*ii*) attraction of male *L. monacha* and *L. fumida* to speakers playing back sound signals from conspecific females, and (*iii*) laser interferometry demonstrating particular sensitivity of tympana to frequency components of conspecific sound signals.

Keywords: *Lymantria dispar*; *Lymantria monacha*; *Lymantria fumida*; *Lymantria mathura*; Lepidoptera; Lymantriidae; acoustic communication; short-range communication; acoustic signals; mate attraction and location; tympanate ear; laser interferometry.

Lymantria dispar Illustration

Dedication

For my Mum and Dad, (Little) Nana and Grandad Roberts, and Nana (With the Purple Hair) and Grandad Rowland. Mo ghrá thú.

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1.1 Acoustic Communication in Insects

Modes of communication used by animals to locate mates, find hosts, and detect predators include vision, chemoreception, tactile sensation, and audition (Yager, 1999; Partan and Marler, 2005). The use of acoustic communication has been widely studied throughout the animal kingdom: dolphins (Whitlow et al., 2009) and bats (Page and Ryan, 2005) use echolocation to locate and detect prey; frogs (Feng et al., 2006) and birds (Patricelli et al., 2007) emit vocal signals that attract mates; and night flying moths detect acoustic signals of predatory bats (Roeder, 1962; Acharya and McNeil, 1998; Miller and Surlykke, 2001). Insect sounds have been documented since the writings of Aristotle and other classical Greek philosophers. Just in the last 50-60 years, however, has the development of sophisticated technology for sound recording and analyses facilitated intense scientific investigations of insect acoustic signals and associated behaviour (Claridge, 2006).

In arthropods, reception of substrate vibrations is common, but reception of sound in the form of pressure waves is an adaptation restricted to insects (Stumpner and Helversen, 2001). There are 19 independent evolutions of audition in the class Insecta and a diversity of body parts are known to contain ears. For example, ears of noctuid

moths are located on the dorsolateral thorax, whereas cricket ears are located on the prothoracic legs. The single ear of praying mantids is located in a deep groove between the metathoracic coxae. Hearing organs of tiger beetles are located on the dorsum of the first abdominal segment under the wings, whereas the ears of scarab beetles are located under the edge of the pronotum. Tachinid flies have ears beneath their "chin" and choerocampiine and acherontiine hawkmoths have ears in the mouth region. Finally, the ears of green lacewings are located on the radial vein of the wings (Yager, 1999).

Hearing plays an important role in the life history of many insects, allowing them to detect and evade predators, home in on singing hosts, and locate and select appropriate mates (Yager, 1999). In addition to the well documented use of acoustic cues by moths to detect and evade bat predators (e.g., Baker and Cardé, 1978; Acharya and McNeil, 1998; Rodriguez and Greenfield, 2004; Jones et al., 2002; Fullard et al., 2008), the hearing structures of tiger beetles (Cicindelidae) (Spangler, 1988), mantids (Dictyoptera) (Yager and Hoy, 1986), and lacewings (Neuroptera) (Miller and Olesen, 1979) have been shown to detect ultrasonic (> 20 kHz) cues from their bat predators.

Several species of ormiine flies (Tachinidae) are "acoustic parasitoids" of singing orthopterans, listening in on mate calling songs of potential hosts. Dipterans of the family Sarcophagidae employ a similar strategy to find their cicada hosts. Host-searching female flies demonstrate acoustic selectivity based on spectral and temporal parameters of the host song (Farris et al., 2008).

Males of the neotropical katydid, *Myopophyllum speciosum* (Morris et al., 1994), and rice moth, *Corcyra cephalonica* (Spangler, 1987), produce ultrasonic signals that attract conspecific females. Males of the whistling moth, *Hecatesia thyridion*,

acoustically advertize mating territories to females using sonic (10 Hz-20 kHz) signals (Alcock et al., 1989), with the highest intensity between 15-20 kHz (Bailey, 1978). Wing fanning males of the lesser wax moth, *Achroia grisella*, produce songs that can attract females up to 1-2 m away. Females choose mates that produce songs with fast pulse rates, high amplitude, and temporal variation (Brandt et al., 2005). Both males and females of the polka-dot wasp moth, *Syntomeida epilais,* locate potential mates by use of acoustic signals (Sanderford and Conner, 1990). Finally, courting males of the common cutworm moth, *Agrotis fucosa*, produce characteristic trembling sounds in the sonic frequency range (Wakamura, 1977).

The behavioural context in which hearing has evolved determines the design and properties of the auditory system. Hearing in the context of communication requires not only the recognition and discrimination of highly specific acoustic patterns but also their localization. Frequency spectra of conspecific signals typically match the sensitivity of the receiver. Directionality is achieved by peripheral auditory structures and is enhanced by neuronal processing (Stumpner and Helversen, 2001). Insects that possess ears known to be pure pressure receivers achieve directional sensitivity by diffraction of sound around the body, which changes the amplitude and phase of the sound and results in a difference in sound pressure between the two ears (Mhatre and Balakrishnan, 2007). These interaural pressure differences are sufficient cues for directional hearing (Robert, 2005). However, even in large insects such as locusts, the diffraction of low frequency sounds (< 8 kHz) is much less effective (Michelsen and Larsen, 1978).

Some insect ears are pressure gradient (difference) receivers, whereby sound propagates to both the external and internal surfaces of the tympanal membrane. The

direction of sound has a substantial effect on the amplitude and/or phase of sounds reaching the ears and as a result provides directionality (Mhatre and Balakrishnan, 2007). Unless the acoustic impedance of the medium on the inner side of the membrane matches that of the external medium, pressure changes of sound waves reaching the tympanum will have little effect (Yager, 1999). Many members of the order Orthoptera, such as bushcrickets (Michelsen et al., 1994; Rheinlaender et al., 2007), crickets (Michelsen et al., 1994; Mhatre and Balakrishnan, 2008), cicadas (Fonseca, 1993), and grasshoppers (Helversen and Helversen, 1995) possess pressure gradient receivers that allow them to locate conspecifics. For example, male congeners of field crickets, *Plebeiogryllus* spp., produce acoustic signals that attract potential mates. Females use these signals to distinguish between con- and heterospecific males and to pin point the location of a mate (Mhatre and Balakrishnan, 2007). Directional sensitivity of ears of notodontid moths (e.g., *Pheosia tremula*; Surlykke, 1984) and some noctuid moths (Payne et al., 1966) has been shown with threshold response curves of ears exposed to sounds from different directions. Phonotaxis, or orientation to a sound source (e.g. Rheinlaender et al., 2007), provides behavioural evidence for sound localization (Michelsen, 1998).

A pre-requisite of hearing is a functional auditory structure. The best known lepidopteran hearing organs are the tympanal structures located on the metathorax of Noctuoidea and on the base of the abdomen of Geometroidea and Pyraloidea (Scoble, 1992). The tympanate ear has three anatomical and functional parts: *i*) the tympanum or tympanal membrane which vibrates in response to sound waves; *ii*) the tracheal sac or air sac behind the membrane which allows the membrane to vibrate with a pressure gradient; and *iii*) the tympanal organ which is a specialized chordotonal organ (stretch receptor)

that transduces the mechanical signal from the tympanal membrane into nerve impulses (Yager, 1999). Lymantriid moths are members of the superfamily Noctuoidea, the monophyly of which is based on the presence of such metathoracic tympanal organs (Speidel et. al., 1996). Scanning electron micrographs show strikingly similar tympanal regions of several noctuoid moths, including those of male gypsy moth, *Lymantria dispar* (Speidel et al., 1996).

Although the tympanal organs in most moth species evidently evolved in response to bat predation (Baker and Cardé, 1978; Bailey, 1991; Heller and Krahe, 1994; Yager, 1999; Stumpner and Helversen, 2001), hearing may also have evolved in the context of mate finding and mate recognition (Stumpner and Helversen, 2001). Several species of pyralid, arctiid and noctuid moths might have first developed the ability to hear (Heller and Krahe, 1994; Conner, 1999) which then set the stage for the evolution of acoustic signaling during courtship (Conner, 1999; Stumpner and Helversen, 2001). The initial selection pressure for the evolution of hearing may differ from the selection pressure for its persistence, with additional functions supplementing or even replacing the original function (Stumpner and Helversen, 2001). The ability to detect and evade bats may be the most significant evolutionary factor in the pre-adaptation of moths to evolve intraspecific acoustic communication systems (Conner, 1999).

1.2 Distribution and Biology of Lymantriid Moths

Lymantriinae is a sub-family of Noctuidae, a diverse taxonomic group, particularly in the Old World tropics. Estimates of the number of lymantriids worldwide range from 2,160 (Holloway et al., 1987) to 2,700 (Scoble, 1992). Heppner (1991) describes an estimated 1,004 species in Afrotropical regions, 742 species in the Oriental east to Moluccas, 255 species in Australasia including New Guinea, 203 species in the Palaearctic, 180 species in Neotropical regions, and 32 species in the Nearctic.

The gypsy moth, *Lymantria dispar* (L.), nun moth, *Lymantria monacha* (L.), pink gypsy moth, *Lymantria mathura* (M.), and *fumida* tussock moth, *Lymantria fumida* (B.) (Lepidoptera: Noctuidae: Lymantriinae) are Eurasian defoliators of coniferous and deciduous trees and shrubs (Pogue and Schaefer, 2007) and are widely distributed throughout Europe and Asia including the Russia Far East, with some species inadvertently introduced into North America (Pogue and Schaefer, 2007).

1.2.1 European Gypsy Moth, *Lymantria dispar*

The European gypsy moth, *Lymantria dispar*, occurs throughout Europe, parts of the Middle East, the Mediterranean islands of Corsica and Sardinia, and North Africa. It was accidently introduced into North America in 1868/69. Larvae are highly polyphagous, favouring trees and shrubs of the genera *Quercus* (oak; Fagaceae), *Salix* (willow; Salicaceae), and *Crataegus* (hawthorn; Rosaceae) (Pogue and Schaefer, 2007).

The European *L. dispar* has one generation per year. Females are fully winged but functionally flightless and thus oviposit close to their pupation site. They lay their eggs in a single mass that contains anywhere from < 100 to > 1000 eggs. They deposit their egg mass in crevices under loose bark of tree trunks, or even on or under stones, and cover it with a thick matting of abdominal hairs. First instar larvae remain within the egg and undergo obligate diapause during winter months (Doane and McManus, 1981; Nealis and Erb, 1993; Pogue and Schaefer, 2007). They hatch in the spring and disperse via "ballooning" to host plants where they feed and develop through 5-6 instars (Nealis and Erb, 1993; Pogue and Schaefer, 2007). Each larva consumes up to 1 m² of foliage. Pupation occurs in early- to mid summer and the stage lasts for about two weeks (Nealis and Erb, 1993). Within several hours of eclosion, adult females release the sex pheromone (7*R*,8*S*)-*cis*-7,8-epoxy-2-methyloctadecane [(+)-disparlure] that attracts males from mid- to late morning into late afternoon (Klimetzek et al., 1976; Cardé et al., 1977; Miller et al., 1977; Plimmer et al., 1977), with peak flight activity 2-5 h before scotophase (Miller and Roelofs, 1978). Females can be found *in copula* before their wings are fully expanded and hardened (Pogue and Schaefer, 2007). Adult moths live for about one week. Their digestive system is not functional and they do not feed (Doane and McManus, 1981).

1.2.2 Nun Moth, *Lymantria monacha*

Lymantria monacha occurs throughout Eurasia, with an almost continuous distribution across Asia. In Europe, most adult moths are melanic, whereas only the non-

melanic or white form is found in the Japanese archipelago and Korea. Host plants of larvae include conifers such as *Abies*, *Larix*, *Picea*, and *Pinus* (all Pinaceae) and hardwoods such as *Acer* (Aceraceae), *Betula* (Betulaceae), *Crataegus*, *Malus*, *Prunus*, *Sorbus* (all Rosaceae), *Fraxinus* (Oleaceae), *Populus*, *Salix* (Salicaceae), and *Vaccinium* (Ericaceae)) (Pogue and Schaefer, 2007).

Lymantria monacha has one generation per year. Females lay 20-300 eggs in masses under bark scales or in cracks on the bole of host trees (Kolk and Starzyk, 1996; Pogue and Schaefer, 2007). Unlike *L. dispar*, eggs are naked within the mass (Pogue and Schaefer, 2007). First instar larvae remain within the egg and undergo obligate diapause during winter months. Larvae hatch in early May and feed on host trees, developing through 5-6 instars. Pupation occurs in July and August and the stage lasts for about two weeks. Adults eclose in late summer (July- August) and both males and females can fly (Kolk and Starzyk, 1996). Sexual communication takes place at night with peak activity between 21:00 and 24:00 hr in Europe and between 02:00 and 05:00 hr in Japan (Wallner et al., 1995; Gries et al., 2001). Females release a sex pheromone blend of (+)-disparlure, (7*R*,8*S*)-*cis*-7,8-epoxy-octadecane [(+)-monachalure], and 2-methyl-(*Z*)-7-octadecene that attracts males (Gries et al, 2001). Adult moths live for about one week and do not feed (Kolk and Starzyk, 1996).

1.2.3 Fumida Tussock Moth, *Lymantria fumida*

Lymantria fumida is found in Japan (Honshu, Shikoku, and Kyushu), Korea and Yunnan Province, and China. Larvae feed on Japanese fir, *Abies firma,* Japanese larch,

Larix leptolepis, *Keteleeria fortunei* (all Pinaceae), and Chinese juniper, *Juniperus chinensis* (Cupressaceae) (Pogue and Schaefer, 2007).

Females insert egg masses into narrow cracks under bark scales on the bole of host trees. Eggs are deposited into a froth of accessory gland fluids which hardens into a tough mass. As with *L. dispar* and *L. monacha*, first instar larvae remain within the egg and undergo obligate diapause during winter months. Larvae hatch in mid-March and feed in the crowns of host trees. Pupation occurs in mid- to late spring and the stage lasts for about two weeks. Adults begin to eclose in May and both males and females can fly (Sato, 1979; Pogue and Schaefer, 2007). Sexual communication occurs between 21:00 and 24:00 hr (Wallner et al., 1995; Gries et al., 2001). Females release a sex pheromone blend of (+)-disparlure and 2-methyl-(Z)-7-octadecene that attracts males (Schaefer et al., 1999; Gries et al., 2001). Adult moths live for about one week and do not feed (Sato, 1979).

1.2.4 Pink Gypsy Moth, *Lymantria mathura*

Lymantria mathura occurs throughout eastern Asia, from Japan and eastern Russian Siberia (Ussuri and Amur), south to Taiwan and Vietnam, and west across China, Thailand, Nepal, India, and Sri Lanka. Host plants of larvae are deciduous hardwoods including *Mangifera*, *Rhus* (Anacardaceae), *Betula* (Betulaceae), *Terminalia* (Combretaceae), *Castanea*, *Quercus* (Fagaceae), *Malus*, *Prunus*, *Pyrus* (Rosaceae), *Shorea* (Dipterocarpaceae), and *Syzygium* (Myrtaceae) (Pogue and Schaefer, 2007).

Females lay eggs under bark scales and cover any exposed eggs with whitish abdominal hairs (Pogue and Schaefer, 2007). First instar larvae remain within the egg and undergo obligate diapause. Larvae hatch in May and develop through 5-6 instars. Pupation occurs in July and the stage lasts about two weeks. Adults eclose at the end of July through August (Opstal, 2005). Both males and females are can fly and engage in sexual communication at night, with peak activity between 01:00 and 03:00 hr (Wallner et al., 1995; Pogue and Schaefer, 2007). Females release the sex pheromone blend of (9*R*,10*S*,3*Z*,6*Z*)-*cis*-9,10-epoxynonadecadiene and (9*S*,10*R*,3*Z*,6*Z*)-*cis*-9,10-epoxynonadecadiene at a 1:4 ratio that attracts males (Gries et al., 1999). Adult moths live for about a week and do not feed (Wallner et al., 1995; Pogue and Schaefer, 2007).

1.3 Acoustic Communication in Lymantriid Moths

Low frequency sounds associated with wing fanning have been described in *L. mathura* (Zlotina, 1999). Median best frequency thresholds of tympana in the closely related *L. dispar* are reported at 49.8 and 48.8 dB SPL (Sound Pressure Level) (10 cm from the source) for males and females, respectively (Cardone and Fullard, 1988). In four species of pyralid moths (*Achroia grisella*, *Galleria mellonella*, *Corcyra cephalonica*, and *Eldana saccharina*), the intensity of acoustic signals is < 80 dB SPL at 10 cm (Heller and Krahe, 1994). Such signals may facilitate close range communication (Heller and Krahe, 1994) helping, for example, to pinpoint the location of a mate (Mhatre and Balakrishnan, 2007). Phonotaxis, or orientation to a sound source (e.g., Rheinlaender et al., 2007), provides behavioural evidence for sound localization (Michelsen, 1998). No

such evidence for sound localization has been described for lymantriid moths. In Chapter 2, I predicted that acoustic signals mediate short-range orientation behaviour in *L. monacha* and *L. fumida*, and that the tympanal membranes of *L. monacha* and *L. mathura* are sensitive to frequencies in the male's acoustic signals.

Insects may exhibit different, context-specific types of flight. In clean air, male *L. dispar* exhibit a casting flight with large side to side sweeps almost perpendicular to the direction of the wind (Kennedy and Marsh, 1974). When they lock on to the pheromone plume of an upwind female, males engage in a more directed zigzagging flight toward the female (Kennedy and Marsh, 1974; Goldsworthy and Wheeler, 1989). It is unknown whether these changes in flight behaviour lead to changes in sounds produced by approaching males. In Chapter 3, I predicted that the wing beat frequency of male *L. dispar* changes in pheromone-laden air, signaling to a calling female that an approaching male has locked on to her pheromone plume.

Tympana of female North American (also known as European) *L. dispar* respond to low frequency sounds (~5-15 kHz; Cardone and Fullard, 1988) that are produced by wing fanning males (Zlotina, 1999). Cardone and Fullard (1988) speculated that these low frequency sounds are involved in courtship. With sound recordings obtained from North American and Asian *L. dispar*, *L. mathura*, and the browntail moth, *Euproctis chrysorrhoea* L., the production of low frequency sound appears common in lymantriids (Zlotina, 1999). However, in previous studies audio and video files of sound and behavioural responses were not recorded at the same time, and thus definitive conclusions regarding acoustic signaling were not possible. Furthermore, because flightless female *L. dispar* are less exposed to bat predation and are less sensitive than

males to ultrasonic frequencies of bat echolocation systems, Cardone and Fullard (1988) proposed that they may possess ears in a state of "evolutionary degeneration". However, comparative audiograms of females and males show that females are more sensitive than males to frequencies < 20 kHz (Cardone and Fullard, 1988), such as those associated with wing fanning males (Zlotina, 1999). In Chapter 3, I predicted (*i*) that low frequency (< 20 kHz) sounds produced by flying males at close range induce movement in conspecific females which, in turn, provides visual signals that can orient males toward females, and (*ii*) that the tympanum of European *L. dispar* is most sensitive to frequencies in the males' acoustic signals.

While visual signals are probably effective in the diurnal *L. dispar* in guiding a prospective mate to the micro-location of a female, such signals may be more difficult to detect by mate-seeking males of nocturnal moths, such as *L. monacha*, *L. fumida*, and *L. mathura*. Thus, males of these three congeners may instead use acoustic signals from conspecific females as a short range orientation signal.

1.4 Research Objectives

Pheromones in the sexual communication of lymantriid moths have been intensely studied (e.g., Miller and Roelofs, 1978; Charlton and Cardé, 1990; Schaefer et al., 1999; Gries et al., 1996, 1999, 2001), but little is known about potential acoustic communication in these moths during mate attraction and location.

Working with congeners of *L. dispar* in Chapter 2, my objectives will be to test the hypotheses that:

(1) male and female Asian *L. monacha*, *L. fumida*, and *L. mathura* produce characteristic acoustic signals within the sonic frequency range;

(2) male *L. monacha* and *L. fumida* exhibit short range orientation behaviour toward sound signals from conspecific females;

(3) the tympanate ears of male and female *L. monacha*, *L. fumida*, and *L. mathura* are located on the metathorax; and

(4) the tympanate ears of male *L. monacha* and *L. mathura* are functional within the sonic frequency range.

Working with European *L. dispar* in Chapter 3, my research objectives will be to test the hypotheses that:

(1) the wing beat frequency of males changes in pheromone-laden air;

(2) females alter their behaviour in response to wing beat sounds of flying males;

(3) similar to males, the tympanum of females is located on the metathorax; and

(4) the tympana of males and females are functional within the sonic frequency range.

2: Morphological, Physiological, and Behavioural Evidence for Close Range Sonic Communication in Lymantriid Moths*

2.1 Abstract

Sexual communication of nun moth, *Lymantria monacha* (L.), pink gypsy moth, *Lymantria mathura* (M.), and fumida tussock moth, *Lymantria fumida* (B.) (all Lepidoptera: Noctuidae: Lymantriinae) is known to be mediated by pheromones. Here I show that it is also mediated by sonic signals. Wing fanning male and female *L. monacha*, *L. mathura*, and *L. fumida* produce species- and sex-specific wing beat and associated click frequencies that could contribute to reproductive isolation or close range communication. Evidence for close range communication in these lymnatriids includes (*i*) scanning electron micrographs revealing metathoracic tympanate ears, (*ii*) laser interferometry recordings showing particular sensitivity of tympana to frequency components of sound signals from conspecifics, and (*iii*) phonotactic attraction of male *L. monacha* and *L. fumida* to speakers playing back sound signals from conspecific females.

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My data support the conclusion that tympanate ears of moths have evolved in response not only to bat predation, but also in the context of mate recognition and mate finding.

Keywords: *Lymantria monacha*; *Lymantria fumida*; *Lymantria Mathura*; Lepidoptera; Noctuidae; Lymantriinae; acoustic communication; short range orientation behaviour; bioacoustic signals; tympanate ear; laser interferometry

2.2 Introduction

Communication is so widespread throughout the biological world that it could be used to characterize life (Hasson, 1997). Animals may locate mates, find hosts, and detect predators using different modes of communication (Yager, 1999), including chemoreception, vision, tactile sensation, and audition (Partan and Marler, 2005).

In arthropods, reception of substrate vibrations is common, but reception of sound in the form of pressure waves is an adaptation restricted to insects (Stumpner and von Helversen, 2001). Hearing plays an important role in the life history of many insects, allowing them to detect and evade predators, home in on singing hosts, and locate and select appropriate mates.

There is ample evidence for sound signals in insect sexual communication systems. Males of the neotropical katydid, *Myopophyllum speciosum* (Morris et al.,

1994), and rice moth, *Corcyra cephalonica* (Spangler, 1987), produce ultrasonic (> 20 kHz) signals that attract conspecific females. Males of the whistling moth, *Hecatesia thyridion*, advertize mating territories to females with sonic (10 Hz-20kHz) signals (Alcock et al., 1989). Males of the lesser wax moth, *Achroia grisella*, produce advertisement songs via stationary wing fanning that attract sexually receptive females up to 1-2 m away. Females, in turn, choose males that produce songs with a fast pulse rates, high amplitude, and temporal variation (Brandt et al., 2005). Finally, both males and females of the polka-dot wasp moth, *Syntomeida epilais,* locate potential mates by means of acoustic signals (Sanderford and Conner, 1990).

In the context of communication, hearing requires not only the recognition and discrimination of highly specific acoustic patterns but also their localization. Frequency spectra of conspecific signals typically match the sensitivity of the receiver. Directionality is achieved with paired peripheral auditory structures and is enhanced by neuronal processing (Stumpner and von Helversen, 2001). In insects that possess ears known to be pure pressure receivers, directional sensitivity is achieved by diffraction of sound around the body, which changes the amplitude and phase of the sound and results in a difference in sound pressure between the two ears (Mhatre and Balakrishnan, 2007). These interaural pressure differences are sufficient cues for directional hearing (Robert, 2005). However, below about 8 kHz even in large insects such as locusts diffraction is much less effective (Michelsen and Larsen, 1978). Other insect ears are pressure gradient (difference) receivers, whereby sound propagates to both the external and internal surfaces of the tympanal membrane. The direction of sound has a substantial effect on the amplitude and/or phase of sounds reaching the ears and thus provides directionality

(Mhatre and Balakrishnan, 2007). Many members of the order Orthoptera, such as bushcrickets (Michelsen et al., 1994; Rheinlaender et al., 2007), crickets (Michelsen et al., 1994; Mhatre and Balakrishnan, 2008), cicadas (Fonseca, 1993), and grasshoppers (von Helversen and von Helversen, 1995) possess pressure gradient receivers that allow them to phonotactically orient toward conspecifics. Male congeners of field crickets, *Plebeiogryllus* spp., produce acoustic signals that attract potential mates. Females use these signals to distinguish between con- and heterospecific males and to pin point the location of a mate (Mhatre and Balakrishnan, 2007). Directional sensitivity of ears of some notodontid moths (Surlykke, 1984) and noctuid moths (Payne et al., 1966) has been demonstrated by computing threshold response curves of ears exposed to sounds from different directions. Phonotaxis, or orientation to a sound source (e.g. Rheinlaender et al., 2007), provides behavioural evidence for sound localization (Michelsen, 1998).

Although the tympanal organs in most nocturnal moth species evidently evolved in response to bat predation (Baker and Cardé, 1978; Bailey, 1991; Heller and Krahe, 1994; Yager, 1999; Stumpner and von Helversen, 2001), hearing also may have evolved in the context of mate finding and mate recognition (Stumpner and von Helversen, 2001). Several species of pyralid, arctiid and noctuid moths might have first developed the ability to hear (Heller and Krahe, 1994; Conner, 1999) which then set the stage for the evolution of acoustic signaling during courtship (Conner, 1999; Stumpner and von Helversen, 2001). The initial selection pressure for the evolution of hearing may differ from the selection pressure for its persistence, with additional functions supplementing or even replacing the original function (Stumpner and von Helversen, 2001).

Paired tympanal structures on the metathorax of Noctuoidea (including lymantriids) are among the best known lepidopteran hearing organs (Scoble, 1992). The tympanate ear has three anatomical and functional parts: *i*) the tympanum or tympanal membrane which vibrates in response to sound waves; *ii*) the tracheal sac or air sac behind the membrane which allows the membrane to vibrate with a pressure gradient; and *iii*) the tympanal organ which is a specialized chordotonal organ (stretch receptor) that transduces the mechanical signal from the tympanal membrane into nerve impulses (Yager, 1999). Scanning electron micrographs show strikingly similar tympanal regions of several noctuoid moths, including those of the male gypsy moth, *Lymantria dispar* (Speidel et al., 1996). No such tympanal regions have yet been imaged in other lymantriids.

Tympana of North American (also known as European) *L. dispar* respond to low frequency sounds (5 kHz; Cardone and Fullard, 1988) that in males are associated with wing fanning (Zlotina, 1999) and that may play a role in courtship (Cardone and Fullard, 1988).Eurasian nun moth, *L. monacha* (L.), fumida tussock moth, *L. fumida* (B.), and pink gypsy moth, *L. mathura* (M.) (all Lepidoptera: Noctuidae: Lymantriinae) are congeners of *L. dispar* with a varied distribution throughout Europe and Asia (Pogue and Schaefer, 2007). Male and female *L. mathura* produce low frequency (3.3-4.2 kHz) sounds associated with wing fanning (Zlotina, 1999). However, there are no studies yet that that describe tympanal sensitivity in congeners of *L. dispar* or that link sound in lymantriid moths with short range phonotactic behaviour.

If hearing in lymantriid moths were to serve in sexual communication, I would expect individuals to posses functional ears that are sensitive to frequency components of

conspecific sounds and mate-seeking males to behaviourally orient towards female-produced sound signals. Working with Japanese *L. monacha*, *L. fumida*, and *L. mathura*, my objectives were to (1) locate and image the tympanal region of males and females, (2) obtain and characterize sound recordings of males and females, (3) determine the functionality of the tympanate ear within the sonic frequency range using laser interferometry, and (4) determine whether acoustic signals mediate phonotactic behaviour of mate-seeking males.

2.3 Materials and Methods

2.3.1 Experimental Insects

Egg masses of *L. monacha*, *L. fumida*, and *L. mathura* were collected in the field in Morioka and Tsukuba, Honshu, Japan in 2007. Adult male *L. monacha* and *L. mathura* were collected in Morioka in July 2008. Eggs were reared to the pupal stage in the quarantine facility of the Beneficial Insects Introduction Research Laboratory, Newark, Delaware. Pupae and adults were shipped to Simon Fraser University (SFU) (permit no. P-2005-02967) and maintained in SFU's Global Forest quarantine facility at 20-25^{o}C, 60-80% relative humidity, and a 16L:8D light regime. Eclosed males and females were kept in separate rooms to prevent exposure of males to the females' pheromone. Insects were retained in 250 ml Ziploc® containers with Snap n' Seal Lids lined with moistened

Kimwipes®. One- to 3-d-old virgin adults were used for sound recordings and behavioural bioassays. All sound recordings and experiments were conducted at 20-25^{o}C.

2.3.2 Imaging Tympana

Dead specimens of *L. monacha*, *L. fumida*, and *L. mathura* were pinned to reveal the metathoracic tympanal region. Scanning electron microscopy (SEM) provided a detailed view of the target area. In preparation for SEM imaging, dead specimens were dried for at least 24 h and excess scales removed with a paintbrush. Mounted specimens were gold sputter coated using a 208 High Resolution Cressington Sputter Coater. A Hitachi S4700 Field Emission Scanning Electron Microscope (University of British Columbia, BioImaging Facility) was used to image the prepared specimens.

2.3.3 Recording and Analyses of Sound Signals

Sounds of male and female *L. monacha*, *L. fumida*, and *L. mathura* were recorded in a mesh cage (83.8 × 35.6 × 35.6 cm) in a portable wind tunnel (91.4 × 48.3 × 48.3 cm) connected to a charcoal filter (Figure 2.1A). The output of AKG CK 61-ULS condenser microphones (sensitivity: 20.0 mV/Pa; frequency response: 20 Hz to 20 kHz +/- 1 dB, AKG Acoustics, Nashville, Tennessee, U.S.A), positioned circa 10 cm from and on a level plane with the moths, was recorded at a sampling frequency of 200 kHz during the insects' peak sexual communication periods. Recordings were saved to a Philips 107-T4

desktop computer, programmed with LabVIEW 7 [National Instruments (NI), Austin, Texas, TX, USA] and equipped with a 12 bit NI data acquisition card (DAQcard-6062E). The signal-to-noise ratio was improved by pre-amplifying sounds (NI SC-2040 amplifier) prior to digitizing via the DAQ card. In experiment 1 (n = 6), a 1- to 3-d-old virgin male was placed into the mesh cage and his sounds recorded for 30 sec. In experiment 2 (n = 6), a 1- to 3-d-old virgin female was placed into the cage upwind from the male and her sounds recorded for 30 sec. During all sound recordings, behavioural observations were noted and videotaped at a rate of 10 frames per second with a Sony digital Handycam (Model DCR-VX1000). Simultaneous acquisition of audio and video files allowed the correlation of sound with behavioural response. Following data acquisition for each species, the mesh cage was replaced and the wind tunnel cleaned with hexane to prevent cross contamination with species-specific pheromone. All sound files were analyzed using the Joint Time Frequency Analysis (JTFA) 7.11 software of LabVIEW to determine the dominant (most intense) frequency (kHz) of sound associated with wing fanning or flight of males and females of all three species.

2.3.4 Laser Interferometry of Sound-Exposed Tympana

To test the sensitivity of tympana to frequency components in the females' sound signals, I used a displacement sensitive homodyne interferometer. It uses a self-mixing effect when laser light reflected from a moving target re-enters the laser cavity resulting in phase dependent changes of the lasing intensity which can be measured with a photodetector and used to assess the movement of the target (Lukashkin et al., 2005).

I used a displacement-sensitive homodyne interferometer similar to that of Lukashkin et al. (2005). It consisted of a 7mW, 670nm wavelength laser diode (Sanyo DL 3149-057) mounted in a collimation tube (LT220P-B, Thorlabs Inc.) and focused with a lens (T45-234, Edmund Optics Ltd.). The lens assembly was mounted on a piezo positioner (P-280.10, Physik Instrumente) which was used to calibrate the laser at a voltage-displacement sensitivity of 30 nm/V. Moths were mounted to a slide with the tympanal membrane and chordotonal organ exposed, onto which the laser beam was focused into a spot of about 5 μm with 45 mm clearance from the focusing lens. The laser set up was placed on a lead plate (30 × 30 × 1 cm) to minimize external vibrations. Vibrations of the tympanal region of male *L. monacha* (experiment 3, n = 6) and male *L. mathura* (experiment 4, n = 6) were measured in response to playback of a sound recording from conspecific females emitted through a Sennheiser 70 headphone speaker (matched +/- 1 dB frequency response: 10 to 39,500 Hz, 0.05% THD; Sennheiser Electronic Corporation, Old Lyme, Connecticut, U.S.A.). A piezoelectric speaker (Buzzer piezo element: CEB-44D06, Digi-Key) was employed to play back pure tone frequencies from 1-20 kHz at increments of 1 kHz up to 10 kHz. All sound stimuli were played back at equal sound pressure levels (SPLs) of 56 dB. Output from the interferometer's photodiode was fed into a current-voltage converter and an A/D converter in LabVIEW 7 and saved to a Philips 107-T4 desktop computer equipped with a NI DAQcard-6062E. Output was recorded as amplitude (dB re 1V) of the tympanum's frequency response. Signals were amplified with a NI SC2040 differential amplifier. The vibration amplitude (dB re 1V), velocity (mm/s), and displacement (μm) of the tympanum of male *L.*

monacha and male *L. mathura* were measured and calculated. Mean responses for each individual at each test frequency were averaged over 10 tests.

2.3.5 Two-Choice Behavioural Experiments: Phonotaxis to Conspecific Sound or White Noise

In experiments 5 (n = 16) and 6 (n = 8), a 1- to 3-d-old virgin male *L. monacha* or *L. fumida* was introduced into a mesh holding cage (10 × 5 × 8 cm) which was then placed within a larger mesh bioassay cage (83.8 × 35.6 × 35.6 cm) in a portable wind tunnel (91.4 × 48.3 × 48.3 cm) (Figure 2.1B). Two speakers were positioned in two corners of the bioassay cage at 45° angles. Each speaker was randomly assigned to play back a recording of female conspecific sounds, a compilation of sounds from six representative females, or a white noise control containing all frequencies within the sonic range. The male was retained in the holding cage for 1 min to settle before the speakers were turned on and an 80-mm diam Whatman No 1 filter paper impregnated with 100 pg of synthetic pheromone (*L. monacha*: (7*R*,8*S*)-*cis*-7,8-epoxy-2-methyloctadecane [(+)-disparlure], (7*R*,8*S*)-*cis*-7,8-epoxy-octadecane, 2-methyl-(*Z*)-7-octadecene; *L. fumida*: (+)-disparlure, 2-methyl-(*Z*)-7-octadecene) was placed circa 10 cm upwind from the holding cage. One min later, the holding cage was opened allowing the male to respond. A response was recorded as the male came within 2 cm of one of the speakers. Each replicate employed a new male and pheromone lure and was video recorded (see above). Between experiments 5 and 6, the mesh cage was replaced and the wind tunnel cleaned with hexane to prevent cross contamination of species-specific pheromone.

Acoustic signals were played back at biologically relevant SPLs of approximately 56 dB C (1-2 cm from the speaker) through two Sennheiser 70 headphone speakers connected to laptop computers equipped with a NI interface (DAQcard-6062E; 12 bit, 500 kHz maximum sampling rate) and software programs developed in LabVIEW 7. Signals were amplified with a NI SC2040 differential amplifier.

2.3.6 Statistical Analyses

Statistical analyses were conducted using JMP® 7 software. Data of experiments 1 and 2 were subjected to separate one-way analyses of variance (ANOVA) to determine whether mean wing beat or click frequencies produced by males or females differ between species. Tukey-Kramer HSD tests were employed to determine the means that were significantly different from one another. In two-choice behavioural experiments 5 (*L. monacha*) and 6 (*L. fumida*), χ^2 tests with Yates correction for continuity were employed to test for differences in the number of males approaching the speaker playing back sound signals from a conspecific female or the speaker playing back white noise. In all statistical tests, the alpha value was set at 0.05 (Zar, 1996).

2.4 Results

2.4.1 Imaging Tympana

Scanning electron micrographs of ears of male and female *L. monacha*, *L. fumida*, and female *L. mathura* (Figure 2.2) reveal the location of the tympanal region on the metathorax. In all three species, the tympanal region has four characteristic structures: (1) prespiracular hood, (2) tympanum, (3) membraneous conjunctiva, and (4) metepimeron.

The tympanum lies within a cavity between the posterior lateral margin of the metathorax and the first abdominal segment (Figure 2.2). The tympanic membrane is composed of thin transparent cuticle backed by tracheal sacs. The attachment site of the auditory chordotonal organ can be seen at the center of the opaque zone of the tympanum. In noctuids, two scolopidia (auditory receptor cells A1 and A2) transduce mechanical signals from the tympanum into neural signals.

2.4.2 Characterization of Sound Signals

In experiment 1, the mean wing beat frequency of male *L. monacha* (34.2 ± 0.5 Hz) was significantly higher than that of male *L. fumida* (30.2 ± 0.3 Hz) and *L. mathura* (30.6 ± 0.3 Hz), with no significant difference between the last two (Figure 2.3: ANOVA: $F_{0.05(1),2,15}=3.68$, $P<0.0001$; Tukey-Kramer HSD: critical value $Q_{0.05,15,3}=3.871$). Associated with the wing beat is a characteristic click (Figure 2.4). The mean dominant

frequency of clicks significantly differed between species, being highest for *L. mathura* (13.9 ± 0.1 kHz), intermediate for *L. monacha* (10.2 ± 0.07 kHz), and lowest for *L. fumida* (9.8 ± 0.02 kHz) (Figure 2.3: ANOVA: $F_{0.05(1),2,15}$ =3.68, P<0.0001; Tukey-Kramer HSD: critical value $Q_{0.05,15,3}$=3.871).

In experiment 2, there were significant differences in the mean wing beat frequency of female *L. monacha* (26.6 ± 0.5 Hz), *L. fumida* (30.3 ± 0.6 Hz), and *L. mathura* (15.4 ± 1.5 Hz) (Figure 2.3: ANOVA: $F_{0.05(1),2,15}$ =3.68, P<0.0001; Tukey-Kramer HSD: critical value $Q_{0.05,15,3}$=3.871). As in males, associated with the wing beat of females was a characteristic click. The mean dominant frequency of clicks produced by female *L. mathura* (14.2 ± 0.04 kHz) was significantly higher than that of female *L. fumida* (10.5 ± 0.07 kHz), and *L. monacha* (10.4 ± 0.06 kHz), with no significant difference between the last two (Figure 2.3: ANOVA: $F_{0.05(1),2,15}$ =3.68, P<0.0001; Tukey-Kramer HSD: critical value $Q_{0.05,15,3}$=3.871).

2.4.3 Laser Interferometry of Sound-Exposed Tympana

Vibrations of the tympanal membranes in response to airborne sound varied in amplitude (dB re 1V) with the frequency (Hz) of the sound stimuli (Figure 2.5). In male *L. monacha*, behaviourally relevant frequencies, including the females' wing beat (20-30 Hz) and a 5-kHz sub-harmonic of the 10-kHz click frequency, are represented as peaks in the spectra of tympanal vibration (Figure 2.5A: Experiment 3). In particular, the tympanum is more sensitive to the 5-kHz sub-harmonic than it is to all other frequencies

tested. The 5-kHz sub-harmonic also induced high velocity (mm/s) vibrations (Figure 2.5C) and increased displacement (μm) (Figure 2.5E,G) of the tympanum.

In male *L. mathura*, the females' wing beat (10-20 Hz) and a 7-kHz sub-harmonic of the 14-kHz mean click frequency are represented as peaks in the spectra of tympanal vibrations (Figure 2.5B: Experiment 4). Compared to all other frequencies tested, tympana were particularly sensitive to the females' mean wing beat frequency. It elicited high velocity vibrations (mm/s) (Figure 2.5D) and pronounced displacement (μm) of male tympana (Figure 2.5F,H).

The vibration velocity (υ) is proportional to the amplitude (dB re 1V) of response ($z_{\circ}*\upsilon$=10^(dB/20), where $z_{\circ}$ equals the acoustic impedance), which varies with frequency. The displacement (*d*) of the tympanum is proportional to the vibration velocity and inversely proportional to frequency (*f*) ($d= \upsilon/2\pi f$). Because the change in frequency of the sound stimulus was larger than the average change in vibration velocity of the tympanum, displacement of the tympanum decreased as frequency increases.

2.4.4 Two-Choice Behavioural Experiments: Phonotaxis to Conspecific Sound or White Noise

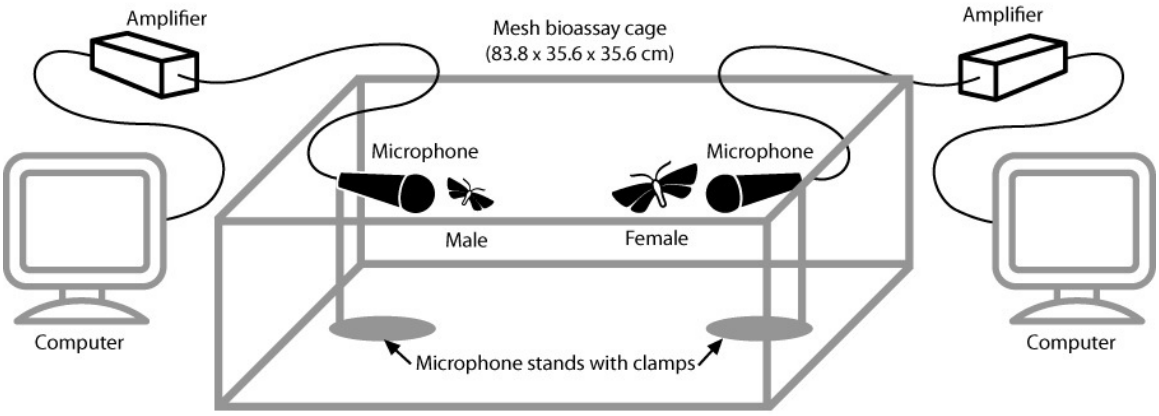
In experiment 5, significantly more male *L. monacha* approached within 2 cm or made contact with the speaker that played back a sound recording from conspecific females than approached the speaker emitting white noise (Figure 2.6: χ^2 test with Yates correction for continuity: P=0.02). A male leaving the holding cage typically walked while wing fluttering towards the pheromone lure, made contact with it, and then proceeded walking while wing fluttering or flying towards the treatment speaker.

In experiment 6, seven out of the eight male *L. fumida* responded to the speaker emitting sound recordings from conspecific females (Figure 2.6). However, the number of males available for testing were not sufficient to obtain a statistically significant result (χ^2 test with Yates correction for continuity: P=0.08).

Our colony of *L. mathura* collapsed, most likely due to a viral disease, not allowing us to complete the two-choice experiment.

Figure 2.1: (A) Design for recording sounds from males and females of *L.monacha*, *L. fumida*, and *L. mathura*; (B) design for two-choice behavioural experiments with speakers emitting treatment or control stimuli equidistant (10 cm) from the pheromone lure. The mesh bioassay cage was placed inside a portable wind tunnel (91.4 × 48.3 × 48.3 cm) (not shown), with the bioassay insect downwind from the pheromone source and speakers.

A


Amplifier
Mesh bioassay cage
(83.8 x 35.6 x 35.6 cm)
Amplifier
Microphone
Microphone
Male
Female
Computer
Computer
Microphone stands with clamps

B

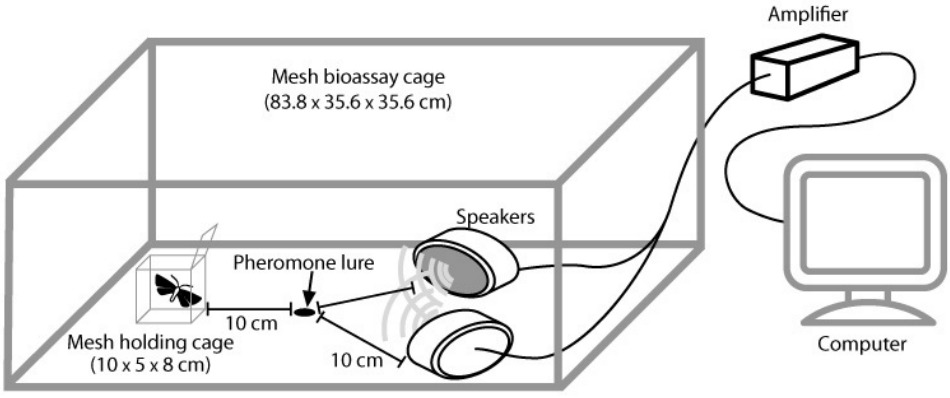

Amplifier
Mesh bioassay cage
(83.8 x 35.6 x 35.6 cm)
Speakers
Pheromone lure
10 cm
Mesh holding cage
(10 x 5 x 8 cm)
10 cm
Computer

Figure 2.2: Schematic drawing (A), and scanning electron micrographs (B-F) of tympanal regions of female *L. mathura* (B), male and female *L. monacha* (C, D), and male and female *L. fumida* (E, F). Abbreviations as follows: sp = first abdominal spiracle; prh = prespiracular hood; t = tympanum; co = membraneous conjunctiva; e(a,b) = metepimeron; wb = hind wing base.

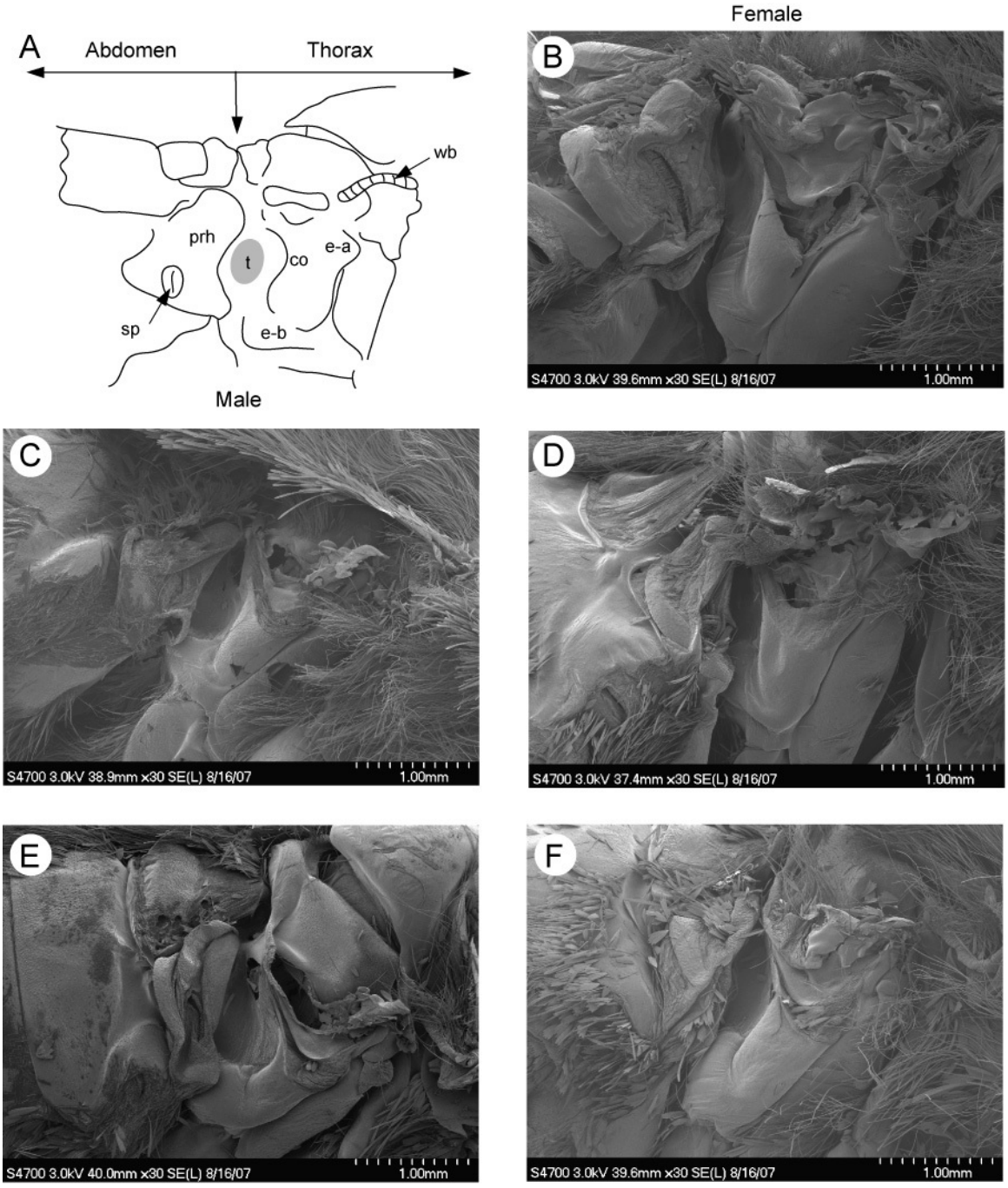

A
Abdomen
Thorax
wb
prh
t
co
e-a
sp
e-b
Male
Female
B
S4700 3.0kV 39.6mm x30 SE(L) 8/16/07
1.00mm
C
S4700 3.0kV 38.9mm x30 SE(L) 8/16/07
1.00mm
D
S4700 3.0kV 37.4mm x30 SE(L) 8/16/07
1.00mm
E
S4700 3.0kV 40.0mm x30 SE(L) 8/16/07
1.00mm
F
S4700 3.0kV 39.6mm x30 SE(L) 8/16/07
1.00mm

Figure 2.3: Mean (± SE) frequencies of wing beat and associated clicks produced in experiments 1 and 2 by wing fanning male and female *L. monacha*, *L. fumida*, and *L. mathura*. For each sound type (wing beat or click) and insect sex, bars with different letters are significantly different (ANOVA followed by Tukey-Kramer HSD, $P<0.05$).

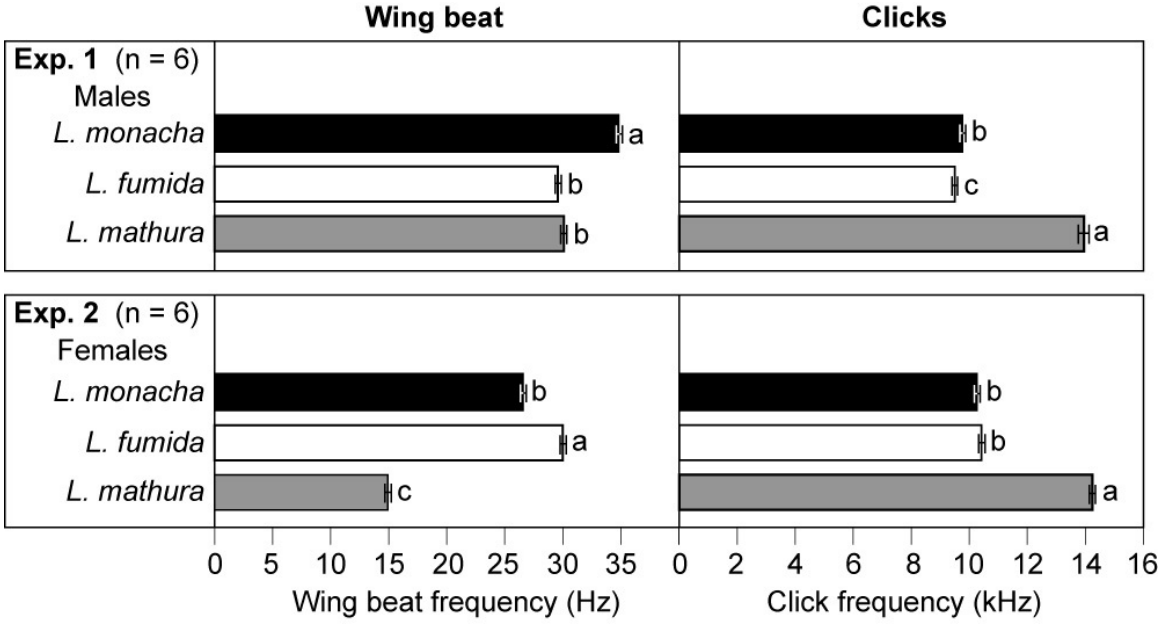

Wing beat
Clicks
Exp. 1 (n = 6)
Males
L. monacha
L. fumida
L. mathura
a
b
b
b
c
a
Exp. 2 (n = 6)
Females
L. monacha
L. fumida
L. mathura
b
a
c
b
b
a
0 5 10 15 20 25 30 35
Wing beat frequency (Hz)
0 2 4 6 8 10 12 14 16
Click frequency (kHz)

Figure 2.4: Analysis of (A) waveform, (B) frequency, and (C) time-frequency sound intensity (sonogram) of sound associated with a single wing beat and an associated click (arrow) in male *L. monacha*. The darker shades in C indicate more intense frequency components.

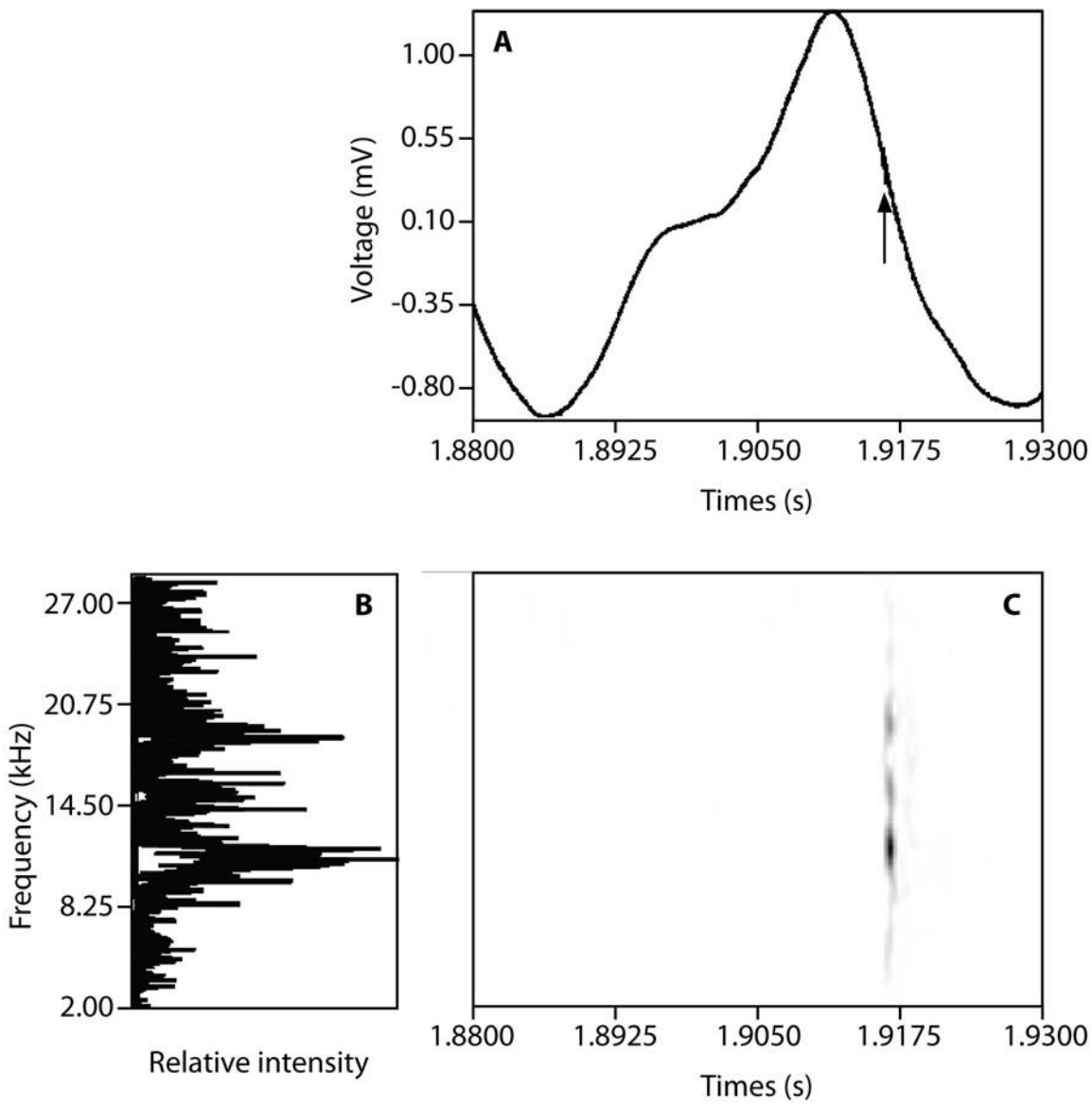

A
1.00
0.55
0.10
-0.35
-0.80
Voltage (mV)
1.8800
1.8925
1.9050
1.9175
1.9300
Times (s)
B
27.00
20.75
14.50
8.25
2.00
Frequency (kHz)
Relative intensity
C
1.8800
1.8925
1.9050
1.9175
1.9300
Times (s)

Figure 2.5: Laser interferometry recordings of vibration amplitude (A,B), velocity (C,D), and displacement (E-H) of tympanal membranes of male *L. monacha* (n = 6) and male *L. mathura* (n = 6) in experiments 3 and 4, respectively, in response to airborne sound stimuli. For each tympanum (specimen) and sound stimulus, recordings were averaged over 10 exposures.

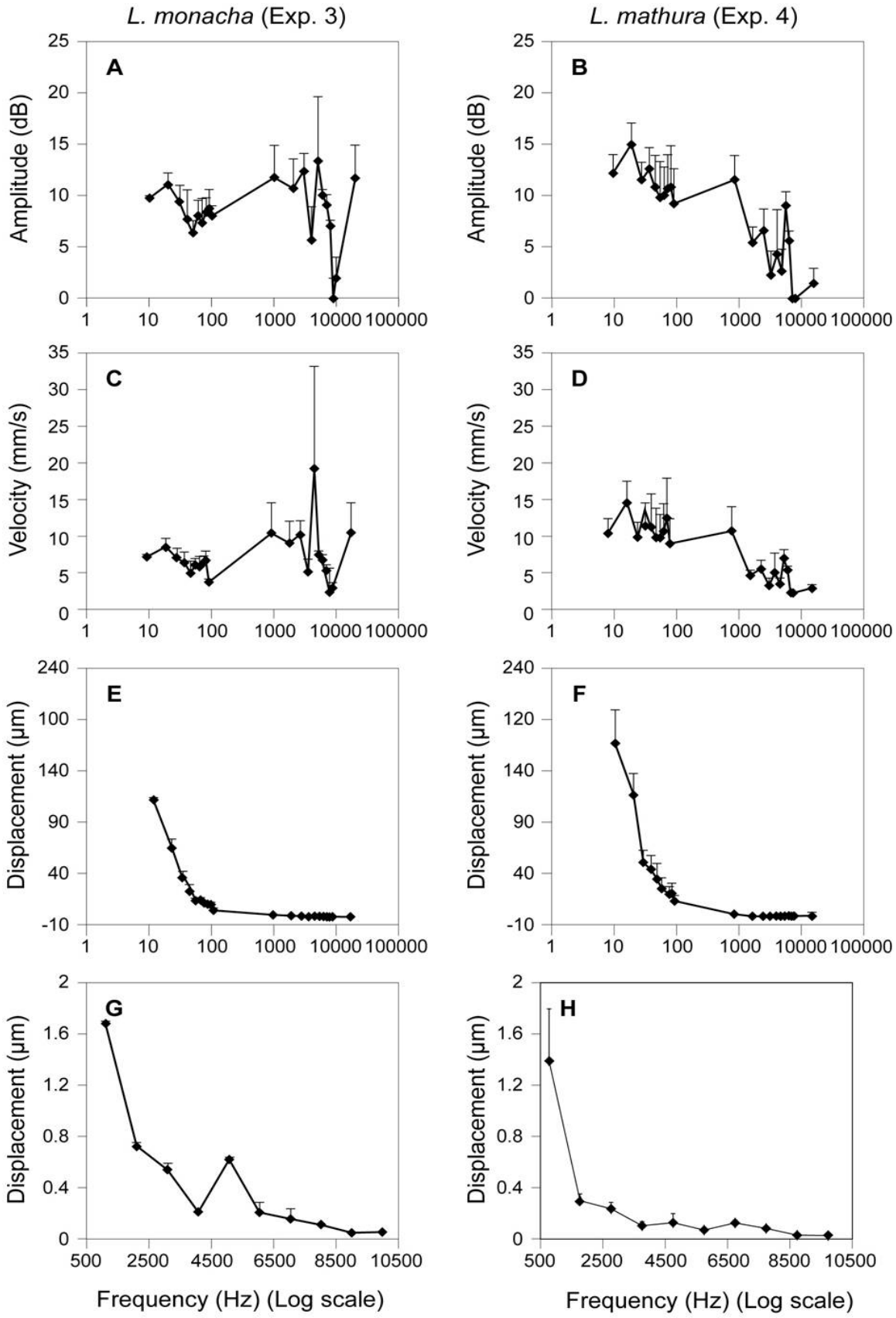
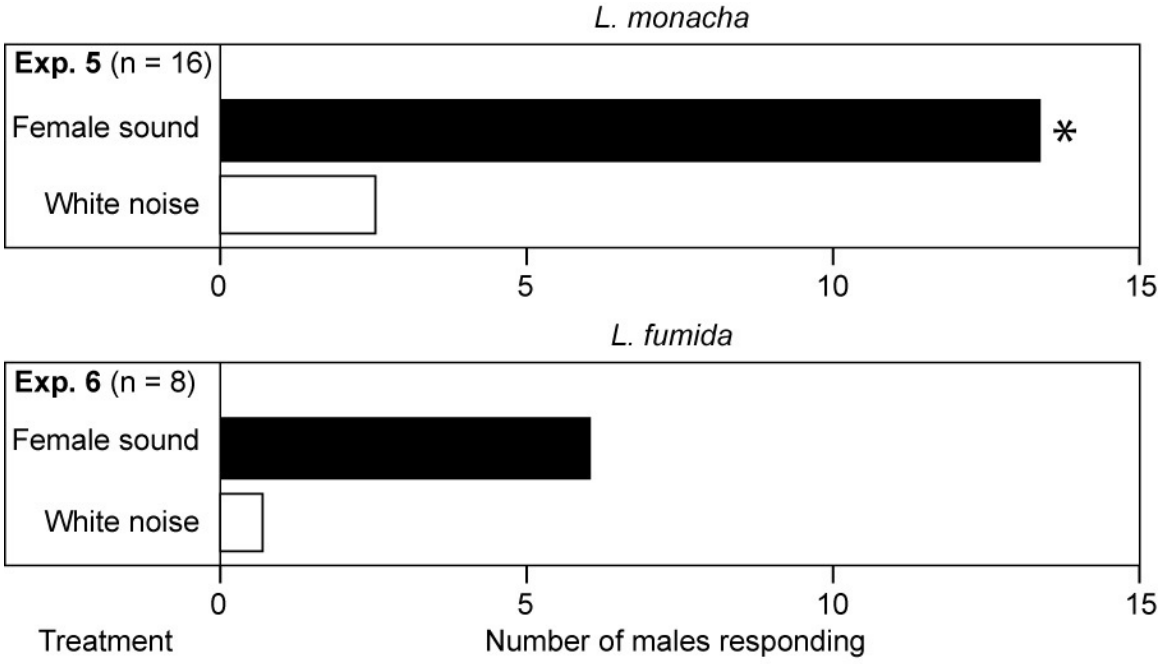

L. monacha (Exp. 3)
L. mathura (Exp. 4)
A
B
C
D
E
F
G
H
Amplitude (dB)
Velocity (mm/s)
Displacement (µm)
Frequency (Hz) (Log scale)

Figure 2.6: Phonotactic response of male *L. monacha* and male *L. fumida* in two-choice experiments 5 and 6 to sound signals from conspecific females or to white noise. In experiment 5, the asterisk (*) indicates a significant preference for the treatment stimulus (χ^2 test with Yates correction for continuity: $P<0.05$).


L. monacha
Exp. 5 (n = 16)
Female sound
White noise
*
0
5
10
15
L. fumida
Exp. 6 (n = 8)
Female sound
White noise
0
5
10
15
Treatment
Number of males responding

2.5 Discussion

My data support the conclusion that female and male *L. monacha* use acoustic signals as part of their sexual communication system. Evidence of tympanate ears in males and females that are fully functional at frequencies well below those used by hunting bats (Figure 2.5), species-specific sound produced by wing fanning males and females (Figure 2.3), particular sensitivity of the males' tympana to frequency components of the wing beat and clicks produced by wing fanning females (Figure 2.5), and phonotactic orientation by males toward sounds produced by females (Figure 2.6), all support the concept that male and female *L. monacha* use sound signals during sexual communication. The same concept likely applies to congeneric *L. fumida* and *L. mathura* but respective experimental results lack some physiological or behavioral data.

Lymantria monacha, L. fumida and *L. mathura* are three of five synchronic and sympatric congeners of mixed forests in the Tohoku region of Honshu, Japan. They maintain reproductive isolation by species-specific communication channels that differ, in part, by their pheromone signal or time of signaling (Wallner et al., 1995; Schaefer et al., 1999; Gries et al., 1999, 2001, 2002, 2005, 2009a, b). Nonetheless, with five congeners emitting communication signals, the forest habitat becomes chemically and acoustically noisy, with selection to improve the signal-to-noise ratio of communication channels (Cardé and Baker, 1984). The species-specific wingbeat frequency produced by wing fanning female *L. monacha, L. fumida* and *L. mathura* (Figure 2.3) may help improve the signal-to-noise ratio during sexual communication and contribute to their reproductive isolation. This concept of sound as a reproductive isolating mechanism is

also applicable to clicks produced by wing fanning males. Females may recognize the species-specific frequency of clicks produced by conspecific males, and may avoid mating with males that produce the "wrong" clicks.

While sound may be the final fail-safe mechanism that ensures mating with conspecific mates, it will be effective only at close range. Wing fanning sound signals from females with an intensity of ca. 56 dB (10 cm from the source) may be detected by foraging males at a distance not exceeding a few meters. Assuming that male *L. monacha, L. fumida* and *L. mathura* can detect pheromone from calling females over a distance of at least 120 m, as shown for *L. dispar* (Willis et al., 1991), the unique sex pheromone blend of a conspecific female (Schaefer et al., 1999; Gries et al., 1999, 2001) appears to be the first and foremost reproductive isolating mechanism.

That pheromone-emitting females do not wing fan unless a conspecific male is approaching or has alighted nearby (personal observation), implies short-range communication as the primary function of sound signals. Both male *L. monacha* and *L. fumida* readily oriented toward, and made contact with, a speaker that played back recordings of conspecific female sounds (Figure 2.6). Because pheromone is not the best type of signal to convey the microlocation of the signaller, due to sensory overload of pheromone receptors (Baker et al., 1981; Willis et al., 1991), and visual signals become obscured and are not efficient during the nocturnal communication periods of these moths, sound appears to be the ideal signal for the male to announce his arrival and for the female to guide him toward her.

Phonotactic orientation of male *L. monacha* and *L. fumida* (Figure 2.4) to the sound of conspecific females is evidence that males have fully functional ears. Additional

evidence includes scanning electron micrographs of the males' tympanal region (Figure 2.2) that are virtually identical to those obtained for male *L. dispar* (Speidel et al., 1996), which have fully functional tympanate ears in the sonic range (Chapter 3). Moreover, laser interferometry revealed that the tympanum of male *L. monacha* and *L. mathura* is particularly sensitive to the wing beat frequency of conspecific females and the sub-harmonic of associated clicks (Figure 2.5).

Based on previous findings (Schaefer et al., 1999; Gries et al., 1999, 2001) and those presented in this study, mate attraction and location in *L. monacha*, and probably in *L. fumida* and *L. mathura*, involve pheromonal and acoustic signals, and appear to proceed in the following sequence: (1) females emit a sex pheromone that attracts males; (2) males fly towards and search for or alight near calling females; (3) females detect the male's sounds, and (4) in turn, sound signals from wing fanning females help males orient towards them.

3: Intraspecific Acoustic Communication and Functionality of the Tympanate Ear of the European Gypsy Moth, *Lymantria dispar* (L.) (Lepidoptera: Noctuidae: Lymantriinae)*

3.1 Abstract

Hearing in the context of communication requires the recognition and discrimination of highly specific acoustic patterns as well as the localization of the signals. Tympanate ears of female gypsy moth, *Lymantria dispar*, are reportedly more sensitive than the ears of conspecific males to sounds in the sonic frequency range (10-20 kHz). I tested the hypothesis that this differential sensitivity is due to sex-specific functional roles of sound during sexual communication, with males sending and females receiving acoustic signals. Analyses of sounds produced by flying males revealed a mean wing beat frequency of 33 Hz and associated clicks of 14 kHz which remained unchanged in the presence of female sex pheromone. While females exposed to sounds (0.3-3.4 kHz) of flying male salt marsh mosquitoes, *Aedes taeniorhynchus*, demonstrated no behavioural response, exposure of females to playback sounds of flying conspecific males elicited wing raising and

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fluttering and walking, generating distinctive visual signals that may be utilized by a mate-seeking male at close range. Laser interferometry recordings revealed that the female tympanum is particularly sensitive to frequencies in the range produced by flying conspecific males, including the 33-Hz wing beat frequency and the 7-kHz sub-harmonic of the 14-kHz clicks. These results support the hypothesis that the female ear is tuned to sounds of flying males. Based on previous findings and data presented here, sexual communication in *L. dispar* may proceed in the following sequence: (1) females emit sex pheromone that attracts males; (2) males fly toward calling females; and (3) sound signals from flying males at close range induce movement in females which, in turn, provides visual signals that could orient males toward females.

Keywords: *Lymantria dispar*; Lepidoptera; Noctuidae; Lymantriinae; intraspecific acoustic communication; behavioural response; acoustic signals; tympanate ear; laser interferometry

3.2 Introduction

Communication occurs when actions(s) or signal(s) on the part of one organism change the pattern of behaviour in a receiver (Wilson, 1975). Modes of communication used by animals to locate mates, find hosts, and detect predators include vision, chemoreception, tactile sensation, and audition (Yager, 1999; Partan and Marler, 2005). Acoustic communication is documented in many vertebrates and invertebrates, including dolphins (e.g., Kyhn et al., 2009), bats (e.g., Page and Ryan, 2005), frogs (e.g., Feng et

al., 2006), birds (e.g., Patricelli et al., 2007) and many insects (e.g., Jones et al., 2002; Rodriguez and Greenfield, 2004).

Hearing in the context of communication requires the recognition and discrimination of highly specific acoustic patterns as well as the localization of the signals. Frequency characteristics of conspecific sound signals typically match the sensitivity of the receiver. Directionality is achieved by the presence of paired peripheral auditory structures and is enhanced by neuronal processing (Stumpner and von Helversen, 2001).

In moths, ears likely evolved in response to bat predation (Baker and Cardé, 1978; Bailey, 1991; Heller and Krahe, 1994; Yager, 1999; Stumpner and von Helversen, 2001), but may have also evolved in the context of mate finding and recognition (Heller and Krahe, 1994; Conner, 1999). This has also been suggested for crickets, bushcrickets, cicadas, and water bugs (Stumpner and von Helversen, 2001). The initial selection pressure for the evolution of hearing may be different from that for its persistence. Selection pressures continuously change and can re-shape the function(s) of hearing (Stumpner and von Helversen, 2001). The ability to detect and evade bats may be the most significant evolutionary factor in the pre-adaptation of moths to evolve intraspecific acoustic communication systems (Conner, 1999).

During courtship, moth species produce signals ranging from trembling sounds in the sonic (10 Hz-20 kHz) frequency range to high frequency ultrasound (> 20 kHz). Ultrasonic (> 20 kHz) signals play a role in the courtship of several pyralid moths. For example, males and females of the polka-dot wasp moth, *Syntomeida epilais* (Sanderford and Conner, 1990), and males of the lesser wax moth, *Achroia grisella* (Spangler et al.,

1984), and rice moth, *Corcyra cephalonica* (Spangler, 1987), produce ultrasonic acoustic signals that attract potential mates. Males of the whistling moth, *Hecatesia thyridion*, advertize mating territories to females using sonic (10 Hz-20 kHz) signals (Alcock et al., 1989), with the highest intensity between 15-20 kHz (Bailey, 1978). Courting males of the common cutworm moth, *Agrotis fucosa*, produce characteristic trembling sounds in the sonic frequency range (Wakamura, 1977). Similarly, female nun moth, *Lymantria monacha*, and *L. fumida* wing fan, producing wing beat frequencies of < 11 Hz and click frequencies of about 10 kHz, which serve as orientation signals for conspecific males (Chapter 2). Furthermore, the tympanal membrane of male *L. monacha* and pink gypsy moth, *L. mathura*, are particularly sensitive to specific frequencies of sound signals from conspecific females (Chapter 2).

Tympana of female North American (also known as European) *L. dispar* respond to low frequency sound (Cardone and Fullard, 1988) associated with wing fanning of courting males (Zlotina, 1999). Electrical activity of flight muscles associated with wing fanning in males increases with dose of synthetic sex pheromone (Obriecht and Hanson, 1989). Because flightless female *L. dispar* are less exposed to bat predation and are less sensitive than males to ultrasonic frequencies of bat echolocation systems, females may possess ears in a state of "evolutionary degeneration" as proposed by Cardone and Fullard (1988). However, comparative audiograms of females and males show that females are more sensitive than males to frequencies < 20 kHz (Cardone and Fullard, 1988), such as those associated with wing fanning males (Zlotina, 1999). I predict that sounds (< 20 kHz) produced by males are recognized by conspecific females, and that the

tympanum of European *L. dispar* is most sensitive to frequencies in the male's acoustic signal.

Sounds associated with the wing beat of a flying insect may provide receivers with important acoustic information. For example, female bolas spiders, *Mastophora hutchinsoni*, exploit the intraspecific communication signals of their moth prey. When the spiders detect the wing beat of an approaching moth, they construct and flick a bolas (a sticky globule at the end of a silk thread) that captures the unsuspecting prey (Haynes et al., 2001). Acoustic information may also be relayed when insects exhibit different, context-specific types of flight. When males of the bark beetle *Pityogenes chalcographus* enter the pheromone plume of conspecifics infesting a host tree, they change their flight pattern and exhibit a very characteristic "Reigenflug" type flight, decreasing their ground speed and hovering like a helicopter slowly changing position (Wichmann, 1953). In clean air, male *L. dispar* exhibit a casting flight with large side to side sweeps almost perpendicular to the direction of the wind (Kennedy and Marsh, 1974). When they lock on to the pheromone plume of an upwind female, males engage in a more directed zigzagging flight toward the female (Kennedy and Marsh, 1974; Goldsworthy and Wheeler, 1989). I predict that the wing beat frequency of male *L. dispar* changes in pheromone-laden air, signaling to a calling female that an approaching male has locked on to her pheromone plume. The male signal may, in turn, lead the female to provide additional acoustic or visual signals that reveal her micro-location.

The objective of this study was to provide evidence for sound communication in European *L. dispar*. Specifically, my objectives were to (1) determine any changes in wing beat frequency of flying males when exposed to female sex pheromone, (2) record

any behavioural changes of females in response to wing beat sounds of flying males, (3) describe and image the tympanal region of females, and (4) determine the functionality of the tympanate ear of male and female European *L. dispar* within the sonic frequency range using laser interferometry.

3.3 Materials and Methods

3.3.1 Experimental Insects

Male pupae were obtained from the United States Department of Agriculture (USDA), Agricultural Research Service, Beneficial Insects Introduction Research Laboratory, Newark, Delaware (permit no. P-2004-01124 and P-2005-02967). Male and female larvae were obtained from the USDA Forest Service, Northern Research Station, Hamden, Connecticut (permit no. P-2007-02143). Insects were reared to adults in the Global Forest quarantine facility at SFU at a temperature of 18-23^{o}C, at 60-80% relative humidity, and a 16L:8D light regime. They were kept in 250 ml Ziploc® containers with Snap n' Seal Lids lined with moistened Kimwipes® and provisioned with artificial diet shipped in dry form from the USDA Forest Service in Ansonia, CT and prepared at SFU. Two- to three-d-old virgin adults were used for sound recordings and behavioural bioassays. All sound recordings and experiments were conducted at 20-25^{o}C.

3.3.2 Flight of Males in Clean versus Pheromone-Laden Air

Sound recordings of males first in clean and then in pheromone-laden air (experiment 1) were conducted in a glass bell shaped olfactometer (28.0 × 14.5 cm diam) with four arms (each 10.0 × 4.0 cm diam) at right-angles to each other 8.0 cm above the base of the bell (Figure 3.1). One arm was connected with Tygon® plastic tubing to a tank of medical air which generated a constant air flow of 24 mL/min, while the opposite arm was attached to a charcoal filter with plastic air ducting. A third arm housed an AKG CK 61-ULS condenser microphone (sensitivity: 20.0 mV/Pa; frequency response: 20 Hz to 20 kHz +/- 1 dB, AKG Acoustics, Nashville, Tennessee, U.S.A). The fourth arm was closed with a glass stopper. Sound recordings were conducted during the last 2-5 h of the photophase, the peak activity period of *L. dispar* (Miller and Roelofs, 1978), using as a light source two Sylvania Daylight Deluxe F40/DX 40 watt, 121.9 cm long tube lights, two Philips Plant and Aquarium F40T12 40 watt, 121.9 cm long tube lights, and a Dolan-Jenner Industries Series 180 Fiber Light cold lamp. Two hours prior to each recording, 1- to 3-d-old virgin male moths were tethered to a Plexiglass stand (Figure 3.1) and placed in the olfactometer. After a 2 min recording of the tethered male flying in clean air, a Whatman Filter Paper (1 cm diam) impregnated with 100 pg of (+)-disparlure was placed upwind from the tethered male (Figure 3.1) before recordings continued for another 2 min.

Recordings were saved to a Philips 107-T4 desktop computer with a NI data acquisition card (DAQcard-6062E; 12 bit, 500 kHz maximum sampling rate), programmed with National Instruments (NI) LabVIEW 7 (National Instruments, Austin, TX, USA). The signal-to-noise ratio was improved by pre-amplifying the sounds with a

NI SC-2040 amplifier. A sampling frequency of 200 kHz was used during recordings. Concurrently, the male's behavioural response was videotaped at a rate of 10 frames per second with a Sony digital Handycam (Model DCR-VX1000). The simultaneous acquisition of audio and video files allowed the correlation of sounds with behavioural responses.

Sound files were analyzed using the Joint Time Frequency Analysis (JTFA) 7.11 software of the LabVIEW program to determine the dominant (most intense) frequency of sounds associated with in-flight wing beats before and after exposure of a male to the synthetic sex pheromone (+)-disparlure.

3.3.3 Behavioural Response of Females to Conspecific Male Acoustic Signals

Acoustic signals were played back through a Sennheiser 70 headphone speaker (matched +/- 1 dB flat frequency response: 10 to 39,500 Hz, 0.05% THD; Sennheiser Electronic Corporation, Old Lyme, Connecticut, U.S.A.), connected to a laptop computer equipped with a NI interface (DAQcard-6062E; 12 bit, 500 kHz maximum sampling rate) and software programs developed in LabVIEW 7. Signals were amplified with a SC2040 differential amplifier (NI Corporation, Austin, Texas, U.S.A., 78759-3504).

For each replicate in experiment 2, a 2- to 3-d-old female was released into a mesh cage (50.5 × 30.5 × 25.5 cm) during the last 2-5 h of the photophase. The female was kept in silence for 1 min to allow her to settle before she was exposed to two consecutive playbacks of a 1 min sound recording. The first was the sound of a flying male salt marsh mosquito, *Aedes taeniorhynchus* (0.3-3.4 kHz (Mankin, 1994)) (source:

Reference Library of Digitized Insect Sounds) played back at a sound pressure level (SPL) of 58 dB C (1-2 cm from the speaker). The second was the sound of a flying male *L. dispar* played back at the same biologically relevant SPL. For each replicate in experiment 3, a 3-d-old virgin female was exposed to two consecutive 1 min playbacks of the mosquito sound at the same SPL. The speakers were placed circa 10 cm away from the female. Her behavioural response was observed and videotaped at a rate of 10 frames per second with the Handycam. Video files were analyzed to determine whether and when during the 60 sec bioassay a female responded to the test stimulus. Any movement by the female was considered a response.

3.3.4 Imaging the Tympanate Ear of Females

Dead females were pinned to reveal the tympanal metathoracic region. Scanning electron microscopy (SEM) provided a detailed view of the target area. In preparation for SEM imaging, dead specimens were dried for at least 24 h, and excess scales removed with a paintbrush. Mounted specimens were gold coated using a 208 High Resolution Cressington Sputter Coater. A Hitachi S4700 Field Emission Scanning Electron Microscope (University of British Columbia BioImaging Facility) was used to image the prepared specimens.

3.3.5 Laser Interferometer Recordings of Sound-Exposed Tympana

A displacement-sensitive homodyne interferometer uses a self-mixing effect when laser light reflected from a moving target re-enters the laser cavity resulting in phase dependent changes of the lasing intensity which can be measured with a photodetector and used to assess the movement of the target (Lukashkin et al., 2005).

I used a displacement-sensitive homodyne interferometer similar to that of Lukashkin et al. (2005). It consisted of a 7 mW, 670 nm wavelength laser diode (Sanyo DL 3149-057) mounted in a collimation tube (LT220P-B, Thorlabs Inc.) and focused with a lens (T45-234, Edmund Optics Ltd.). The lens assembly was mounted on a piezo positioner (P-280.10, Physik Instrumente) which was used to calibrate the laser at a voltage-displacement sensitivity of 30 nm/V. The laser beam was focused on the tympanum into a spot of ~5 μm in diam at a distance of ~45 mm from the surface of the focusing lens. Vibrations of the exposed tympanal membrane of male ears (experiment 4) and female ears (experiment 5) were measured in response to playback of sound recordings of males (10-100 Hz) and to pure tone frequencies (1-20 kHz at increments of 1 kHz up to 10 kHz). A Sennheiser 70 headphone speaker delivered the conspecific sound. A piezoelectric speaker (Buzzer piezo element: CEB-44D06, Digi-Key) was used to play the pure tone frequencies. Sound stimuli were played back at equal SPLs of 58 dB C. The output from the photodiode was fed into a current-voltage converter and into an A/D converter in LabVIEW 7 (NI) and saved to a Philips 107-T4 desktop computer equipped with a NI data acquisition card (DAQcard-6062E; 12 bit, 500 kHz maximum sampling rate). Output was recorded as amplitude (dB re 1V) of the frequency response

of the tympanum. Signals were amplified with a NI SC2040 differential amplifier. The laser set up was placed on a lead plate (30 × 30 × 1 cm) to minimize external vibrations.

Vibration amplitude (dB re 1V), velocity (mm/s), and displacement (μm) of the tympanum of male and female moths were measured and calculated. Mean responses of each tympanum to each test frequency were averaged over 10 tests.

3.4 Results

3.4.1 Flight of Males in Clean versus Pheromone-laden Air

Males flying in clean air produced a wing beat with a mean dominant (2^{nd} harmonic) frequency of 32.63 ± 1.0 Hz (Figure 3.2). Associated with each wing beat was a click with a mean dominant frequency of 13.74 ± 0.38 kHz (Figure 3.3).

Males flying in pheromone-laden air produced a mean dominant (2^{nd} harmonic) wing beat frequency of 32.51 ± 0.78 Hz. The click associated with the wing beat had a mean dominant frequency of 13.73 ± 0.50 kHz. The presence of pheromone did not significantly alter the mean dominant frequency of wing beats or clicks (Figure 3.4A,B: Wilcoxon signed-ranks test: wingbeats: $P = 0.84$; clicks: $P = 0.84$).

3.4.2 Behavioural Response of Females to Conspecific Male Acoustic Signals

Females did not respond to playback sounds of a flying mosquito, but did respond to playback sounds of a flying conspecific male (Figure 3.5A: Experiment 2, Wilcoxon signed-ranks test: P=0.002). Females typically responded by raising and fluttering their wings, followed by walking up the side of the mesh cage. Females exposed twice for 1 min each to playback sounds of a flying mosquito did not respond (Figure 3.5B: Experiment 3, Wilcoxon signed-ranks test: P=1.0).

3.4.3 Imaging the Tympanate Ear of Females

The tympanal region is characterized by the presence of a prespiracular hood, tympanum, membraneous conjunctiva, and metepimeron (Figure 3.6A). Scanning electron micrographs (Figure 3.6B) of the female tympanal region on the metathorax are strikingly similar to SEM images of males (Speidel et al. 1996). Compound microscope images reveal details of the male (Figure 3.6C) and female (Figure 3.6D) tympanal membranes and chordotonal organs. The tympanum lies within a cavity between the posterior lateral margin of the metathorax and the first abdominal segment. The tympanic membrane is composed of thinned transparent cuticle (circled in Figure 3.6C, D) backed by tracheal sacs. The attachment site of the auditory chordotonal organ can be seen at the center of the opaque zone of the tympanum (arrow in Figure 3.6C, D). In noctuids, two scolopidia (auditory receptor cells A1 and A2) transduce mechanical signals from the tympanum into neural signals.

3.4.4 Laser Interferometer Recordings of Sound-exposed Tympana

Vibrations of male and female tympanal membranes in response to airborne sound varied in amplitude (dB re 1V) with the frequency of the sound stimuli (Figure 3.7A). Behaviourally relevant frequencies, including the mean dominant (2^{nd} harmonic) male wing beat frequency (~30 Hz) and a sub-harmonic (7 kHz) of the mean dominant frequency of an associated click (~14 kHz) are represented as peaks in the spectra of the tympanal vibration. In particular, the tympanum is more sensitive to the 7-kHz sub-harmonic than it is to 0.01-10 kHz frequencies tested with males (experiment 4), and than it is to all other frequencies tested with females (experiment 5). The 7-kHz sub-harmonic also induced high-velocity vibrations (Figure 3.7B) and increased displacement (Figure 3.7C,D) in tympana of male and female ears.

The vibration velocity (υ) is proportional to the amplitude (dB re 1V) of response ($z_{\circ}*\upsilon$=10^(dB/20), where $z_{\circ}$ equals the acoustic impedance), which varies with frequency. The displacement (d) of the tympanum is proportional to the vibration velocity and inversely proportional to frequency (f) ($d= \upsilon/2\pi f$). The displacement of tympana decreased as the frequency increased because the change in frequency of the sound stimulus exceeded the average change in vibration velocity of the tympana.

Figure 3.1: Experimental design for recording sound produced by a tethered male *L. dispar* flying in clean air or air laden with synthetic sex pheromone. For graphical clarity, the fourth arm of the olfactometer which is located opposite the arm housing the microphone and which was plugged with a glass stopper during recordings is not shown.

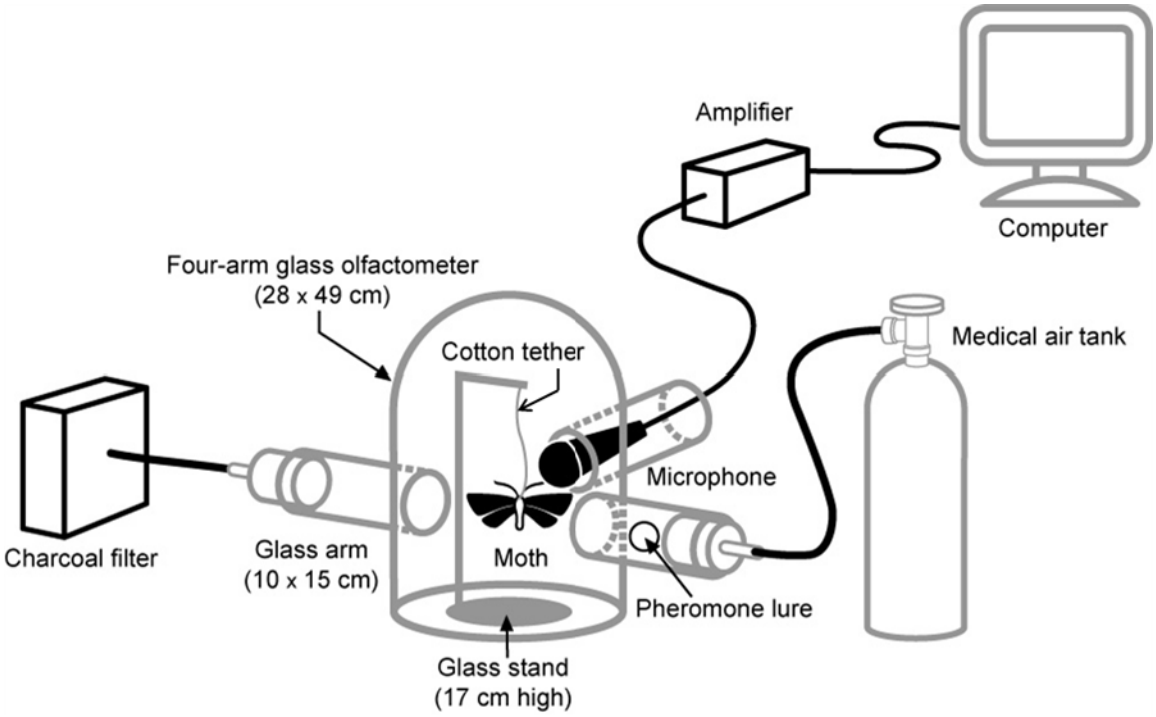

Amplifier
Computer
Four-arm glass olfactometer
(28 x 49 cm)
Cotton tether
Medical air tank
Microphone
Charcoal filter
Glass arm
(10 x 15 cm)
Moth
Pheromone lure
Glass stand
(17 cm high)

Figure 3.2: Analysis of (A) waveform, (B) frequency, and (C) time-frequency sound intensity (sonogram) of sound caused by three wing beats of a tethered flying male *L. dispar*. The darker the shading in C, the more intense the frequency component of the recorded sound signal.

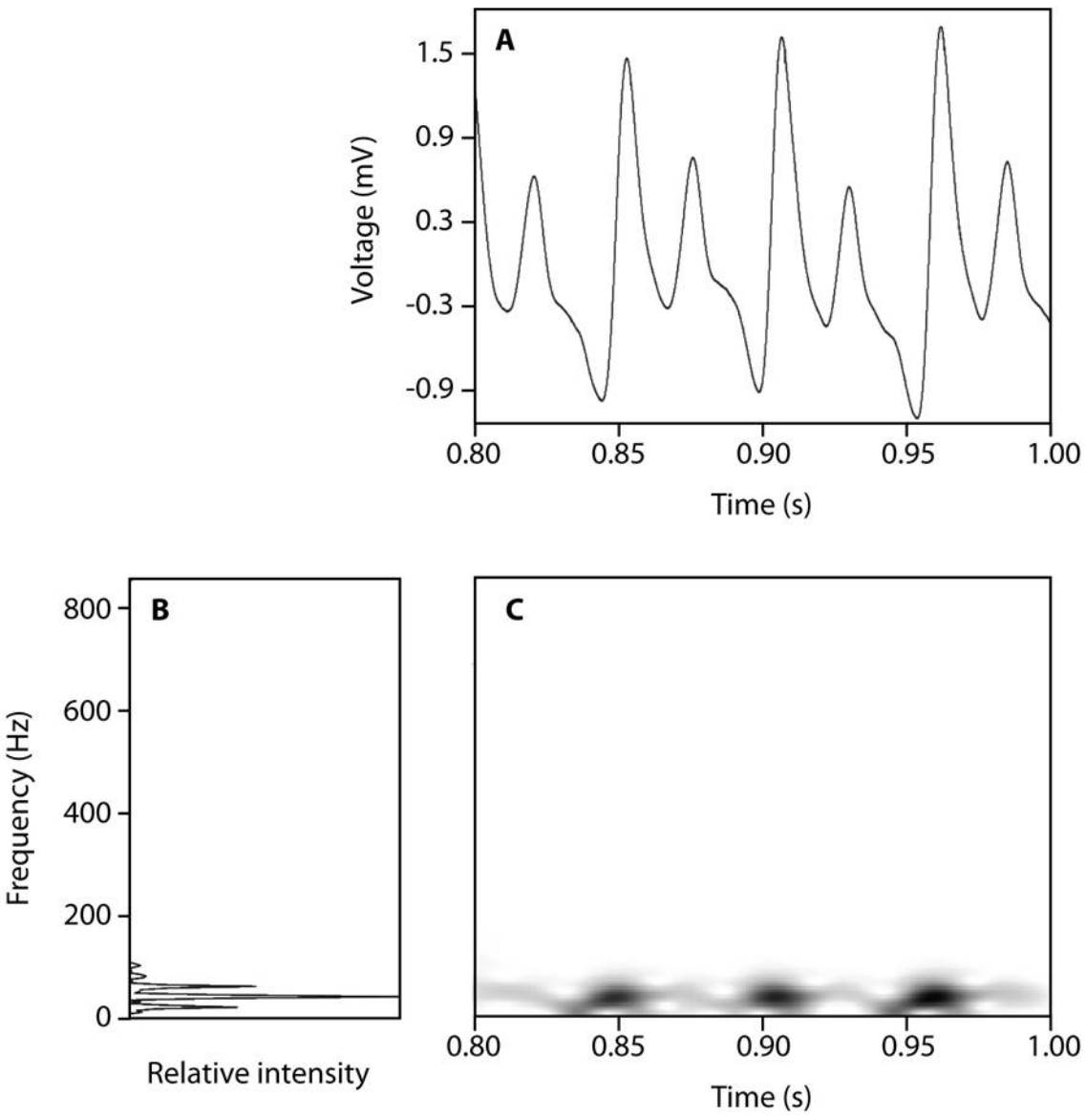

A
1.5
0.9
0.3
-0.3
-0.9
Voltage (mV)
0.80
0.85
0.90
0.95
1.00
Time (s)
B
800
600
400
200
0
Frequency (Hz)
Relative intensity
C
0.80
0.85
0.90
0.95
1.00
Time (s)

Figure 3.3: Analysis of (A) waveform, (B) frequency, and (C) time-frequency sound intensity (sonogram) of a single click (marked by an arrow) associated with the wing beat (see Figure 3.2) of a tethered flying male *L. dispar*. The darker the shading in C, the more intense the frequency component of the recorded sound signal.

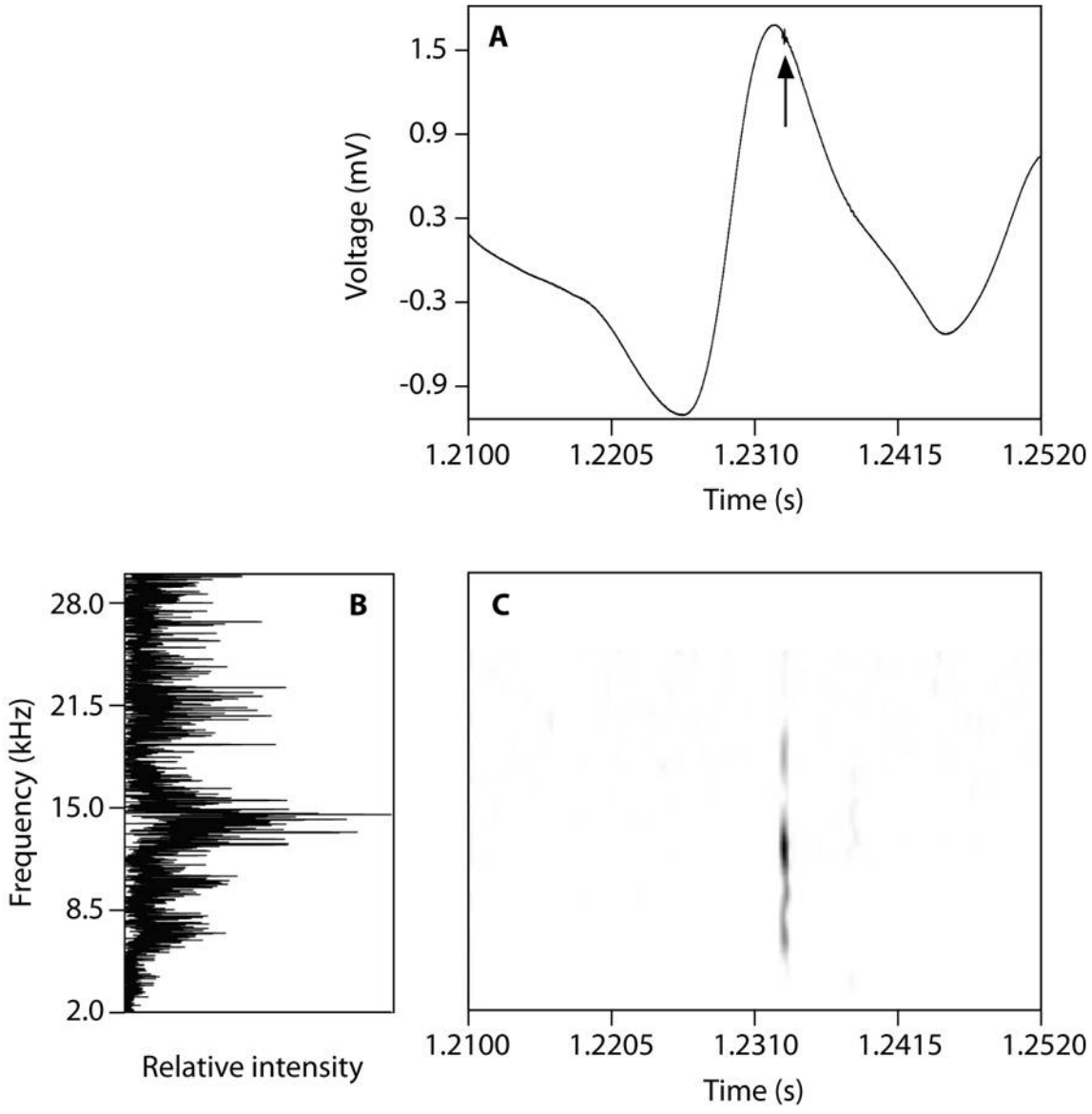

A
1.5
0.9
0.3
-0.3
-0.9
Voltage (mV)
1.2100
1.2205
1.2310
1.2415
1.2520
Time (s)
B
28.0
21.5
15.0
8.5
2.0
Frequency (kHz)
Relative intensity
C
1.2100
1.2205
1.2310
1.2415
1.2520
Time (s)

Figure 3.4: Comparison of mean (± SE) wing beat frequency (A) and click frequency (B) produced by tethered male *L. dispar* (n=6) flying in clean or pheromone-laden air. The presence of pheromone had no significant effect on A or B (Wilcoxon signed-rank test: $P>0.05$).

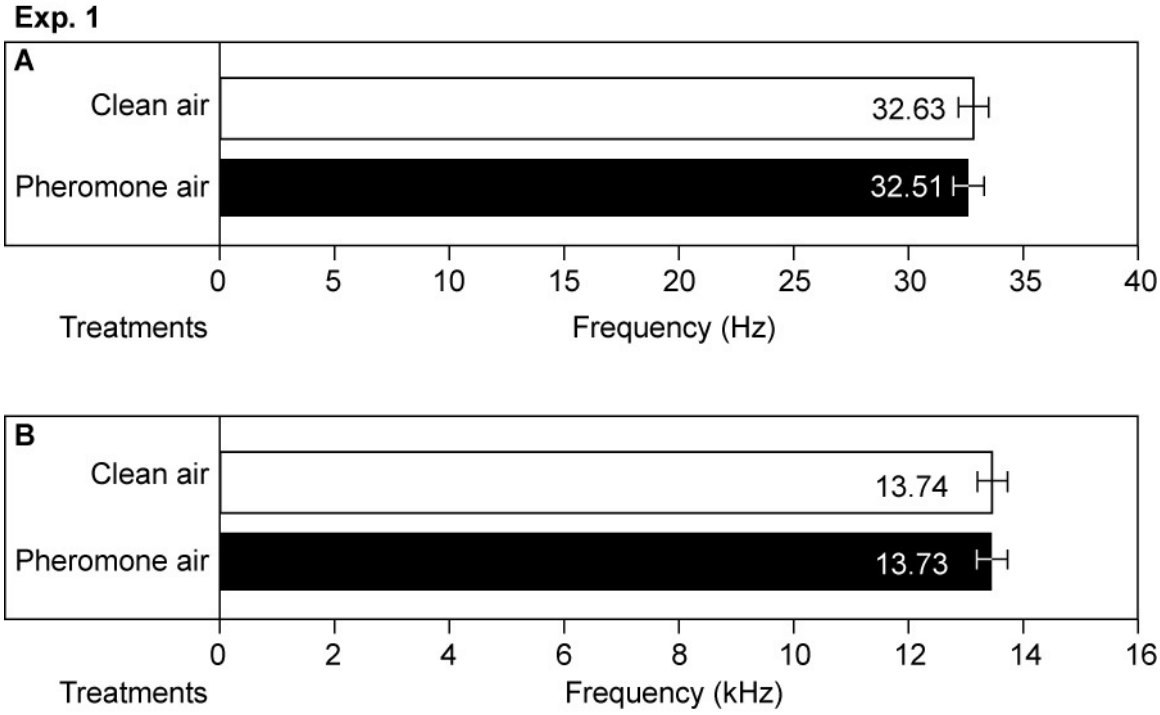

Exp. 1
A
Clean air
Pheromone air
32.63
32.51
0
5
10
15
20
25
30
35
40
Treatments
Frequency (Hz)
B
Clean air
Pheromone air
13.74
13.73
0
2
4
6
8
10
12
14
16
Treatments
Frequency (kHz)

Figure 3.5: Comparison of response latency (s) by female *L. dispar* exposed to a 1 min playback of sound from flying male salt marsh mosquitoes followed by (A) exposure to a 1 min playback of sound from flying male *L. dispar* (see Figure 1) (experiment 2; n=10), or (B) another 1 min playback of flying male salt marsh mosquitoes (experiment 3; n=10). In A, females responded to the male *L. dispar* sound within ~6 s (Wilcoxon signed-rank test: $P \leq 0.05$), but in B did not respond to either stimulus within 60 s.

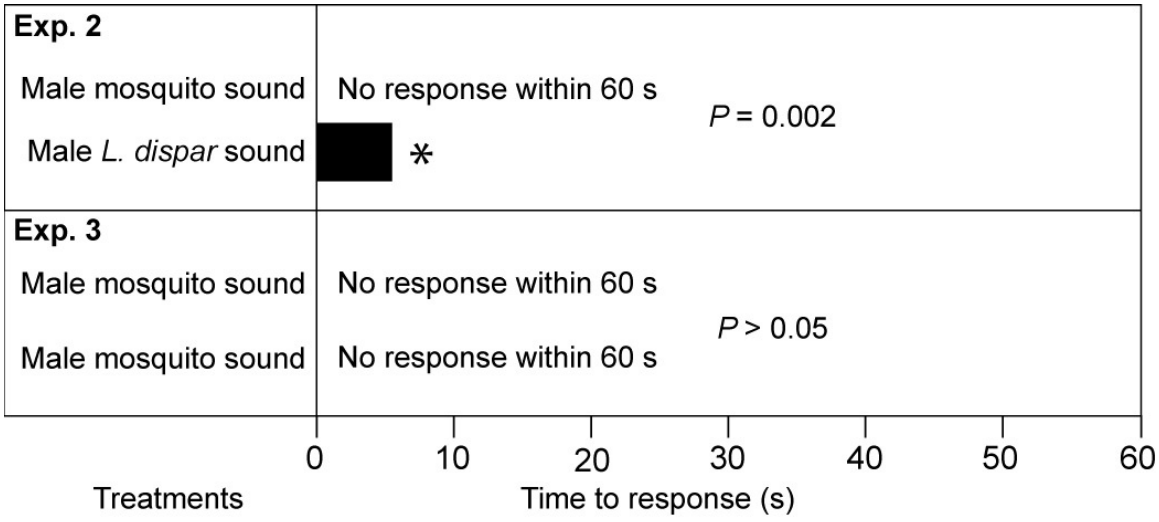

Exp. 2
Male mosquito sound
No response within 60 s
P = 0.002
Male L. dispar sound
*
Exp. 3
Male mosquito sound
No response within 60 s
P > 0.05
Male mosquito sound
No response within 60 s
0
10
20
30
40
50
60
Treatments
Time to response (s)

Figure 3.6: Schematic drawing (A), scanning electron microscopy (SEM) image of female *L. dispar* tympanal region (B), and compound microscope images of male (C) and female (D) *L. dispar* tympanal membrane. Tympanal membranes are circled and arrows point to scolopidia; prh = prespiracular hood; t = tympanum; co = membraneous conjunctiva; e-a & e-b = metepimeron.

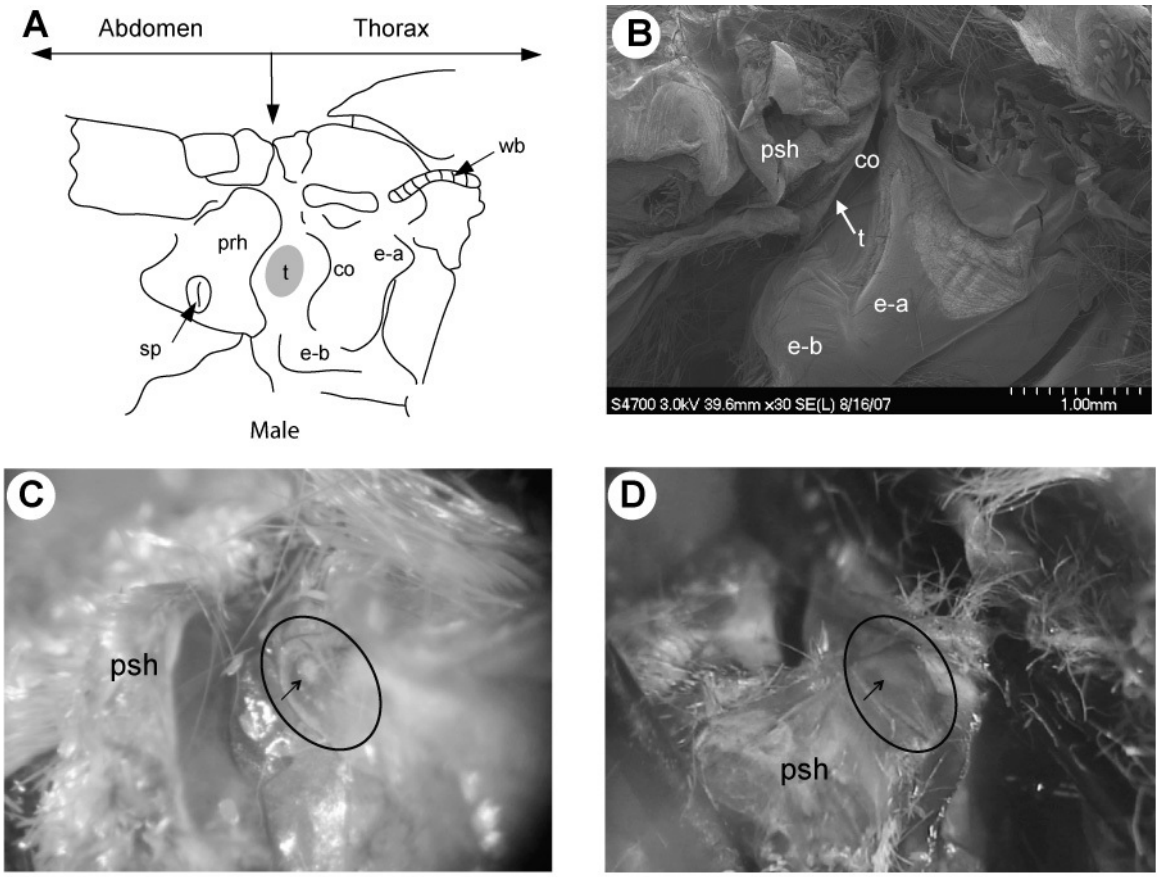
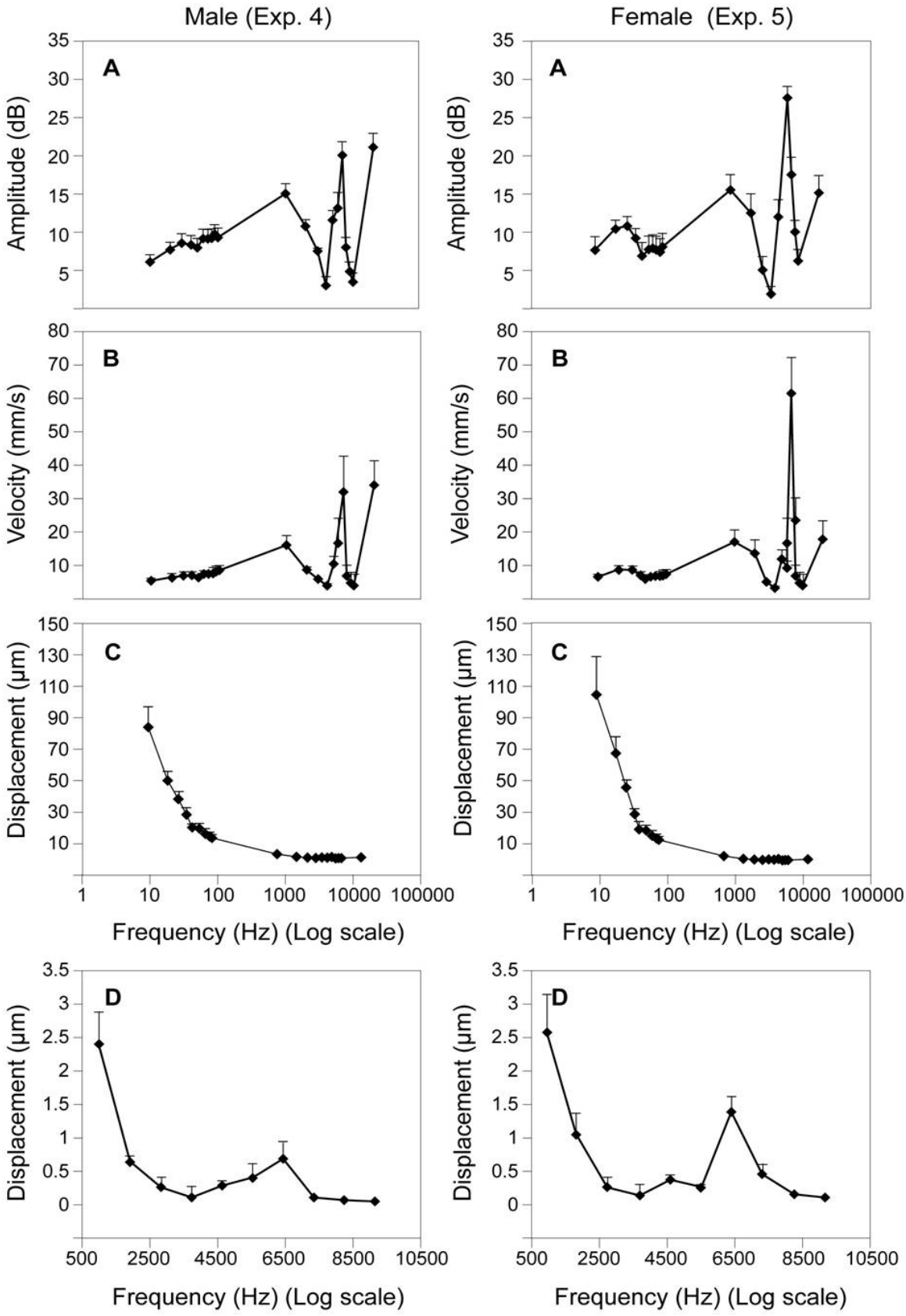

A
Abdomen
Thorax
wb
prh
t
co
e-a
sp
e-b
Male
B
psh
co
t
e-a
e-b
S4700 3.0kV 39.6mm x30 SE(L) 8/16/07
1.00mm
C
psh
D
psh

Figure 3.7: Laser interferometer recordings of (A) amplitude, (B) velocity, and (C,D) displacement of tympanal membranes of male (experiment 4; n=12) and female (experiment 5; n=8) *L. dispar* in response to airborne sound stimuli. For each tympanum (specimen) and sound stimulus, recordings were averaged over 10 exposures.


Male (Exp. 4)
Female (Exp. 5)
A
B
C
D
Amplitude (dB)
Velocity (mm/s)
Displacement (µm)
Frequency (Hz) (Log scale)

3.5 Discussion

My data support the conclusion that female *L. dispar* physiologically receive and behaviourally respond to sound signals from conspecific males. Wing raising or fluttering followed by vertical movement of females in response to playback sounds of a flying male (Figure 3.5), sensitivity of the female tympanum to specific frequency components of the male in-flight sound, and evidence of ears in females and males that are fully functional outside the frequency range used by hunting bats (Figure 3.7; Cardone and Fullard, 1988), all support the concept of the use of sound signals by *L. dispar* as part of its sexual communication system.

The distinct types of flight displayed by males before and after they lock on to the pheromone plume of a calling female (Kennedy and Marsh, 1974; Miller and Roelofs, 1978; Goldsworthy and Wheeler, 1989) suggested that different types of sound could be generated. It follows that sounds of pheromone-mediated flight could convey information to a female that a male has received her pheromone message and is approaching. However, sound characteristics of the male flight in the absence or presence of pheromone were virtually identical (Figure 4), indicating that the different types of flight are attributable to characteristics other than the wing beat frequency, such as changes in the angle or pitch of the wings (Zlotina, 1999), changes in body angle with reference to the ground, or slight left and right rolling and yawing movements made by the male (Goldsworthy and Wheeler, 1989).

Females are more sensitive than males to sounds in the sonic frequency range (5-20 kHz) (Cardone and Fullard, 1988), but behaviourally do not respond to just any sound

within that range. Repeated exposure of females to the wing beat of flying male salt marsh mosquitoes did not elicit a behavioural response. In contrast, females exposed to sounds of flying conspecific males raised and fluttered their wings and walked upward generating distinctive visual signals to a mate-seeking male (Figure 3.5). The use of visual signals by males to locate females has previously been suggested by Doane (1968) who reported that males oriented toward and attempted copulation with non-calling females located downwind of calling females. While visual signals are likely effective in the diurnal *L. dispar* in guiding a prospective mate to the micro-location of a female, such signals may be more difficult to detect by mate-seeking males of nocturnal moths, such as *L. monacha*, *L. fumida*, and *L. mathura*. This may explain why males of these three congeners use sound signals from conspecific females as a short range orientation signal (Chapter 2).

Male *L. dispar* can detect the sex pheromone of calling females over a distance of at least 120 m (Willis et al., 1991). However, at close range the concentration of pheromone can be sufficiently high as to cause flying males to become arrested before reaching the source (Baker et al., 1981; Willis et al., 1991). Pheromone receptors become overloaded, effectively eliminating signal transduction to the central nervous system (Willis et al., 1991). By shifting to visual communication signals at close range, females could reduce the constraints of olfactory signals and readily guide males to their micro-location.

The behavioural response of females to the sounds of flying conspecific males implied that females possess a fully functional ear. Supporting evidence includes SEM and compound microscope images of the female tympanal region which is virtually

identical to that described for males (Speidel et al., 1996). Moreover, laser interferometry revealed that the female tympanum is particularly sensitive to frequency components of the male wing beat (33 Hz) and associated clicks (14 kHz) (Figure 3.7). This applies specifically to the 7-kHz sub-harmonic of the mean click frequency, which induced high velocity (mm/s) vibrations and significant displacement (μm) of female tympana. These results are consistent with previous findings that the female tympanum is more sensitive than the male's to sounds in the sonic range (5-20 kHz) (Cardone and Fullard, 1988). The results also imply a sex-specific function, with males sending and females receiving the sound signal. There is no obvious advantage for a male to be particularly sensitive to the sound of another flying male as it is very unlikely for two males to simultaneously orient towards and compete for the same female. For a female, however, the sensitive detection of a male's sound signal is advantageous because she may then provide visual signals that securely guide the male toward her.

Based on previous findings and those presented in this study, mate attraction and location in *L. dispar* appear to involve not only chemical but also bioacoustic and visual signals, and appear to proceed in the following sequence: (1) females emit a sex pheromone that attracts males; (2) males fly towards calling females; and (3) sound signals from flying males at close range induce movement in females which, in turn, provides visual signals that function in the orientation of males toward females.

4: Conclusion

I have investigated acoustic communication in four species of lymantriid moths: Japanese *L. monacha*, *L. fumida*, and *L. mathura*, and European *L. dispar*. From the results, I conclude:

(1) There are significant species- and sex-specific differences in wing beat and associated click frequencies produced by Japanese *L. monacha*, *L. fumida*, and *L. mathura*. These differences may contribute to reproductive isolation of these biologically and ecologically similar congeners.

(2) Male *L. monacha* and *L. fumida* orient towards speakers emitting playback of sound signals from wing fanning conspecific females, demonstrating the use of the signals for short range communication.

(3) SEM images reveal the tympanate ears of male and female *L. monacha*, *L. fumida*, and female *L. mathura* on the metathorax.

(4) Laser interferometry recordings demonstrate that tympanate ears of male *L. monacha* and *L. mathura* are functional within the sonic (10 Hz-20 kHz) frequency range and are particularly sensitive to sound signals from wing fanning conspecific females.

(5) Flying male *L. dispar* have a mean wing beat frequency of 33 ± 1.0 Hz and associated clicks of 14 ± 0.38 kHz which are unchanged in the presence of synthetic female sex pheromone.

(6) Female *L. dispar* exposed to sounds (0.3-3.4 kHz) of flying male salt marsh mosquitoes, *A. taeniorhynchus*, demonstrate no behavioural response, but exposure to playback sounds of flying conspecific males elicits wing raising and fluttering and vertical movement, generating distinctive visual signals that may reveal her micro-location to a mate-seeking male at close range.

(7) SEM and compound microscope images reveal details of the female *L. dispar* metathoracic tympanal region which is virtually identical to that described by Speidel et al. (1996) for males.

(8) Laser interferometry recordings demonstrate that tympanate ears of male and female *L. dispar* are functional within the sonic frequency range and are particularly sensitive to frequency components of the male wing beat and associated clicks.

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