THE JOURNAL OF COMPARATIVE NEUROLOGY 508:677–686 (2008)

Hearing in a Diurnal, Mute Butterfly, *Morpho peleides* (Papilionoidea, Nymphalidae)

KARLA A. LANE, KATHLEEN M. LUCAS, AND JAYNE E. YACK*
Department of Biology, Carleton University, Ottawa, Ontario K1S 5B6, Canada

ABSTRACT

Butterflies use visual and chemical cues when interacting with their environment, but the role of hearing is poorly understood in these insects. Nymphalidae (brush-footed) butterflies occur worldwide in almost all habitats and continents, and comprise more than 6,000 species. In many species a unique forewing structure—Vogel's organ—is thought to function as an ear. At present, however, there is little experimental evidence to support this hypothesis. We studied the functional organization of Vogel's organ in the common blue morpho butterfly, *Morpho peleides,* which represents the majority of Nymphalidae in that it is diurnal and does not produce sounds. Our results confirm that Vogel's organ possesses the morphological and physiological characteristics of a typical insect tympanal ear. The tympanum has an oval-shaped outer membrane and a convex inner membrane. Associated with the inner surface of the tympanum are three chordotonal organs, each containing 10–20 scolopidia. Extracellular recordings from the auditory nerve show that Vogel's organ is most sensitive to sounds between 2-4 kHz at median thresholds of 58 dB SPL. Most butterfly species that possess Vogel's organ are diurnal, and mute, so bat detection and conspecific communication can be ruled out as roles for hearing. We hypothesize that Vogel's organs in butterflies such as *M. peleides* have evolved to detect flight sounds of predatory birds. The evolution and taxonomic distribution of butterfly hearing organs are discussed. J. Comp. Neurol. 508: 677–686, 2008.

Indexing terms: butterfly; auditory; sensory; neuroethology; tympanum; chordotonal organ; Papilionoidea; insect; evolution

A sense of hearing has been widely studied in moths over the past several decades. Tympanal ears have evolved independently at least six times and, due to the nocturnal lifestyle of most moths, function primarily in detecting ultrasonic echolocation calls of predatory bats (Fullard, 1998; Minet and Surlykke, 2003). Much less is known about a sense of hearing in butterflies. Indeed, it is commonly believed that butterflies lack a sense of hearing. In recent years there has been accumulating evidence that hearing in butterflies may be widespread in some taxonomic groups, but the phenomenon has received little experimental attention.

Butterflies comprise 3 of 46 superfamilies in the Lepidoptera: Hedyloidea, Hesperoidea, and Papilionoidea (Ackery et al., 1999). The remaining 43 superfamilies are moths. There is no evidence that hearing occurs in the skipper butterflies (Hesperoidea). The Hedyloidea are a small group of neotropical nocturnal butterflies believed to represent the 'living ancestors' of the two other butterfly superfamilies (Aiello, 1992). Recent morphological, physiological, and behavioral studies demonstrate that the Hedyloidea possess ultrasound-sensitive ears on their forewings that function as bat detectors (Yack and Fullard, 2000; Yack et al., 2007). Within the Papilionoidea (true butterflies) there is increasing evidence that hearing may be prominent in the Nymphalidae, a large family comprising around 6,000 species worldwide (Ackery et al., 1999). The proposed site of hearing in this group is a structure on the forewing called the Vogel's organ (VO).

Grant sponsor: National Science and Engineering Research Council (USRA to K.L., and Discovery Grant to J.E.Y.); Grant sponsor: Canadian Foundation for Innovation; Grant sponsor: Ontario Trust Fund (to J.E.Y.).

*Correspondence to: Dr. Jayne E. Yack, Department of Biology, Carleton University, 1125 Colonel By Drive, Ottawa, ON, Canada, K1S 5B6.
E-mail: jyack@ccs.carleton.ca

Received 6 November 2007; Revised 22 December 2007; Accepted 9 January 2008

DOI 10.1002/cne.21675

Published online in Wiley InterScience (www.interscience.wiley.com).

VO was first described in the subfamily Satyrinae (Nymphalidae) by Vogel (1911, 1912) as a membranous structure located at the base of the forewing, associated with tracheal air sacs and chordotonal organs. Given the morphological resemblance to tympanal ears in other insects, Vogel (1912) named this structure a "Tympanum", although there was no direct evidence at the time that it functioned in hearing. Subsequent studies of the VO have demonstrated that it is widespread among certain Nymphalidae subfamilies, but with varying degrees of development (LeCerf, 1926; Otero, 1990; Mahony, 2006; Yack, unpubl.). Although the VO has morphological features of a tympanal ear, there is little experimental evidence that it even functions in hearing. Only one physiological study has confirmed that nerve branches supplying the VO respond to sound (Yack et al., 2000). However, the species studied, *Hamadryas feronia* (Nymphalidae, Biblidinae), is atypical of most butterflies in that it produces sounds, and hearing is thought to have evolved for conspecific communication. Rydell et al. (2003) demonstrated that *Manataria maculata* (Nymphalidae, Satyrinae), a tropical crepuscular butterfly, responds to ultrasound (≈26 kHz) by exhibiting evasive flight maneuvers. However, the role of the VO in hearing was not examined in this study. Ribaric and Gogala (1996) described various behavioral responses, including wing flicks and taking flight, to low-frequency (≈1 kHz) sounds in two species of diurnal wood nymphs, *Erebia euryale* and *E. manto* (Nymphalidae, Satyrinae). Response thresholds were increased when VO was covered in wax, providing indirect evidence that VO is involved in hearing. Although these studies provide important initial steps toward understanding the role of VO in butterflies, many questions remain about its function and evolution. Most important, it is necessary to establish whether or not VOs even function as ears in most butterflies that possess them—namely, those that are diurnally active and mute. The purpose of this study therefore was to test the hypothesis that the VO in a common, diurnally active, mute butterfly, *Morpho peleides* Kollar (Fig. 1A), functions as an ear. We describe the morphological and neurophysiological characteristics of the VO in *M. peleides* and discuss hypotheses on its function in 'typical' Nymphalidae butterflies.

MATERIALS AND METHODS

Animals

Morpho peleides Kollar were obtained as pupae from London Pupae Supplies (Oxfordshire, UK; Permit number P-2004-01807). The pupae were kept in a wire mesh enclosure inside a greenhouse at high humidity with temperatures ranging between 24°C and 35°C. Adults were fed on fermented fruit until they were used in experiments, which was typically within 1–3 days following eclosion.

External morphology of Vogel's organ

The external anatomy of the VO was examined to describe the general location, size, and surface topography, in addition to assessing any sexual dimorphism in these characteristics. A forewing of each animal (males, n = 10; females, n = 11) was removed and pinned ventral side up in a Petri dish lined with Sylgard (Dow Corning, Midland, MI). Light micrographs of the VO were obtained using an Olympus (Tokyo, Japan) SZX12 light microscope equipped with a Zeiss (Oberkochen, Germany) AxioCamMRc5 camera (1.4 mega pixels, 1388 × 1040). Measurements of the length, width, and surface area of both inner and outer membranes were made using AxioVision AC (rel. 4.1) software. In addition, the width of the thorax and length of the forewing were measured as body size indicators. An unpaired Student's *t*-test for unequal variances was used to test for significant differences between males and females. Analysis of covariance (ANCOVA) was used to test for significant differences in the size of the VO while accounting for body size. For scanning electron microscopy, wing bases were dissected out of dried specimens, mounted on aluminum stubs, sputter-coated with gold-palladium, and examined with a JEOL (Tokyo, Japan) JSM-6400 scanning electron microscope. A paired *t*-test was used to test for differences in density and length of microtrichs between the inner and outer membranes.

Innervation of Vogel's organ

Innervation of the VO was characterized using two nerve stains, Janus Green B and Cobalt-Lysine. Nomenclature used to describe the nerve roots and musculature follows Nüesch (1953, 1957), and for the wing base nerve branches, Vogel (1911, 1912).

Janus Green B (Yack, 1993) was used to follow nerve branches during dissections in live specimens and those that had been previously injected with C&C (Chauthani and Callahan, 1966) fixative (n = 8). The thoracic nervous system was exposed using the dorsal dissection approach previously described in Yack and Fullard (1990). Nerve IIN1c was followed from the pterothoracic ganglion to the VO. As the dissection neared the VO, the main nerve branch was severed proximally and the wing base was removed from the animal. The preparation was transferred to saline (Paul, 1974) in a Sylgard-lined Petri dish and the dissection continued until the chordotonal organs were located. The chordotonal organs were removed from the ear and placed in a few drops of saline on a microscope slide, coverslipped, and viewed with a Zeiss Axio Imager.M1 compound microscope equipped with a Zeiss AxioCam MRm camera (1.4 mega pixels, 1388 × 1040).

Cobalt-Lysine retrograde fills were performed to help locate and identify the chordotonal organs (n = 4). The head and legs of the butterfly were removed and the animal was pinned dorsal side up on a block of modeling clay. The tegula and underlying membrane were removed, exposing IIN1c. IIN1c was cut proximally and the nerve ending was draped into a saline-filled 'boat' made from a small disc of parafilm and silicon grease. The saline was then replaced with Cobalt-Lysine solution (Lázár, 1978) and the exposed nerve branch and tegular opening were covered with silicon grease to prevent desiccation. The animal was kept at 4°C for 24–48 hours in a container lined with moist paper towels. The nerve branches and sensory structures in VO were intensified and processed following the method of Davis (1982). Chordotonal organs were dissected out of the animal, mounted in saline (prior to dehydration) or methyl salicylate (following dehydration) on a microscope slide, and examined and photographed using a compound microscope as described above. Cell counts and measurements were made using AxioVision AC (rel. 4.4) software.

Histology

Animals were injected directly into the mesothorax where the fixative could infiltrate up into the forewing through the ear and adjoining wing veins. The wing base was removed and placed in fixative under vacuum for 30 minutes. Specimens were fixed with alcoholic Bouin's (Pantin et al., 1946), rinsed with a solution of 70% ethanol/$LiCO_2$, and dehydrated in ethanol. Tissues were then embedded in Spurr's epoxy (Canemco, Lakefield, QC) and sectioned at 1–2 μm using a Reichert-Jung Ultracut E with glass knives produced from an LKB 7800 B KnifeMaker. The sections were stained with 0.1% Toluidine blue in 1% M borax solution and photographed. All photographic plates were assembled and adjusted to optimize brightness and contrast using Adobe Photoshop 7.0 (San Jose, CA).

Physiology

Animals were placed dorsal side up on a piece of modeling clay with the wings uncoupled and the forewing placed on top of the hindwing to prevent the latter from obstructing the VO. A groove was made in the modeling clay so that the sound could reach the VO with minimal interference. Nerve IIN1c was accessed by removing the tegula and the underlying membrane. The nerve trifurcates into N I, N II, and N III (see Fig. 3). Activity of all three branches was tested using a stainless steel hook electrode referenced to a second stainless steel electrode placed in the abdomen. Once a neural response was obtained petroleum jelly was applied to the opening in order to prevent desiccation. Preparations typically remained active for several hours following dissection. Neural signals were amplified with a Grass P15 AC preamplifier (West Warwick, RI) and displayed on a Tectronix (Beaverton, ON) THS720A digital oscilloscope. Neural activity and stimuli were recorded at a sampling rate of 48 kHz and stored as .wav files on a Fostex FR-2 field memory recorder (Gardena, CA) for later analysis. All recordings were performed inside a Faraday cage (1.22 × 0.89 × 0.84 m) with the preparation placed on a platform so that the ear was aligned with the speakers.

Responses to sounds ranging between 500 Hz and 40 kHz were tested using trapezoidal sound pulses (30 ms duration, 5 ms rise/fall, linear ramp) that were shaped using a PC with Tucker Davis software (RPvdsEx, v. 5.4; Alachua, FL) and synthesized by a Tucker Davis Technologies (TDT) digital signal processor (RX6 multifunction processor). In order to determine the phasic/tonic nature of the neural response, the stimulus duration was increased up to a maximum of 2 seconds. Sound pulses between 500 and 4,000 Hz were attenuated by a TDT SA1 stereo power amp and broadcast from a generic six-inch woofer. Stimuli between 3 and 40 kHz were attenuated with a TDT PA5 programmable attenuator and broadcast from a two-inch cone tweeter (Motorola, model KSN1078A; Schaumburg, IL). Thus, stimuli between 3 and 4 kHz were presented using both speakers. The speakers were placed 0.8 m from the butterfly on a stand separate from the Faraday cage. Within 24 hours of each experiment the intensity of the sound stimuli were converted to dB SPL (reference pressure 20 μPa) by broadcasting a 1-second sine wave, generated with the TDT digital signal processor, to a Brüel & Kjaer Type 2239 sound level meter (Naerum, Denmark) for frequencies up to 18 kHz. Sounds above 18 kHz were calibrated using a Brüel & Kjaer Type 4135 6.35 mm microphone and type 2610 Brüel & Kjaer measuring amplifier.

The dynamic range of the ear was determined by stimulating at one frequency with intensities ranging from around 5 dB below threshold to maximum output of the speaker, usually corresponding to around +50 dB above threshold. The response was recorded to five stimuli at minimum steps of 3 dB. These ramped responses were recorded as .wav files on a Fostex FR-2 field memory recorder for later analysis using Sony (Tokyo, Japan) Sound Forge 8.0 software.

Audiograms were constructed to characterize the auditory range of *M. peleides'* VO. At each sound frequency, stimuli were initially presented above threshold intensity. The intensity was then systematically lowered using the attenuator of the TDT system. Only frequencies up to 20 kHz were used in constructing audiograms, as we had previously determined that there was no response to higher frequencies. Frequencies were tested in random order and at the end of the audiogram procedure we returned to the original test frequencies to ensure that the thresholds had not changed. Responses remained stable throughout the procedure. The auditory threshold for each frequency was determined to be the lowest intensity at which neural spikes could consistently be heard in synchrony with the sound stimulus by two independent observers. Thresholds were converted to dB SPL as described above; if there was no response to a particular frequency at the maximum output of our speakers (≈110 dB SPL), an arbitrary threshold of 110 dB SPL was recorded. Twelve animals (six males and six females) were used for constructing audiograms. Both left and right auditory organs were tested for two of the females, giving eight female audiograms. Mann–Whitney *U*-tests were performed for each frequency to determine if there was a significant difference between the thresholds of males and females.

RESULTS

Morphology

External morphology. The VO is located on the ventral surface of the forewing at the bases of the cubital, subcostal, and anal veins (Figs. 1–3). It comprises the typical morphological characteristics of an insect tympanal ear, including enlarged trachea, a tympanal membrane, and chordotonal sensory organs. The trachea at the wing base form the tympanal chamber, a common airspace that lies behind the ear drum (=tympanal membrane). A protective fringe of elongate scales extends from the cubital vein along the posterior edge of the tympanum (Fig. 1C). The tympanum comprises an inner and an outer membrane (Figs. 1, 2, 4A). Both are translucent when observed with a light microscope (Fig. 1). The outer membrane is whitish in appearance, oval-shaped, and bordered by a thick ridge of cuticle. The inner membrane is light brown in color, and protrudes as a convex 'bubble' from the outer membrane. It is centered lengthwise on the outer membrane, but located closer to the anterior than the posterior edge (Figs. 1, 2). When viewed with a scanning electron microscope at high magnification, both membranes are seen to be covered with non-socketed hairs, known as microtrichs (Ghiradella, 1998) (Fig. 2). The mi-

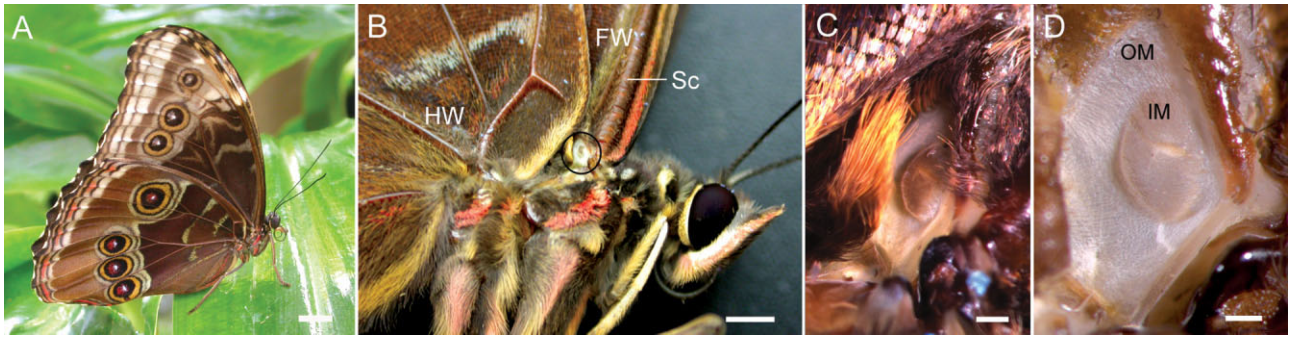

Fig. 1. **A:** *Morpho peleides* in its natural resting position. **B:** Vogel's organ (circled) lies at the base of the forewing (FW) between the cubital and anal veins. **C:** Close-up of the circled region in B. **D:** Higher magnification of the tympanal membrane with protective scales removed. HW, hindwing; Sc, subcostal vein. Scale bars = 1 cm in A; 2.5 mm in B; 300 μm in C; 200 μm in D.

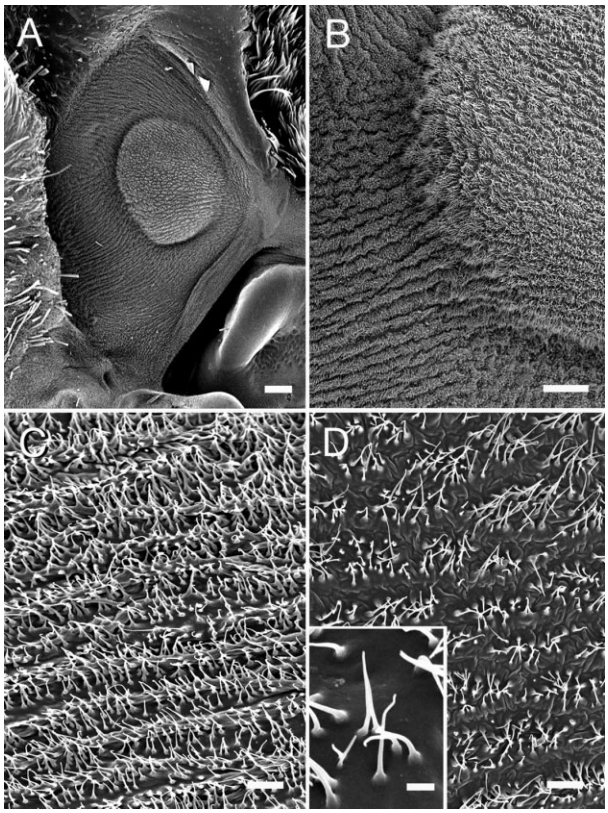

Fig. 2. Scanning electron micrographs of the ear. **A:** Whole view of the right tympanal membrane, with protective scales removed. Anterior is to the right. **B:** Junction between the posterior edge of the inner membrane and anterior edge of the outer membrane, taken at approximately the same orientation as in part A. **C:** Close-up the outer membrane surface. **D:** Close-up of the inner membrane surface. Inset: Higher magnification of the microtrichs on inner membrane. Scale bars = 100 μm in A; 50 μm in B; 10 μm in C,D; 2 μm in D, inset.

crotrichs covering the inner membrane are longer than those on the outer membrane (IM, 6.46 μm; OM, 3.60 μm, $P < 0.0001$, n = 9 ears, 45 measurements), but denser on the outer than on the inner membrane (OM, 170.07 setae/1,000 μm^2; IM, 92.81 setae/1,000 μm^2, $P < 0.0001$, n = 9). There were no significant differences between males and females with respect to the length of the microtrichs on either membrane ($P = 0.264$ and $P = 0.208$, respectively, n = 4 males/20 measurements, 5 females/25 measurements), or the density of the microtrichs on either membrane ($P = 0.098$ and $P = 0.664$, respectively, n = 4 males, 5 females). The lengths of both the outer and inner membranes are similar between males and females, whereas the width of both membranes is significantly greater in females than males (Table 1). Thus, the average area of the female outer membrane was significantly larger than the male outer membrane, as was the inner membrane. However, the body of the female is also significantly larger than the male (Table 1). When body size is accounted for, the only measure that showed a significant difference between genders was the width of the outer membrane ($F = 5.226$, $P = 0.035$). The power of the ANCOVA was low for all measures, so there remains some question regarding sexual dimorphism.

Sensory innervation of Vogel's organ. The main forewing nerve, IIN1c, extends laterally from the pterothoracic ganglion and curves around the anterior edge of the dorsoventral flight muscles. IIN1c then passes under the tegular arm before separating into four branches: N Teg, N I, N II, and N III (Fig. 3). N Teg innervates the tegula. N I, the most anterior branch of the three supplying the wing, runs up the subcostal vein without innervating the ear. N II runs along the cuticle at the base of the tympanal chamber (i.e., the dorsal side of the wing). A thin branch gives rise to chordotonal organ II (COII) (Figs. 3, 4B), which attaches to the inner tympanal membrane, near the center. COII is anchored to the cuticle at the base of the tympanal chamber. Estimates of 10 to 15 scolopidia in COII were based on counts of scolopale caps and sensory cell bodies in four preparations.

N III enters the tympanal chamber from its posterior side and divides into two branches. The first branch gives rise to chordotonal organs IIIa and IIIb (COIIIa and COIIIb) (Figs. 3, 4B–D). COIIIa attaches distally to the trachea that underlies the outer membrane and is anchored to the base of the tympanal chamber. Estimates of 10–12 scolopidia in COIIIa were based on counts of caps and cell bodies from three preparations. COIIIb is the largest of the three organs, containing between 15–20 scolopidia estimated from four preparations. It attaches distally to the posterior edge of the outer tympanal mem-

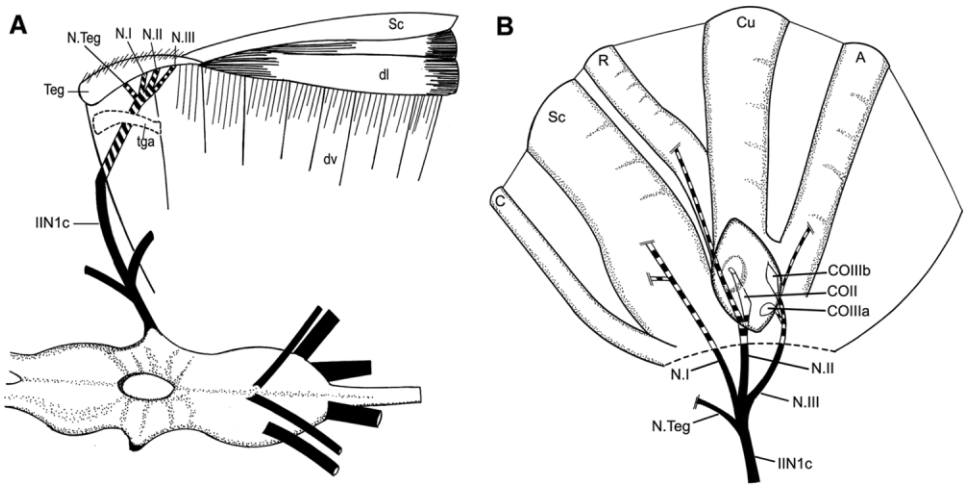

Fig. 3. Schematic illustrations showing the peripheral projection patterns of the nerves innervating Vogel's organ in *Morpho peleides*. **A:** The right half of the thorax is represented, showing the main nerve branch (IIN1c) that innervates the mesothoracic wing base. IIN1c projects laterally around the anterior edge of the dorsoventral (dv) flight musculature. The dashed lines illustrate the nerve branches and structures that lie behind (lateral to) the muscles. IIN1c divides into four branches: N Teg, N I, N II, and N III. **B:** A ventral view of the left mesothoracic wing base (anterior is to the left). The three primary wing nerve branches lie beneath the membranous cuticle and hence are shown as dotted lines. N I travels up the subcostal (Sc) vein and does not enter the Vogel's organ. N II innervates CO II, which attaches to the center of the inner membrane, and then continues up the radial (R) vein. N III innervates CO IIIa and CO IIIb, that are associated with the outer membrane, and then continues up the anal (A) vein. tga, tegular arm; C, costal vein.

brane and rests on a large trachea so that its long axis is 'sandwiched' between the trachea and outer membrane. The second branch of N III continues up the anal vein. All of the scolopidia observed in this study can be categorized as being monodynal, meaning that there is only one sensory cell per scolopale cell, and mononematic, meaning that the dendritic tip is associated with a scolopale cap rather than a tube (see Yack, 2004).

Physiology. All three nerve branches, N I, N II, and N III, were tested during our initial experiments (n = 8–10) where we tested generally for a response to sound but did not conduct full audiograms. The anterior branch (N I) did not respond to acoustic stimuli between 500 Hz to 40 kHz at maximum intensity. The middle branch (N II) responded to high sound pressures (around 75 dB SPL) at ≈3 kHz in two preparations. However, this response was sporadic and the thresholds were much higher than those recorded from the posterior branch (N III). N III responded consistently to frequencies between 500 Hz and 19 kHz, at sound pressures as low as 52 dB SPL. Thus, our physiological recordings in this study focused on N III responses, which were used to construct the intensity–response relationship and the audiograms. Neural responses to sound stimuli were abolished after N III was severed distal to the recording electrode. Conversely, the neural response was unaffected when N III was severed proximally to the recording electrode, confirming that the recorded neural activity originated from primary sensory neurons.

N III responded to acoustic stimuli between 500 Hz and 19 kHz. The response consisted of compound action potentials from multiple units (Fig. 5A), and was tonic for all stimulus durations tested (30 ms to 2 sec) (Fig. 5B). The amplitude of the compound action potentials increased with increasing stimulus intensity, indicating that more sensory neurons were stimulated at higher intensities (Fig. 6). The duration of the response increased and the latency decreased with increasing stimulus intensities (Fig. 6). The latency of the response ranged from 2–15 ms after taking into account the time required for the sound to reach the ear (c = 345 m/s). At 5–10 dB above threshold, latencies ranged between 5 and 15 ms, and at 30–40 dB above threshold this decreased to 2–4 ms. The range of latencies suggests a dynamic range of about 20–30 dB.

Extracellular recordings from N III were used to construct audiograms for six male and six female *M. peleides*. Considering all audiograms, *M. peleides* is most sensitive to low-frequency sounds of 2–4 kHz, with median thresholds around 60 dB SPL. For females the best frequency was 2 kHz, with a median threshold of 58 dB SPL. The best threshold in an individual was 54 dB SPL at 3 kHz (Fig. 7A). For males the best frequency was 3.5 kHz, with a median threshold of 64 dB SPL. The best threshold in an individual was 52 dB SPL at 2 kHz (Fig. 7B). As can be seen in Figure 7 and confirmed using Mann–Whitney *U*-tests at each frequency, there are no significant differences between the audiograms of males and females. Based on the median thresholds, the female audiograms had a Q10 value of around 0.8, while males had a Q10 of around 0.9, where Q10 is defined as the best frequency divided by the bandwidth at 10 dB above threshold.

DISCUSSION

Is the *Morpho* Vogel's organ a tympanal ear?

Vogel's organ morphology. The *M. peleides* VO possesses morphological features common to other insect tympanal ears, which are specialized for detecting the far field (pressure) component of sound (Yager, 1999; Yack, 2004). It comprises a thin membrane supported by a chiti-

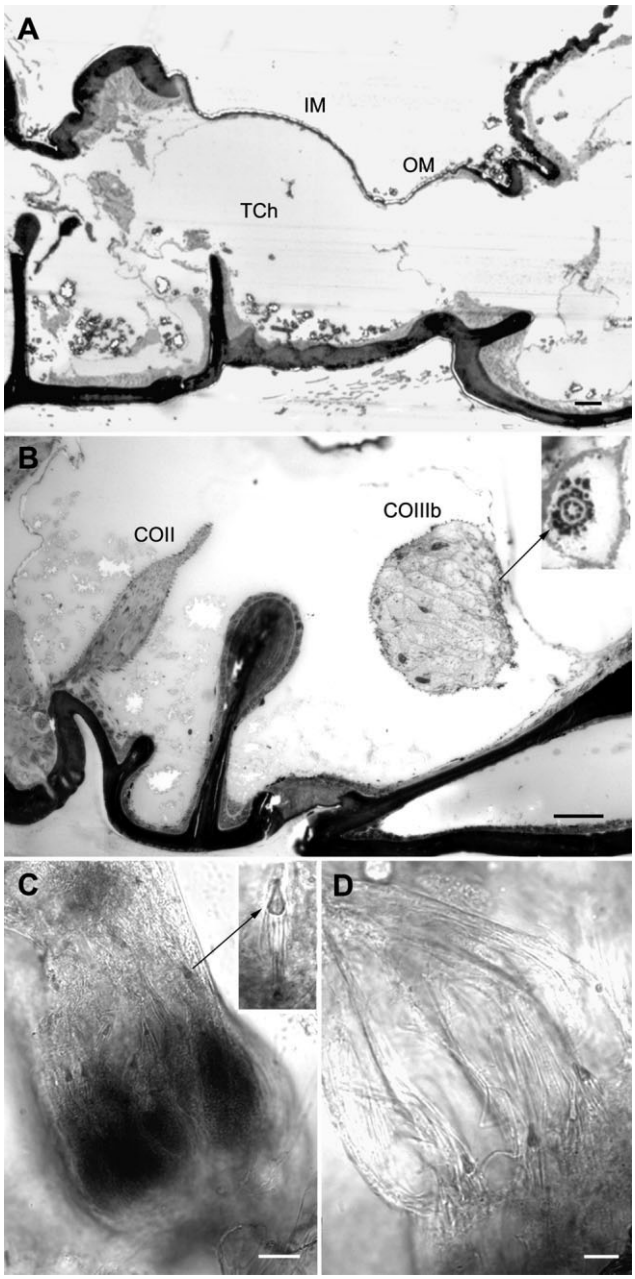

Fig. 4. Light micrographs of the tympanal chamber and associated chordotonal organs. **A:** A histological cross section through the tympanal chamber (T Ch) slightly distal to the chordotonal organs. Note the dome-like shape of the inner membrane (IM) surrounded by the outer membrane (OM). **B:** A cross section through the ear showing two of the three chordotonal organs, COII and COIIIb. Note that COII is sectioned longitudinally and COIIIb is sectioned transversely, due to their different orientations in the tympanal chamber. The inset shows a close-up cross-section of a single scolopidium, photographed from the general region of COIIIb indicated by the arrow. **C:** A whole-mount of COIIIb filled with cobalt lysine showing several scolopale caps at the distal region of the organ, and sensory neuron cell bodies at the proximal region of the organ. The inset shows a close-up of a single scolopidium. **D:** A whole mount of the distal region of COIIIa. Scale bars = 100 μm in A; 50 μm in B; 20 μm in C; 10 μm in D.

TABLE 1. Measurements of *M. peleides'* Vogel's Organ (Mean ± Standard Deviation)

	Male (n = 10)	Female (n = 11)	*P*
OM length (μm)	1546.6 ± 79.0	1600.3 ± 103.5	0.065
OM width (μm)	877.7 ± 47.7	960.7 ± 67.5	<0.001*
OM Area (mm^2)	0.870 ± 0.089	0.982 ± 0.108	<0.001*
IM length (μm)	730.9 ± 310.5	660.3 ± 66.7	0.331
IM width (μm)	455.8 ± 27.0	493.6 ± 55.9	0.008*
IM area (mm^2)	0.215 ± 0.030	0.239 ± 0.045	0.015*
Forewing (cm)	6.1 ± 0.4	6.5 ± 0.4	0.016*
Thorax (mm)	1.9 ± 0.1	2.1 ± 0.2	0.021*

*Significant difference between males and females, determined using an unpaired Student's *t*-test for unequal variances ($P < 0.05$ taken as significant). OM, outer membrane; IM, inner membrane.

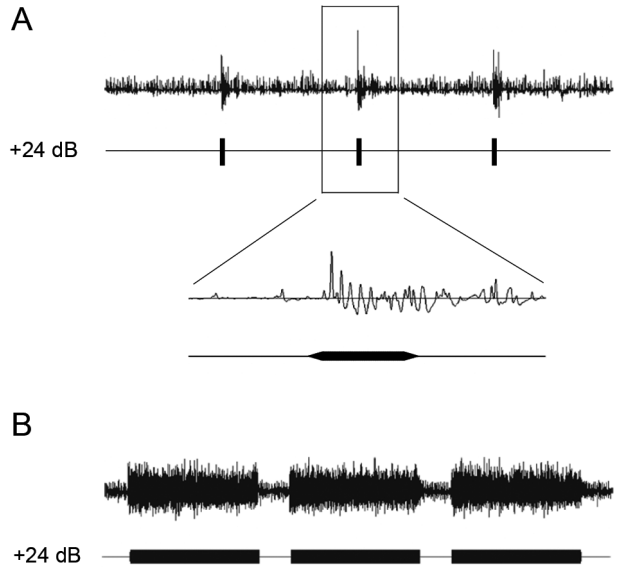

Fig. 5. Representative traces of extracellular physiological recordings of N III. **A:** Response of N III to 30 ms, 1.5 kHz acoustic stimuli presented at 24 dB SPL above threshold. Enlarged region shows the complex peaks of the compound action potential. **B:** Response of N III to 2 seconds, 2.5 kHz acoustic stimuli at 24 dB above threshold.

nous ring, enlarged trachea that enable the membrane to vibrate, and chordotonal organs associated with the vibrating membrane or trachea.

An interesting feature of the *M. peleides* ear is the prominent dome-shaped inner membrane. To our knowledge, this characteristic has not been described for other insect tympanal ears outside of the Nymphalidae. An inner membrane has been noted in some Satyrinae species (e.g., *Pararge aegeria*: Satyrini), but is absent in others (e.g., *Caligo memnon*: Brassolini) and appears variable in its size and shape (Le Cerf, 1926; Otero, 1990; Mahony, 2006). We speculate that the inner and outer membranes vibrate at different frequencies, functioning to expand the frequency range of the ear. For example, frequency discrimination in Acrididae (grasshoppers) has been explained by the 'place principle,' whereby tuning of the chordotonal organs is achieved in part by the different vibration properties of the regions of tympanal membrane to which they attach (Michelsen, 1971; Stephen and Bennet-Clark, 1982; Breckow and Sippel, 1985). If the

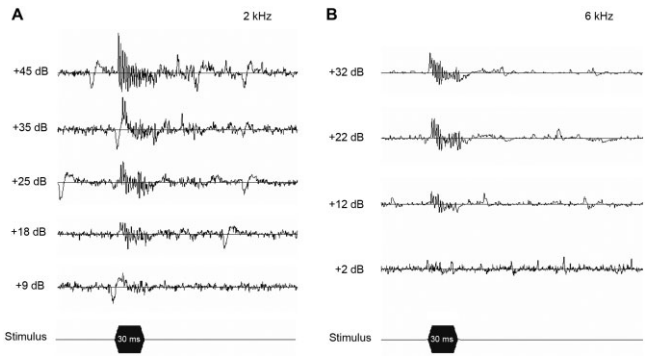

Fig. 6. Intensity-response relationships recorded from N III. Stimuli were shaped pulses of 2 kHz (**A**) and 6 kHz (**B**) presented at one pulse per second. Neural recruitment increases with increasing stimulus intensity. The neural response continues for a short period of time after the stimulus ends, and this is particularly noticeable at higher intensities.

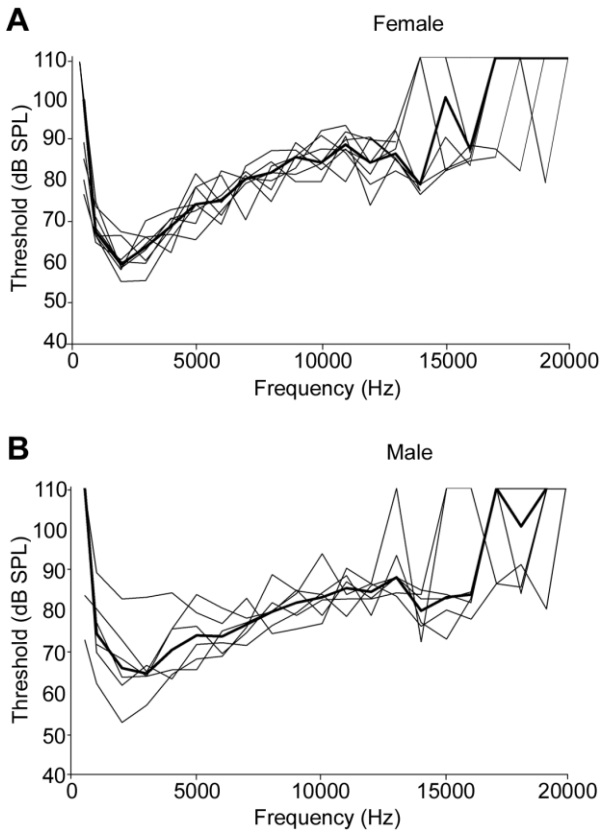

Fig. 7. Audiograms for *Morpho peleides*. **A:** Female (n = 6 animals, n = 8 ears). **B:** Male (n = 6). Best frequency for both males and females was between 2 and 4 kHz, with minimum thresholds of 52 dB SPL (M) and 54 dB SPL (F). Bold lines represent median thresholds. Points at 110 dB SPL are arbitrary thresholds assigned where no auditory response was detected, and these points are included in our calculation of the median values.

inner and outer membranes in *M. peleides* have different vibration properties, we would expect their respective chordotonal organs (COII and COIIIa,b) to respond physiologically to different frequencies. In our study we did not obtain direct evidence for this. However, our focus was on the nerve branch that innervated chordotonal organs associated with the outer membrane only (COIIIa,b). Studies are currently under way to examine the vibrational properties of the inner and outer tympanal membranes using scanning laser vibrometry.

Another interesting feature of the *Morpho* ear is that the surfaces of both the inner and outer tympanal membranes are covered with non-socketed, non-innervated microtrichs. Similar structures have been noted on the tympanal membranes of other insects, including locusts (*Locusta migratoria* Fuhr et al., 1999), crickets (*Acheta domesticus,* Mason, 1991), moths (*Archieris parthenias,* Cook and Scoble, 1992; *Trichoplusia ni, Heliothis zea,* Callahan and Carlysle, 1972; *Plodia interpunctella*, Mullen and Tsao, 1971), hedylid butterflies (Yack et al., 2007), flies (*Ormia ochracea*, Robert et al., 1994; *Therobia leonidei*, Lakes-Harlan and Heller, 1992), and mantids (*Mantis religiosa*, Yager and Hoy, 1987), although there has been little speculation on their function. In a comparative study of the tympanal surface in two noctuid moth species, Callahan and Carlysle (1972) suggested that differences in the size and distribution of these 'micronodules' contributed to filtering or attenuation of specific sound frequencies. In our survey of the literature, we have noted that many insect ears that are ultrasound-sensitive possess microtrichs that are reduced to 'nodules' or 'bumps,' while ears responsive to lower frequency sounds tend to have longer microtrichs. In *M. peleides* the microtrichs on the inner membrane are significantly longer, but less dense than those on the outer membrane, and we speculate that these physical differences may contribute to differences in vibrational properties of the two membranes.

VO is innervated by chordotonal organs arising from N II and N III of the main wing nerve, IIN1c. N II supplies COII, which attaches to the inner membrane in an orientation similar to that seen in other insect tympanal ears (e.g., many Lepidoptera, Acrididae) (see Yager, 1999; Yack, 2004). Given that COII attaches directly to the inner tympanal membrane, we expected N II to be more sensitive to sound. N III innervates COIIIa,b, which are indirectly associated with the tympanal membrane via a large trachea, similar to that seen in the ears of crickets and katydids (see Yager, 1999; Yack, 2004). All scolopidia that we observed in the *M. peleides* VO are characterized as being monodynal and mononematic. These features are common to all tympanal ear scolopidia that have been studied to date, and are thought to play a role in transducing rapid membrane vibrations (Yack, 2004).

Physiology. Before conducting audiogram experiments we carried out initial experiments (n = 8–10) where we recorded from all three nerve branches, N I, N II, and N III, that innervated the wing base near the VO. N I failed to respond to any sound stimuli, and this observation is consistent with our observations that N I neither enters the tympanal chamber, nor innervates a CO in this region. The poor physiological response from N II, however, is inconsistent with our observation that this branch innervates a chordotonal organ that attaches to the inner membrane. One explanation for these results is that N II is a large nerve branch that, in addition to innervating CO II, continues up the radial vein to innervate numerous seta on the wing (Vogel, 1911), and therefore has a higher level of background noise compared to that of N III. This

background activity may have contributed to the observed elevated thresholds. Further work on the role of COII in *M. peleides* are being performed to confirm its role in hearing, if any.

Physiological recordings from N III provide evidence that VO responds to sound, and that these responses are typical of other insect tympanal ears. Neurons in N III responded with short latencies (2–15 ms) to a sound stimulus. These responses were eliminated by severing the nerve branch between the recording electrode and the ear, but were retained if the branch was de-efferented. The amplitude of the compound action potential increased as the stimulus intensity increased, establishing a further correlation between the sound stimulus and the neural response. The median thresholds recorded from male and female *M. peleides* were 58 and 60 dB SPL respectively, and minimum thresholds obtained were 52 dB SPL. Thus, *M. peleides* ears may be somewhat less sensitive than the tympanal ears of well-studied organisms such as the cricket, locust, or noctuid moths, whose best thresholds are reported to be 30–50 dB SPL (Yager, 1999). On the other hand, *M. peleides* auditory thresholds are comparable to those of other insects, including mantids (Yager, 1999), scarab beetles (Forrest et al., 1997), and lacewings (Miller, 1984). *Morpho peleides* thresholds are also lower than those obtained for another butterfly, *H. feronia,* that is believed to use its hearing for conspecific communication (Yack et al., 2000). At present, it is not possible to conclude whether or not the *M. peleides* ear is sensitive to sound frequencies and intensities that are biologically relevant since we do not yet know the function of hearing. However, the responses are well within the range of functional insect ears (Yack and Fullard, 1993).

What is the function of the *Morpho* VO?

Little is known about the function and taxonomic distribution of the VO in Nymphalidae butterflies. In the sound-producing *H. feronia* (Yack et al., 2000) and the crepuscular *M. maculata* (Rydell et al., 2003) there is evidence that hearing functions in social communication and bat evasion, respectively. However, these functions are unlikely to explain hearing in *M. peleides* or the majority of other Nymphalidae species that possess VO. We have no evidence to date that the *M. peleides* ear functions in bat detection. *Morpho* species are active during the daytime (Penz and DeVries, 2002) and there is no evidence to our knowledge that either aerial hawking or gleaning bats eat them. Our neural recordings demonstrate that ears are most sensitive to low-frequency sounds, but not to sounds above 20 kHz. We therefore conclude that bat detection is unlikely. A second hypothesis is that the ears function in conspecific communication. However, sound production has never been reported in the many field studies of this species, and our attempts to record sounds both in the sonic and ultrasonic range while *M. peleides* was at rest or flying freely in a greenhouse yielded no results. Therefore, it is unlikely that VO functions in conspecific communication. Another hypothesis is that the VO has no function in *M. peleides*, and that it is a vestigial organ. However, the sensitivity of the ear makes this an unlikely possibility. So the question remains: what is the function of the VO in *M. peleides* and other nonsound-producing, diurnal butterflies?

We propose the hypothesis that *M. peleides* is listening to the flight sounds of approaching avian predators. The following indirect evidence suggests that this hypothesis would be worthwhile pursuing in future studies. First, *M. peleides* is heavily preyed upon by birds, including jacamars (*Jacamerops aureus* and *Galbula ruficauda*), flycatchers (*Conopias parva* and *Megarhynchus pitangua*) and motmots (*Baryphthengus ruficapillus* and *Electron platyrhynchum*) (Young, 1971). Researchers in Costa Rica have found piles of *M. peleides* wings beneath the roosts of these tropical birds (Young, 1971; DeVries, 1983), indicating that they may impose a strong selection pressure on *M. peleides*. Second, when birds fly up to a target they produce audible sounds with their wings. In preliminary studies on North American insectivorous bird species, including blue jays (*Cyanocitta cristata*), chickadees (*Poecile rufescens*), and the common grackle (*Quiscalus quiscula*), we found that the flapping wings of an approaching bird contain sound frequencies of sufficient intensity to match the sensitivity of the *M. peleides* ear. For example, at 14 cm the average sound pressure level of the 1-kHz component of the grackle wing beat is 78 dB SPL, while the 3-kHz component is 74 dB SPL (Yack lab, unpubl.). These values are well above the hearing threshold of *M. peleides* at these frequencies. We suggest that the ability to hear might provide a selective advantage for *M. peleides* when it is at rest. In its natural resting position, the camouflaged ventral sides of its wings are exposed (Young, 1973; DeVries, 1983), and the ear is also exposed, presumably facilitating the ease with which these butterflies could hear approaching birds. Thus, the butterfly can remain hidden in the forest, but if a bird does see the butterfly, and is flying in to capture the insect, *M. peleides* could hear the approach and presumably initiate an escape response.

A more elaborate hypothesis includes the possibility that *M. peleides* can discriminate between birdcalls and approaching flight sounds. The idea that butterflies may be listening to calling sounds of birds has been previously proposed by Ribaric and Gogala (1996). They suggested that *Erebia euryale* and *E. manto* (Nymphalidae, Satyrinae) were listening to the vocalizations of predatory birds. The most sensitive behavioral threshold in these species (49 dB SPL at 1 kHz) lies within the range of bird vocalization frequencies. However, neurophysiological recordings were not performed on the VO in these species. Further studies aimed at characterizing the flight sounds of insectivorous birds, and recording from butterfly ears using playbacks of flight sounds and vocalizations of natural predators will be necessary to further test these hypotheses.

Evolution of hearing in butterflies

To date, evidence for hearing in butterflies is restricted to two groups: the superfamily Hedyloidea, and one large family of the Papilionoidea, the Nymphalidae (Fig. 8A). A tympanal ear structure occurs at the base of the forewing in all Hedyloidea species examined to date (Scoble, 1986; Yack et al., 2007), and physiological and behavioral experiments on nine species demonstrate that hearing functions primarily in detecting the ultrasound cries of bats (Yack and Fullard, 2000; Yack et al., 2007). In the Nymphalidae, VO also occurs at the base of the forewing, but does not occur in all subfamilies (Fig. 8B). Based on nerve branch patterns and wing venation, it appears that the VO and the Hedyloidea ear are homologous structures. This presents interesting speculation on how hearing may

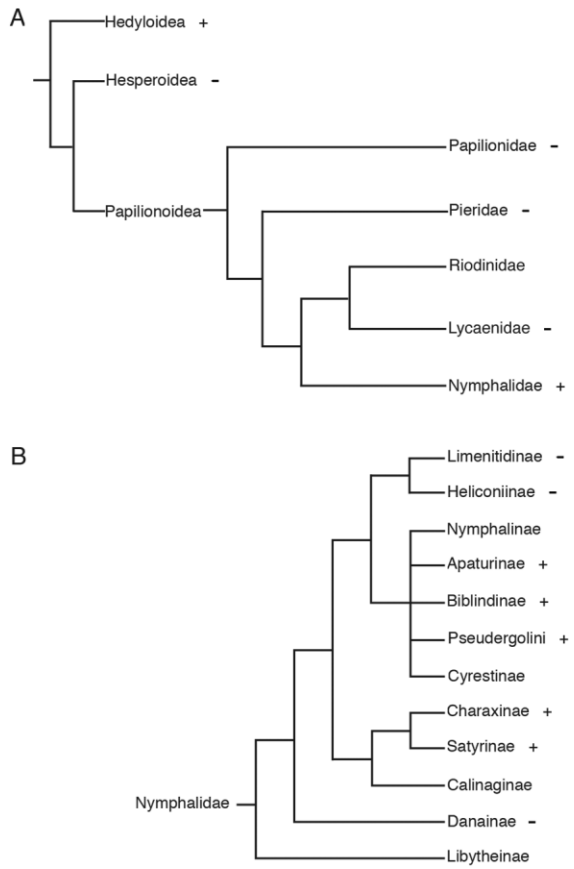

Fig. 8. Phylogenetic relationships of butterfly groups showing where Vogel's organs have been noted in at least one species. A plus (+) or minus (−) sign indicates the presence or absence of a Vogel's organ, and groups that are unmarked represent those for which we did not have information. **A:** Phylogenetic relationship of the butterfly superfamilies Hedyloidea, Hesperoidea, and Papilionoidea (based on Kristensen and Skalski, 1999), and the families contained within the Papilionoidea (based on Ackery et al., 1999; Campbell et al., 2000; Wahlberg et al., 2005). **B:** Phylogenetic relationship of the Nymphalidae subfamilies (based on Ackery et al., 1999; Brower, 2000; Freitas and Brown, 2004; Wahlberg et al., 2003). Evidence for the presence or absence of a potential hearing organs were based on the following sources: Hedyloidea (Scoble, 1986; Yack et al., 2007); Hesperoidea (Otero, unpubl.); Papilionidae (Otero, unpubl.); Lycaenidae (Robbins, pers. commun.); Pieridae (Otero, unpubl.); Limenitidinae (Otero, 1990); Apaturinae (Otero, 1990); Biblindinae (Otero, 1990 [cited as Eurytelinae]; Yack et al., 2000; Le Cerf, 1926); Charaxinae (Otero, 1990); Pseudergolini (Le Cerf, 1926); Satyrinae (Vogel, 1911; Mahony, 2006).

have evolved in butterflies. It has been proposed that Hedyloidea represent the nocturnal moth-like ancestor of the Papilionoidea and Hesperoidea (Fig. 8A) (Aiello, 1992; Kristensen and Skalski, 1999). One hypothesis explaining the origins of hearing in butterflies is that VO in the Nymphalidae derives from an ancestral bat detector in Hedyloidea (Yack and Fullard, 2000). However, since the Nymphalidae are a highly derived group, we would have to explain the loss of VOs in the Hesperoidea, and all other Papilionoidea families that lack a VO (Fig. 8A). Within the Nymphalidae (Fig. 8B), VO does not occur in the most basal groups, and is scattered among the more derived groups (Fig. 8B), suggesting that the common ancestor to all Nymphalidae probably did not possess a VO. A more conservative hypothesis is that hearing in the Hedyloidea and Nymphalidae evolved independently. However, if hearing evolved independently in several butterfly taxa, it is surprising that all of these ears are composed of homologous nerve branches and wing veins. Tympanal hearing organs in moths has also evolved multiple times, but in most cases, on different body parts, including the thorax, different positions on the abdomen, and mouthparts (see Minet and Surlykke, 2003). A more extensive survey of Nymphalidae VOs is underway to establish better how hearing evolved in this group.

We have provided physiological and morphological evidence that the VO in *M. peleides* is a functional tympanal ear, and we suggest that these and other diurnal, nonsound-producing Nymphalidae butterflies are listening to the flight sounds of avian predators. Future studies of the VO in *M. peleides* and other Nymphalidae should explore the vibration properties of the inner and outer tympanal membranes, and how this relates to the response properties of their respective chordotonal organs. Behavioral and neurophysiological responses to natural acoustic stimuli will be important to assess the role of hearing in predator avoidance.

ACKNOWLEDGMENTS

We thank Dr. Jeff Dawson, Shannon Mahony, Ed Bruggink, and Lewis Ling for field and technical assistance during the course of this project. We thank Dr. A. Michelsen and an anonymous reviewer for helpful suggestions to the article.

LITERATURE CITED

Ackery PR, de Jong R, Vane-Wright RI. 1999. The butterflies: Hedyloidea, Hesperoidea and Papilionoidea. In: Kristensen NP, editor. Handbook of zoology, vol. 4. Arthropoda: Insecta. Lepidoptera, moths and butterflies, vol. 1. Evolution, systematics, and biogeography. Berlin: Walter de Gruyter. p 263–300.

Aiello A. 1992. Nocturnal butterflies in Panama, Hedylidae (Lepidoptera: Rhopalocera). In: Arias DQ, Aiello A, editors. Insects of Panama, Mesoamerica: selected studies. Oxford: Oxford University Press. p 549–553.

Breckow J, Sippel M. 1985. Mechanics of the transduction of sound in the tympanal organ of adults and larvae of locusts. J Comp Physiol A 157:619–629.

Brower AVZ. 2000. Phylogenetic relationships among the Nymphalidae (Lepidoptera), inferred from partial sequences of the wingless gene. Proc R Soc Lond B 267:1201–1211.

Callahan PS, Carlysle TC. 1972. Comparison of the epaulette and micronodules on the tympanic membrane of the corn earworm moth with those of the cabbage looper. Ann Entomol Soc Am 65:918–925.

Campbell DL, Brower AVZ, Pierce NE. 2000. Molecular evolution of the wingless gene and its implications for the phylogenetic placement of the butterfly family Riodinidae (Lepidoptera: Papilionoidea). Mol Biol Evol 17:684–696.

Chauthani AR, Callahan PS. 1966. A dissection technique for studying internal anatomy of different stadia of Noctuidae. Ann Entomol Soc Am 59:1017–1018.

Cook MA, Scoble MJ. 1992. Tympanal organs of geometrid moths: a review of their morphology, function, and systematic importance. Syst Entomol 17:219–232.

Davis NT. 1982. Improved methods for cobalt filling and silver intensification of insect motor neurons. Stain Tech 57:239–244.

DeVries PJ. 1983. *Morpho peleides* (Nymphalidae). In: Janzen DH, editor. Costa Rican natural history. Chicago: University of Chicago Press. p 741–742.

Forrest TG, Read MP, Farris HE, Hoy RR. 1997. A tympanal hearing organ in scarab beetles. J Exp Biol 200:909–919.

Freitas AVL, Brown KS. 2004. Phylogeny of the Nymphalidae (Lepidoptera). Syst Biol 53:363–383.

Fuhr G, Ronacher B, Krahe R, Fest S, Shirley SG, Rogaschewski S. 1999. UV-laser ablation of sensory cells in living insects. Appl Phys A 68: 379–385.

Fullard JH. 1998. The sensory coevolution of moths and bats. In: Hoy RR, Popper AN, Fay RR, editors. Comparative hearing: insects. Heidelberg: Springer. p 279–326.

Ghiradella H. 1998. Hairs, bristles, and scales. In: Harrison FW, Locke M, editors. Microscopic anatomy of invertebrates, vol. 11A. Insecta. Wilmington: Wiley-Liss. p 257–287.

Kristensen NP, Skalski AW. 1999. Phylogeny and palaeontology. In: Kristensen NP, editor. Handbook of zoology, vol. 4. Arthropoda: Insecta. Lepidoptera, moths and butterflies, vol. 1. Evolution, systematics, and biogeography. Berlin: Walter de Gruyter. p 7–25.

Lakes-Harlan R, Heller K-G. 1992. Ultrasound-sensitive ears in a parasitoid fly. Naturwissenschaften 79:224–226.

Lázár G. 1978. Application of cobalt-filling technique to show retinal projections in the frog. Neuroscience 3:725–736.

LeCerf F. 1926. Contribution à l'étude des organs sensoriels des Lépidoptères. Encyclop Ent 3:133–146.

Mahony S. 2006. Hearing in the speckled wood butterfly, *Parage aegeria* (Nymphalidae: Satyrinae). MSc thesis, Carleton University, Ottawa, Canada.

Mason AC. 1991. Hearing in a primitive ensiferan: the auditory system of *Cyphoderris monstrosa* (Orthoptera: Haglidae). J Comp Physiol A 168: 351–363.

Michelsen A. 1971. The physiology of the locust ear. II. Frequency discrimination based upon resonances in the tympanum. Z Vergl Physiol 71:63–101.

Miller LA. 1984. Hearing in green lacewings and their responses to the cries of bats. In: Canard M, Séméria Y, editors. Biology of Chrysopidae. The Hague: Dr. W. Junk. p 134–149.

Minet J, Surlykke A. 2003. Sound producing and auditory organs. In: Kristensen NP, editor. Handbook of zoology. Lepidoptera, moths and butterflies, morphology and physiology, vol. 2. Berlin: Walter de Gruyter. p 289–323.

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). Int J Insect Morphol Embryol 1:3–10.

Nüesch H. 1953. The morphology of the thorax of *Telea polyphemus* Cr. (Lepid.) I. Skeleton and muscles. J Morphol 93:589–608.

Nüesch H. 1957. Die Morphologie des Thorax von *Telea polyphemus* Cr. (Lepid.) II. Nervensystem. Zool Jb Abt Anat 75:615–642.

Otero LD. 1990. Estudio de algunos caracteres para su uso en la classificación de Eurytelinae (Lepidoptera: Nymphalidae). Bol Ent Venez 5:123–128.

Pantin CFA. 1946. Notes on microscopical techniques for zoologists. Cambridge, MA: Cambridge University Press.

Paul D. 1974. Responses to acoustic stimulation of thoracic interneurons in noctuid moths. J Insect Physiol 20:2205–2218.

Penz CM, DeVries PJ. 2002. Phylogenetic analysis of *Morpho* butterflies (Nymphalidae, Morphinae): implications for classification and natural history. Am Mus Novit 3374:1–33.

Ribaric D, Gogala M. 1996. Acoustic behaviour of some butterfly species of the genus *Erebia* (Lepidoptera: Satyridae). Acta Ent Slov 4:5–12.

Robert D, Read MP, Hoy RR. 1994. The tympanal hearing organ of the parasitoid fly *Ormia ochracea* (Diptera, Tachinidae, Ormiini). Cell Tissue Res 275:63–78.

Rydell J, Kaerma S, Hedelin H. 2003. Evasive response to ultrasound by the crepuscular butterfly *Manataria maculata*. Naturwissenschaften 90:80–83.

Scoble MJ. 1986. The structure and affinities of the Hedyloidea: a new concept of the butterflies. Bull Br Mus Nat Hist 53:251–286.

Stephen RO, Bennet-Clark HC. 1982. The anatomical and mechanical basis of stimulation and frequency analysis in the locust ear. J Exp Biol 99:279–314.

Vogel R. 1911. Über die Innervierung der Schmetterlingsflügel und über den Bau die Verbreitung der Sinnesorgane auf Denselben. Z Wiss Zool 98:68–134.

Vogel R. 1912. Über die Chordotonalorgane in der Wurzel der Schmetterlingsflügel. Z Wiss Zool 100:210–244.

Wahlberg N, Weingartner E, Nylin S. 2003. Towards a better understanding of the higher systematics of Nymphalidae (Lepidoptera: Papilionoidea). Mol Phylogenet Evol 28:473–484.

Wahlberg N, Braby MF, Brower AVZ, de Jong R, Lee M-M, Nylin S, Pierce NE, Sperling FAH, Vila R, Warren AD, Zakharov E. 2005. Synergistic effects of combining morphological and molecular data in resolving the phylogeny of butterflies and skippers. Proc R Soc Lond B 272:1577–1586.

Yack JE. 1993. Janus Green B as a rapid, vital stain for peripheral nerves and chordotonal organs in insects. J Neurosci Methods 49:17–22.

Yack JE. 2004. The structure and function of auditory chordotonal organs in insects. Microsc Res Tech 63:315–337.

Yack JE, Fullard JH. 1990. The mechanoreceptive origin of insect tympanal organs: a comparative study of similar nerves in tympanate and atympanate moths. J Comp Neurol 300:523–534.

Yack JE, Fullard JH. 1993. What is an insect ear? Ann Entomol Soc Am 86:677–682.

Yack JE, Fullard JH. 2000. Ultrasonic hearing in nocturnal butterflies. Nature 403:265–266.

Yack JE, Otero LD, Dawson JW, Surlykke A, Fullard JH. 2000. Sound production and hearing in the blue cracker butterfly *Hamadryas feronia* (Lepidoptera, Nymphalidae) from Venezuela. J Exp Biol 203:3689–3702.

Yack JE, Kalko EKV, Surlykke A. 2007. Neuroethology of ultrasonic hearing in nocturnal butterflies (Hedyloidea). J Comp Phys A 193:577–590.

Yager DD. 1999. Structure, development, and evolution of insect auditory systems. Microsc Res Tech 47:380–400.

Yager DD, Hoy RR. 1987. The midline metathoracic ear of the praying mantis, *Mantis religiosa*. Cell Tissue Res 250:531–541.

Young AM. 1971. Wing colouration and reflectance in *Morpho* butterflies as related to reproductive behaviour and escape from avian predators. Oecologia 7:209–222.

Young AM. 1973. Studies on comparative ecology and ethology in adult populations of several species of *Morpho* butterflies (Lepidoptera: Morphidae). Studies Neotrop Fauna 8:17–50.