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Auditory mechanics and sensitivity in the tropical butterfly *Morpho peleides* (Papilionoidea, Nymphalidae)

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SUMMARY

The ears of insects exhibit a broad functional diversity with the ability to detect sounds across a wide range of frequencies and intensities. In tympanal ears, the membrane is a crucial step in the transduction of the acoustic stimulus into a neural signal. The tropical butterfly *Morpho peleides* has an oval-shaped membrane at the base of the forewing with an unusual dome in the middle of the structure. We are testing the hypothesis that this unconventional anatomical arrangement determines the mechanical tuning properties of this butterfly ear. Using microscanning laser Doppler vibrometry to measure the vibrational characteristics of this novel tympanum, the membrane was found to vibrate in two distinct modes, depending on the frequency range: at lower frequencies (1–5 kHz) the vibration was focused at the proximal half of the posterior side of the outer membrane, while at higher frequencies (5–20 kHz) the entire membrane contributed to the vibration. The maximum deflection points of the two vibrational modes correspond to the locations of the associated chordotonal organs, suggesting that *M. peleides* has the capacity for frequency partitioning because of the different vibrational properties of the two membrane components. Extracellular nerve recordings confirm that the innervating chordotonal organs respond to the same frequency range of 1–20 kHz, and are most sensitive between 2 and 4 kHz, although distinct frequency discrimination was not observed. We suggest that this remarkable variation in structure is associated with function that provides a selective advantage, particularly in predator detection.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/212/21/3533/DC1

Key words: butterfly, tympanal hearing, scanning laser vibrometry, extracellular nerve recording, chordotonal organ.

INTRODUCTION

Insects display an impressive diversity of acoustic sensory organs that play key roles in detecting predators and prey, and in social communication. Insect tympanal hearing organs share three structural elements, including a thinned region of cuticle acting as a tympanal membrane, innervating chordotonal organs, and associated tracheal air sacs (for reviews, see Yager, 1999; Yack, 2004). Tympanal ears vary widely in their ability to detect a broad range of frequencies and intensities, localize sounds and process temporal patterns (Robert and Göpfert, 2002; Robert and Hoy, 2007; Yack and Dawson, 2008). This functional diversity is achieved by impressive morphological diversity. Insect tympanal ears have been found to occur on almost any part of the body, and to vary in their morphological structure with respect to both the number and location of sensory receptors (Yack, 2004; Yager, 1999).

As the first stage of sound reception, the membrane is crucial in the transduction of a physical sound to a neural signal, and variation in its structure can have important implications for filtering sounds that stimulate receptor organs. Insect tympanal membranes exhibit wide variation with respect to their size, shape, thickness and surface topography. For example, the structure of the tympanal membrane has been found to be a key factor in frequency discrimination in locusts and cicadas (Michelsen, 1971a; Windmill et al., 2005; Sueur et al., 2006). In each of these species (locusts: *Schistocerca gregaria* and *Locusta migratoria*; cicada: *Cicadatra atra*), the structure of the tympanal membrane results in the propagation of travelling waves that decompose incoming sound stimuli into high and low frequency components (Windmill et al., 2005; Sueur et al., 2006). The importance of membrane structure is even more pronounced in the cicada *Cicada orni*, whose sexually dimorphic tympanal membranes respond differently to an incoming sound stimulus, such that the female membrane is sharply tuned to the male calling song, while the male membrane is not (Sueur et al., 2008). The study of membrane mechanics in noctuid moths has shown that this 'simple' membrane displays a non-linear mechanical response to ultrasound stimuli (Windmill et al., 2007), such that the resonant frequency of the membrane increases with increasing sound intensity (Windmill et al., 2006). The role of membrane mechanics can therefore provide important insight into the significance of the morphological diversity of insect ears.

This study focuses on the functional organization of a morphologically unusual tympanal membrane found in the tropical butterfly *Morpho peleides*. Although butterflies were once thought to be deaf, increasing literature shows that the presence of a tympanal ear is not only widespread among some taxa but also morphologically diverse. This morphological diversity is particularly apparent in their tympanal membranes. The ear of Nymphalidae (Papilionoidea) butterflies is called Vogel's organ (VO) (Vogel, 1912), and is characterized by a tympanal membrane located at the base of the cubital vein of the forewing associated with tracheal air sacs and chordotonal organs (Vogel, 1912). A homologous ear is found in a group of nocturnal butterflies belonging to the superfamily Hedyloidea (Scoble, 1986; Yack and Fullard, 2000; Yack et al., 2007). These tympanal membranes vary from very small, thin and

transparent, as in the nocturnal Hedyloidea, to topographically complex, non-uniform membranes that presumably function for conspecific communication or predator detection (Ribaric and Gogala, 1996; Yack et al., 2000; Lane et al., 2008) (J.E.Y., unpublished). The tropical Blue Morpho butterfly, *M. peleides*, is of particular interest because of its unusual tympanal morphology (Lane et al., 2008). The *M. peleides* VO has an oval-shaped membrane with a discrete dome in the middle of the structure, dubbed the outer and inner membranes, respectively (Fig. 1). There is also preliminary evidence that individual chordotonal sensory organs are associated with each of these membranes (Lane et al., 2008). We hypothesized that the inner and outer membranes vibrate differently, imparting different frequency sensitivities to the corresponding chordotonal sensory organs. This hypothesis was tested by (1) characterizing the morphological properties of VO and associated chordotonal organs, (2) testing the vibrational properties of the ear using laser Doppler vibrometry, and (3) testing the physiological properties of the nerve branches by performing extracellular neural recordings.

MATERIALS AND METHODS

Animals

Morpho peleides Kollar were obtained as pupae from London Pupae Sales (Oxford, UK: Permit number P-2007-01460 for use at Carleton University). At Carleton University, the pupae were kept in a wire mesh enclosure inside a greenhouse with high humidity and daily temperature fluctuations between 24 and 35°C. At the University of Bristol, pupae were kept in mesh cages inside an incubator set at 28°C and 80% humidity. Upon eclosion, animals were transferred to a large (approximately 0.5 m×0.5 m×1 m) cage at room temperature (24–25°C). At both locations, butterflies were provided with fermented fruit until they were used for experiments, typically 1–5 days following emergence.

Morphology

The external morphology of the *M. peleides* VO was imaged using light microscopy and scanning electron microscopy. A *M. peleides* specimen was pinned with the ventral side of the wing up, and light micrographs were taken using an Olympus (Tokyo, Japan) SZX12 light microscope equipped with a Zeiss (Oberkochen, Germany) AxioCamMRc5 camera (1.4 mega pixels, 1388×1040). Scanning electron micrographs were obtained by mounting dried, dissected wing bases on aluminium stubs, which were sputter-coated with gold-palladium, and examined with a JEOL (Tokyo, Japan) JSM-6400 scanning electron microscope.

To study the anatomy of the VO, wing bases were removed from the forewing of each animal and pinned ventral side up in a Petri dish lined with Sylgard (Dow Corning, Midland, MI, USA). The same Olympus SZX12 light microscope equipped with a Zeiss AxioCamMRc5 was used to observe and take pictures of the dissections.

For histological analysis of the VO, butterflies were injected with 3% glutaraldehyde in Sorensen's buffer (Glauert, 1975) into the mesothorax where the fixative could infiltrate the ear and adjoining wing veins. The wing base, including the VO, was removed, submerged in fixative, and placed under vacuum for 30 min to remove air bubbles. The specimens were dehydrated in ethanol, then embedded in Spurr's epoxy (Canemco, Lakefield, QC, Canada). Embedded VOs were sectioned at 1–2 µm with a Reichart-Jung Ultracut E microtome using glass knives produced from an LKB 7800 B Knife-Maker (LKB Instruments, Croydon, Surrey, UK). Sections were stained with 0.1% Toluidine blue in 1 mol l^{-1} borax solution, and photographed using a Zeiss (Oberkochen, Germany) Axio Imager.M1 compound microscope equipped with a Zeiss AxioCam MRm camera (1.4 mega pixels, 1388×1040) and AxioVision AC (Release 4.6) software.

Laser vibrometry

Tympanal vibrations of the VO were examined in response to wideband (chirp) signals between 0.1 and 30 kHz. This frequency range was chosen to encompass the hearing range of *M. peleides* that had been determined in preliminary neurophysiological recordings.

Sound signals were generated using a PCI data acquisition board (PCI-4451; National Instruments, Austin, TX, USA), amplified (Amplifier Model TAFE570; Sony, Tokyo, Japan), and projected from one of two loudspeakers [(1) 1–30 kHz, ESS AMT-1; ESS Laboratory, Sacramento, CA, USA; (2) 100–1000 Hz, AP100MO, diameter 117 mm; AUDAX, Château-du-Loir, France]. Vibration velocities were measured using a microscanning laser Doppler vibrometer (PSV-300-F; Polytec, Waldbronn, Germany) with an OFV-056 scanning head fitted with a close-up video attachment, allowing for the laser spot (~1 µm diameter) to be positioned with an accuracy of ~5 µm. All experiments were carried out on a vibration isolation table (TMC 784-443-12R; Technical Manufacturing, Peabody, MA, USA) at room temperature (24–26°C) with a relative humidity of 40–62%, in an acoustic isolation booth (IAC series 1204A; Industrial Acoustics, Bronx, NY, USA).

The butterflies were positioned upright and attached ventral side down to a brass bar (6 mm×1 mm×16 mm) using BLU-TACK (Bostik-Findley, Stafford, UK), with wings clasped above using a paper clip to expose the VO. The brass bar was connected to a metal rod, which provided control in correctly positioning the animal for scanning. Scan arrays were set to encompass the entire tympanal membrane of the VO, which was set perpendicular to the direction of the loudspeaker. When possible, both the left and right VOs were scanned for an individual butterfly. A single scan consisted of >200 measurement points, and each point was sampled 16 times throughout the scan, resulting in scan durations of approximately

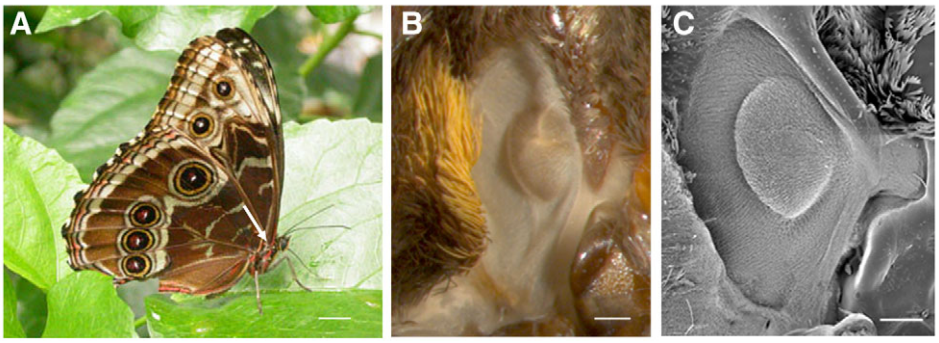

Fig. 1. Location and external morphology of Vogel's organ (VO) on the butterfly *Morpho peleides*. (A) The natural resting position of *M. peleides*, with the VO exposed on the forewing (location indicated by the arrow). Scale bar, 1 cm. (B) Close-up of VO on right wing (posterior is on the left); scale bar, 200 µm. (C) Scanning micrograph of VO with the same orientation as in B; scale bar, 200 µm.

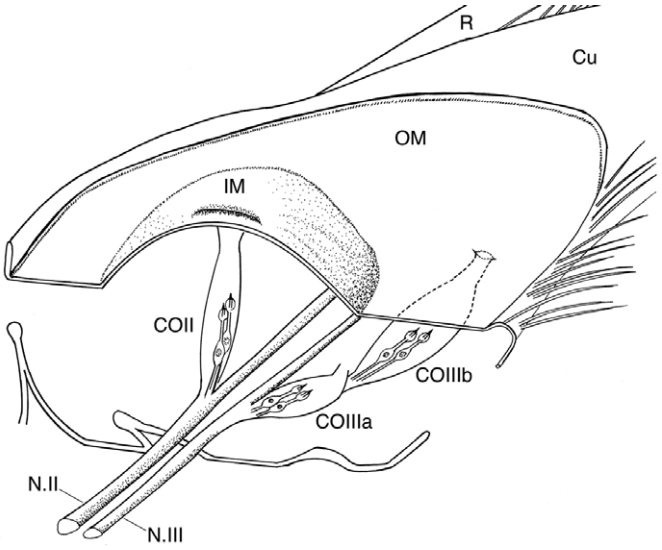

Fig. 2. A schematic diagram of the left mesothoracic wingbase and VO in *M. peleides* (anterior is to the left). The drawing shows a proximal view of the tympanic membrane, with a cut-away view to reveal the nerve branches innervating the wing and ear. N.II innervates the chordotonal organ COII, which attaches to the middle of the inner membrane (IM), then continues up the radial (R) vein. N.III innervates the chordotonal organs COIIIa and COIIIb, which are associated with the proximal half of the posterior outer membrane (OM) at the base of the cubital (Cu) vein, then continues up the anal vein. The direction of the sensory cells is denoted in each organ by two scolopidia.

7–10 min. The sound pressure level (SPL) of the acoustic stimulus, projected from a loudspeaker positioned approximately 20 cm from the specimen, was recorded next to the VO of the specimen using a B&K Type 4138 pressure-field microphone and a B&K 2633 preamplifier (Brüel & Kjær, Nærum, Denmark). The microphone was calibrated at 1 kHz, 94 dB SPL using a Brüel & Kjær 4231 sound level calibrator. Recordings were made when the sound stimulus was at approximately 18–20 mPa (20 mPa corresponds to 60 dB SPL) with constant amplitude across the range of frequencies [computer corrected, as described by Windmill et al. (Windmill et al., 2007)]. The signals were sampled at 102.4 kHz, and a frequency spectrum was produced for each signal with a resolution of 12.5 Hz using a FFT (fast Fourier transform) with a rectangular window. The amount of unrelated noise was estimated by calculating the magnitude-squared coherence (see Windmill et al., 2005), where a value was assigned between 0 and 1 (a value of 1 indicates the absence of external, unrelated noise). Data were only used when coherence exceeded 0.85.

Neurophysiology

Two branches, N.II and N.III, innervate the *M. peleides* VO, from the main wing nerve IIN1c (Fig. 2). Previous extracellular nerve recordings focused only on the posterior N.III branch (Lane et al., 2008). Our neurophysiological analysis focused on the middle N.II branch in order to determine whether the two branches respond differently to the same auditory stimulus.

Animals were tethered dorsal side up on a piece of modelling clay. In order to ensure the VO was unobstructed, the forewing was placed on top of the hind wing, the wings were trimmed to ~2 cm in length, and a groove was made in the modelling clay to allow acoustic stimuli to reach the hearing organ from speakers aligned

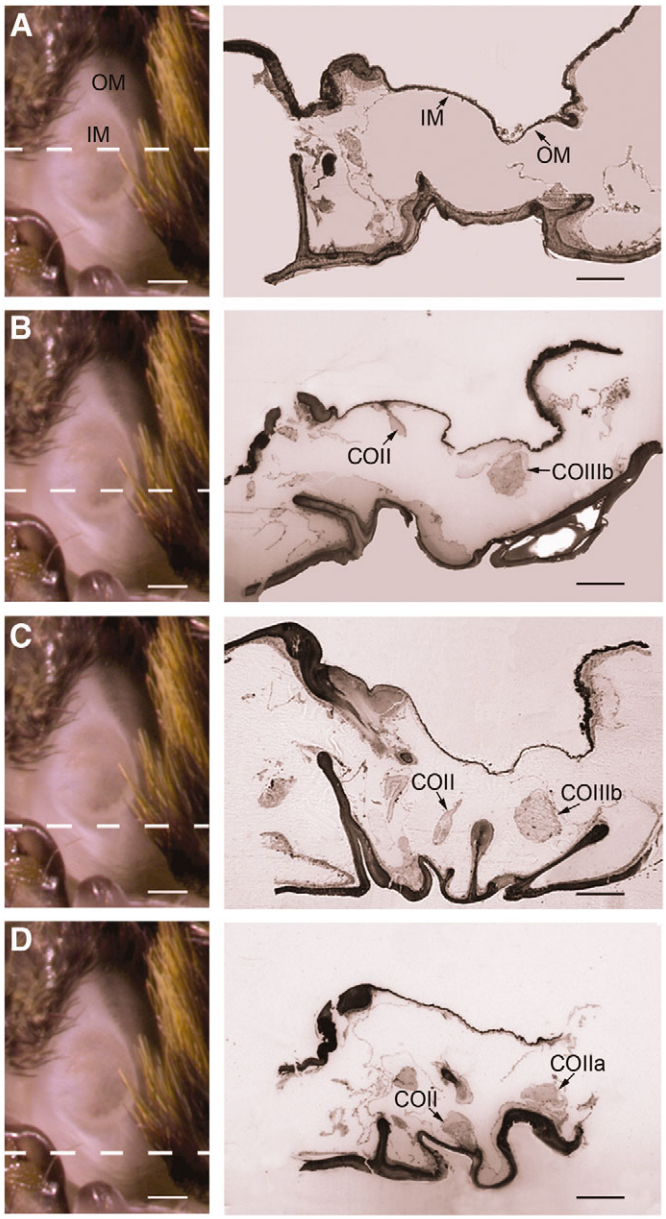

Fig. 3. Histological sections through the left forewing base of *M. peleides*, oriented such that the anterior edge of the ear is on the left. The approximate locations of the cross-sections are indicated by the dashed line through the light micrograph of VO (left), moving from distal to proximal from A to D. (A) Section through the distal portion of the inner membrane showing both the inner (IM) and outer (OM) membranes. (B) Section through the proximal end of the inner membrane, showing both the attachment strand of the chordotonal organ COII extending from the inner membrane, and the beginning of the chordotonal organ COIIIb resting below the outer membrane, surrounded by associated tracheal tissue. (C) Section through the base of the outer membrane, showing the intermittent structure of COII as it extends towards the base of the tympanal chamber, as well as the end of COIIIb. (D) Section through the base of the outer membrane, showing COII attached to the base of the tympanal chamber, as well as the chordotonal organ COIIIa at the base of the tympanal chamber. All scale bars, 200 μm.

at the same height as the specimen. The main wing nerve IIN1c was accessed by removing the tegula and associated membrane. The activity of the two branches arising from IIN1c that innervate the VO, N.II and N.III, were recorded using a stainless steel hook

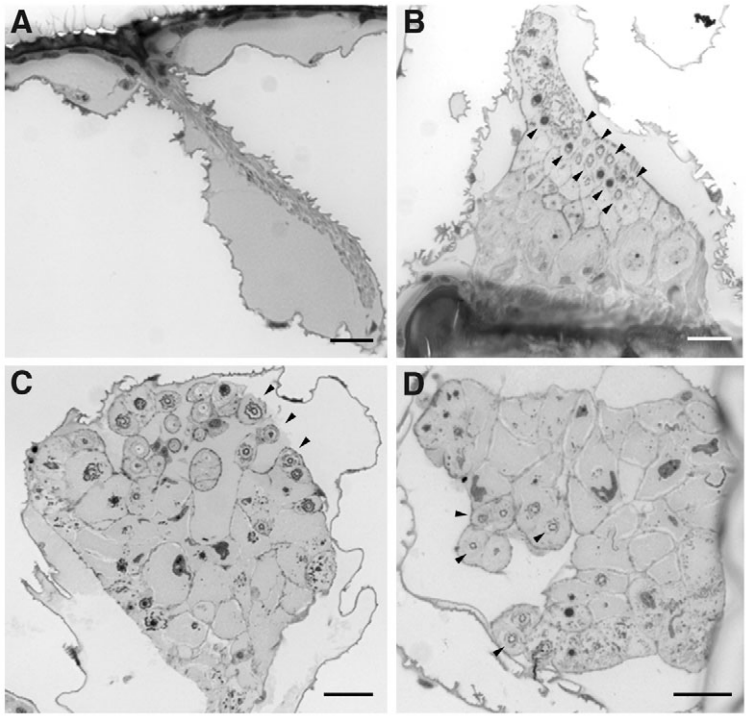

Fig. 4. Histological sections of the three chordotonal organs in the *M. peleides* VO. (A) The attachment strand of the chordotonal organ COII attaches directly to the inner membrane. (B) Cross-section through the proximal region of COII where it attaches to the base of the tympanal chamber. Arrowheads indicate the parallel arrangement of the sensory cells. (C) Cross-section through the chordotonal organ COIIIb, showing scolopale caps at the distal end of the section. Arrowheads point to three examples of scolopale caps within the organ. (D) Cross-section through the chordotonal organ COIIIa showing scolopale caps. Arrowheads point to four examples of scolopale caps within the organ. Scale bars, 20 μm.

electrode referenced to a second stainless steel electrode placed in the thorax. When a single branch was successfully hooked (N.II or N.III), all other branches of IIN1c were severed to ensure recorded signals were only from the focus branch. To prevent desiccation, the preparation was covered in petroleum jelly. Neural signals were amplified with a Grass P15 AC preamplifier (West Warwick, RI, USA) and displayed on a Tectronix (Beaverton, ON, Canada) THS720A digital oscilloscope. Neural activity and the associated sound stimulus were recorded on a Fostex FR-2 field memory recorder (Gardena, CA, USA) at a sampling rate of 48 kHz and stored as .wav files. All preparation and recordings were performed within a Faraday cage lined with acoustic foam (1.22 m×0.89 m×0.84 m).

Acoustic stimuli between 0.5 and 36 kHz were presented as trapezoidal sound pulses (30 ms duration, 5 ms rise/fall, linear ramp) shaped using PC Tucker Davis software (RpvdsEx, v. 5.4; Alachua, FL, USA) and synthesized by a Tucker Davis Technologies (TDT) digital signal processor (RX6 multifunction processor). Sound pulses between 500 and 3000 Hz were attenuated by a TDT SA1 stereo power amp and broadcast from a generic 6 in woofer at 80 cm from the specimen. The sound intensity of these stimuli was calibrated to dB SPL (reference pressure 20 μPa) by casting a 10 s sine wave, generated with the TDT digital signal processor, to a Brüel & Kjær Type 2239 sound level meter. Stimuli between 3 and 36 kHz were attenuated using a TDT PA5 programmable attenuator and broadcast from a 2 in cone tweeter (GT-1016, Q-components, Waterloo, ON, Canada), also broadcast at 80 cm. The speaker was calibrated using a Brüel & Kjær Type 4135 6.35 mm microphone and a type 2610 Brüel & Kjær measuring amplifier.

Audiograms were constructed to characterize the auditory range of the N.II nerve branch of the *M. peleides* VO. Frequencies between 0.5 and 36 kHz were tested in random order at 1 kHz intervals with occasional sampling in between. Threshold was calculated by gradually decreasing the sound intensity of the stimulus, and 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. To determine whether the neural responses recorded were sensory, we measured the latency of the neural response to the sound stimulus from .wav files recorded on a Fostex FR-2 field memory recorder for later analysis using Audacity 1.2.5 software (Free Software Foundation, Boston, MA, USA). An average latency from four neural responses was calculated per specimen. This was performed at the same sound intensity for each individual.

RESULTS

Morphology

The *M. peleides* VO is located on the ventral side of the forewing at the base of the cubital, subcostal and anal veins (Figs 1 and 2). In a previous study, Lane and colleagues (Lane et al., 2008) demonstrated that the VO comprises the three typical morphological characteristics of an insect ear: a tympanal membrane, enlarged trachea and chordotonal sensory organs. In this study, we focused specifically on the tympanal membrane and its association with the three chordotonal organs.

The oval-shaped tympanal membrane is made up of two distinct morphological regions. The dome-like inner membrane rests in the middle of the outer membrane, and is slightly thicker (mean±s.d.; 4.94±0.80 μm and 3.44±0.89 μm, respectively, N=3; Fig. 3A). The inner membrane is innervated by the chordotonal organ COII, while the outer membrane is innervated by the two chordotonal organs COIIIa and COIIIb (Figs 2, 3 and 4). COII, which is supplied by the N.II branch of the main wing nerve IIN1c (Lane et al., 2008), innervates the inner membrane by attaching directly to the middle of the inner membrane *via* an attachment strand (Fig. 2, Fig. 3B, Fig. 4A). The attachment strand extends through the tympanal chamber towards the more proximal location where the organ attaches at the base (Fig. 2, Fig. 3D, Fig. 4B). Scolopale caps can be observed in the base of the organ in a linear array, oriented such that the distal ends of the dendrites extend towards the membrane (Fig. 2, Fig. 4B). The two other chordotonal organs, COIIIa and COIIIb from the nerve branch N.III, innervate the outer membrane at the base of the posterior end. The larger of the two, COIIIb, attaches to the membrane *via* an attachment strand, and is anchored

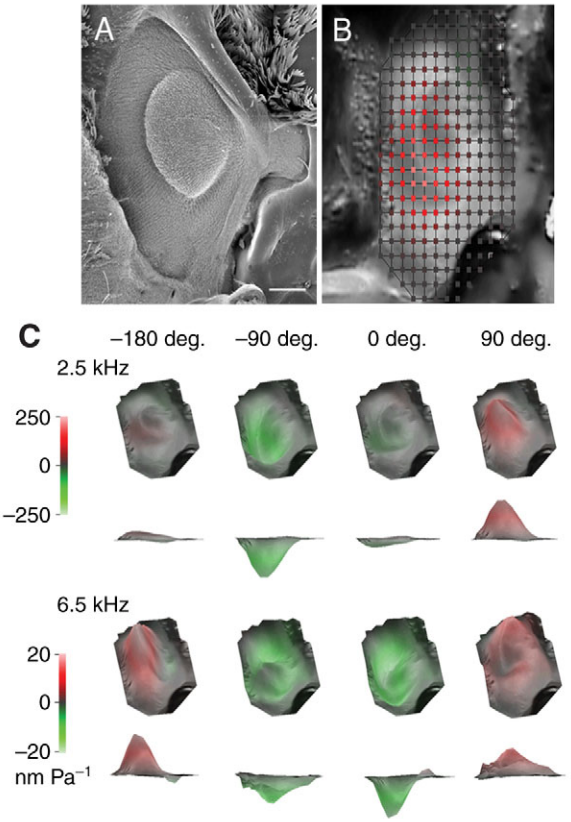

Fig. 5. Overall displacement of the *M. peleides* tympanal membrane in response to pure tones at 2.5 and 6.5 kHz. (A) Scanning electron micrograph of the *M. peleides* VO; whole view of the right tympanal membrane. Scale bar, 100 µm. (B) Orientation image of the *M. peleides* tympanal membrane displaying the scan points included in the area scan. (C) Tympanal displacement in response to sound frequencies at 2.5 kHz (top) and 6.5 kHz (bottom) at 60 dB SPL. Each set is shown at four different phases throughout the oscillation cycle. The profile is shown from both a top view and a side view.

to the sclerotization that divides the cubital and anal veins at the base of the tympanal chamber (Fig. 2). The smaller organ, COIIIa, is situated more proximally, and is indirectly associated with the membrane *via* trachea and its attachment to COIIIb (Fig. 2).

Vibrational analyses

The VOs of 24 *M. peleides* were scanned with a laser Doppler vibrometer to determine whether the inner and outer membranes and their respective chordotonal organ attachment sites have different vibrational properties. Overall, the membrane was found to vibrate in two modes at 60 dB SPL, depending on the frequency range: at lower frequencies (1–5 kHz) the vibration was focused on the proximal half of the posterior side of the outer membrane, while at higher frequencies (5–20 kHz) the entire membrane contributed to the vibration (Fig. 5; supplementary material Movies 1 and 2). These two modes correspond to the relative placements of the two sets of chordotonal organs on the inner and outer membranes. This response was linear, behaving in the same way at all sound intensities tested (27–60 dB SPL).

The average displacement gain of the membrane at the two specific attachment sites was analysed to determine how the chordotonal organs are stimulated by the membrane vibration (Fig. 6A). These two points were labelled X_1 and X_2, where X_1 corresponds to the attachment point of COII to the inner membrane, and X_2 corresponds to the approximate resting location of COIIIa and b beneath the outer membrane. Mechanical measurements were performed for nine individuals, seven females and two males. While both points responded with the greatest amplitude between 1 and 5 kHz, the displacement gain at X_2 was approximately twofold that at X_1 at the lower frequency range (mean±s.e.m.; 173.3±57.8 nm Pa^{-1} and 82.6±13.1 nm Pa^{-1} at 2.5 kHz, respectively). At higher frequencies, the average displacement gain decreased to levels between 10 and 30 nm Pa^{-1} at both X_1 and X_2. Therefore, the mechanical response of the membrane greatly varies with frequency, with a best frequency between 2 and 3 kHz. The shape of the mechanical response across the attachment points, described as the deflection envelope of the membrane, also varies with the stimulus frequency (Fig. 6B). In the lower frequency range, the membrane uniformly vibrates around X_2. At higher frequencies, the membrane appears to vibrate around two nodes, one at the very base of the proximal posterior end of the membrane (at its most proximal point), and the other at the inner membrane (at X_1). Therefore, not only does the membrane oscillate over a much greater distance at low frequencies but also the shape of the whole membrane deflection changes with frequency.

Neurophysiology

The hearing range of six female *M. peleides* was tested by performing extracellular nerve recordings from N.II. The N.II branch responded consistently to pure tone acoustic stimuli between 0.7 and 20 kHz. The response consisted of compound action potentials and was tonic for all stimulus durations tested (30 ms to 1 s). The amplitude of the compound action potentials increased with increasing sound intensity, suggesting that more sensory neurons were stimulated at higher sound intensities. At approximately 8 dB SPL above threshold, latencies ranged between 6 and 9 ms, indicating that the neural response was sensory with a similar range found in other lepidoptera (see Göpfert and Wasserthal, 1999; Skaals and Surlykke, 2000). Nine audiograms were generated from the six specimens for the N.II branch (Fig. 7A). It was found to be most sensitive at frequencies around 4 kHz, with a best median threshold of 54 dB SPL at 4 kHz. The best threshold for an individual was 45 dB at 4 kHz. There was some response of N.II to frequencies between 21 and 32 kHz at high sound intensities (generally >90 dB SPL). When comparing the median threshold response recorded from the N.II branch *versus* the N.III branch [data from Lane et al. (Lane et al., 2008)], no difference was apparent with respect to either the sensitivity or the frequency range (Fig. 7B; see Appendix).

DISCUSSION

The variation exhibited in tympanal membrane morphologies among insects has important implications for species-specific auditory abilities. The tympanal membrane, as the first structure to intercept an incoming sound, transforms the energy of an acoustic stimulus into mechanical energy and transfers it to the mechanosensory organs proper. The peripheral processing carried out by the tympanum and other associated structures (e.g. the acousto-mechanical converter) essentially determines what sound frequencies and intensities an insect can detect. For some insects, tympanal morphology also plays an important role in the generation of acoustic and mechanical cues for directional hearing, such as in parasitoid flies (Robert, 2008). The unusual dome-shaped tympanal ear of the *Morpho* butterfly has apparent implications for the vibrational properties of the membrane, and the relationship between the sensory organ attachments and their neurophysiological response. We found that

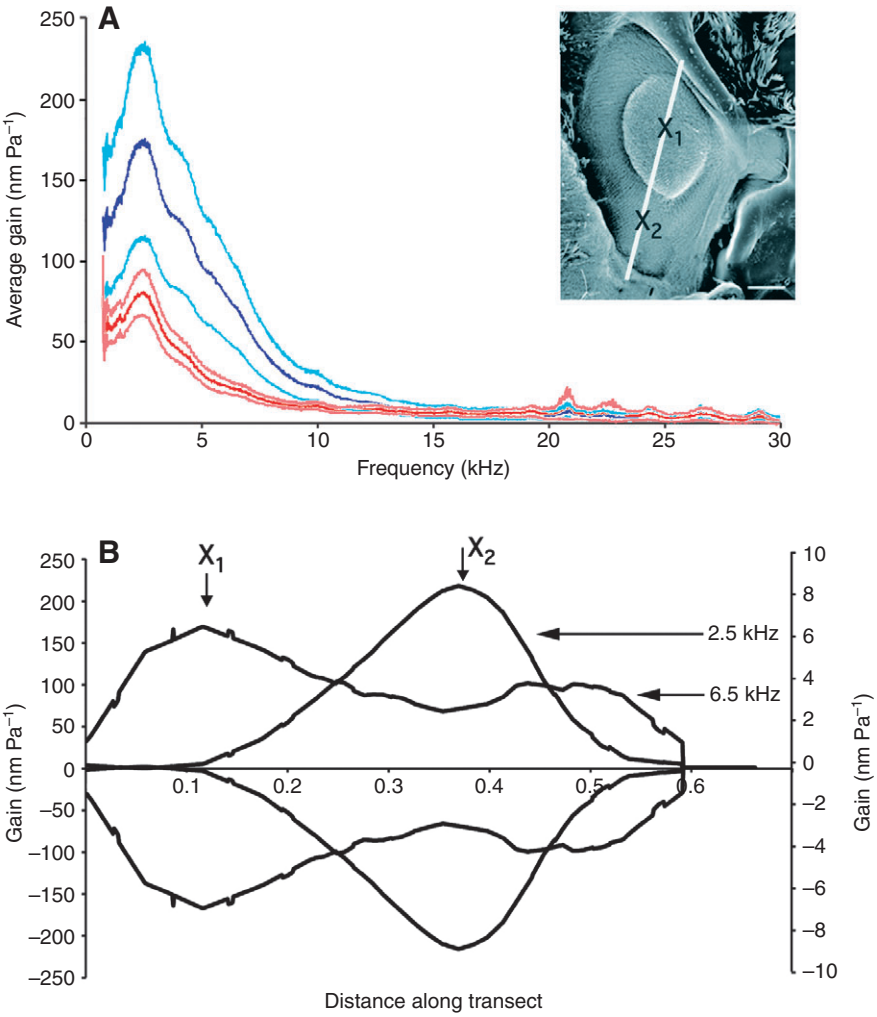

Fig. 6. Mechanical response of the *M. peleides* membrane to sound. (A) The average amplitude gain response of two points on the outer (blue) and inner (red) tympanal membrane in response to frequencies between 1 and 30 kHz (*N*=9, 7 females and 2 males). Specific points tested are denoted on the scanning electron micrograph of VO as X_1 on the inner membrane and X_2 on the outer membrane (inset). The standard error of the mean is depicted as the light blue lines above and below the outer membrane average amplitude gain and as the pink lines above and below the inner membrane average amplitude gain. (B) The deflection envelopes of the amplitude gain response. The left *y*-axis corresponds to the response at 2.5 kHz, while the right *y*-axis corresponds to the response at 6.5 kHz. The transect measured is shown on the scanning electron micrograph of the VO with a white line (inset), where the most distal point of the line corresponds to 0 and the most proximal point corresponds to 0.7 arbitrary units on the *x*-axis. Scale bar in inset, 200 μm.

the heterogeneous topography of the tympanal membrane creates two distinct vibrational modes, which in turn correspond to the location and response of the associated chordotonal organs.

Vibrational properties of the tympanum

Using laser Doppler vibrometry, we found that the tympanal membrane of *M. peleides* vibrates in two frequency-dependent modes within the frequency range of approximately 1–20 kHz. At frequencies below 5 kHz, the mechanical response is focused at the proximal region of the posterior end of the outer membrane, while at frequencies above 5 kHz, both the inner and outer membranes vibrate in response to acoustic input. This could contribute to coarse frequency partitioning by essentially dividing frequencies into two groups, indicating to the animal whether the sound stimulus is at a high or a low frequency, simply based on the mechanical tympanal response. The transition between the inner and outer membranes is very abrupt (Figs 1–3) and most likely creates the divide between the vibrational modes. Interestingly, the attachment sites of the two sets of chordotonal organs correspond with the focal points of the two vibrational modes. One organ, COII, attaches to the middle of the inner membrane, while the other two organs, COIIIa and COIIIb, are associated with the posterior region of the outer membrane. Immediate consequences of this arrangement may be either (1) a broadening of the frequency range detected by the *M. peleides* VO and/or (2) the ability to detect differences between frequencies. Indeed, frequency discrimination in locusts is achieved by the differential membrane response at different sensory attachment points (Michelsen, 1971a; Römer, 1976). The sensory organs in the locust Muller's organ are organized into four groups of scolopidia that attach to the membrane *via* attachment strands (Gray, 1960), which are further categorized into separate functional groups based on their different frequency sensitivities (Michelsen, 1971a; Michelsen, 1971b). The variable vibrational properties of the locust membrane correspond to the attachment points of the scolopidia, such that different sets of sensory cells are activated in response to the driving frequencies (Windmill et al., 2005). A similar situation is also observed in the cicada *Cicadatra atra*, where frequency discrimination is achieved *via* a travelling wave that is propagated in the heterogeneous membrane in response to sound (Sueur et al., 2006). In another cicada species *Tettigetta josei*, frequency discrimination has been shown to be conserved into the CNS *via* finely tuned interneurons (Fonseca et al., 2000). The heterogeneous membrane in *M. peleides* may also transfer different frequency information by activating chordotonal organs differently. Altogether, because it is also endowed with distinctive and multiple innervation, the tympanal membrane of *M. peleides* may constitute another method of partitioning or expanding the range of audible frequencies.

Response of the sensory organs

Overall, the physiological response patterns from both nerve branches (N.II and N.III) match the auditory mechanics. Both the outer and inner membranes vibrate best in response to low

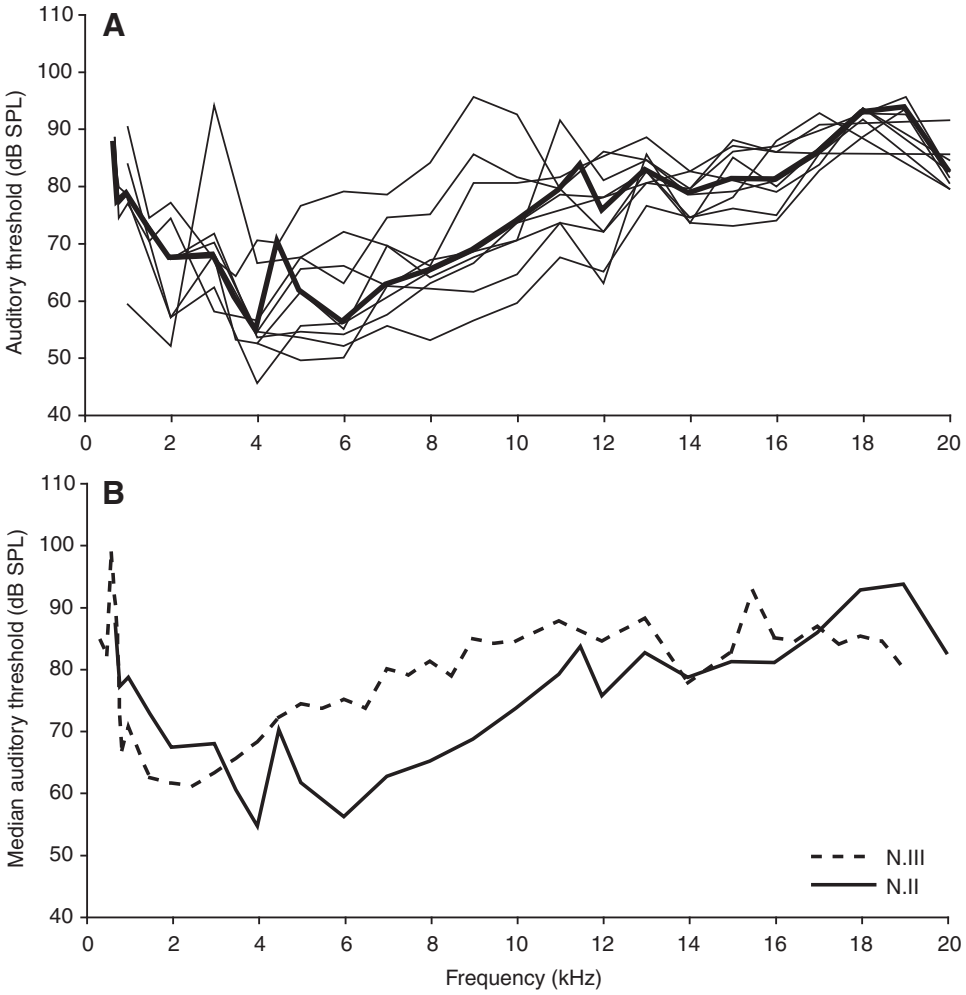

Fig. 7. Audiograms for *M. peleides*. (A) Response of neural branch N.II (N=6 animals, N=9 ears). The bold line represents the median threshold response. (B) The median threshold response of the branch N.II (solid line; N=9 ears) and N.III [dashed line; data from Lane et al. (Lane et al., 2008)].

frequencies (Fig. 6A) and, correspondingly, sensory organs associated with both membranes respond best to low frequencies (Fig. 7).

But does the complex mechanical behaviour of the *M. peleides* tympanal membrane impart frequency partitioning as suggested by the vibrational modes? Whole-nerve extracellular recordings from the two nerve branches that innervate the inner and outer membranes showed no significant difference in their response, suggesting that the sensitivity thresholds of these branches do not provide frequency information to the animal. The number of units firing within each nerve branch may instead be providing more detailed information in response to the frequency-dependent vibrational response of the membrane. Course frequency discrimination may be attained through an analysis of firing ratios between the two nerve branches. At low frequencies, the proximal half of the posterior side of the outer membrane moves twice as much as the inner membrane, suggesting that more units within the associated chordotonal organs (COIIIa and b) would be firing than in COII. At higher frequencies, the displacement of both membranes decreases, suggesting substantively fewer units altogether would be firing, and the ratio of units between COIIIa/b and COII would be smaller. When detecting broadband sounds, the responses to high and low frequencies would be additive, and overall a large number of units would be responding. In this way, the VO may provide course frequency information to the animal. Another explanation could be that we were unable to see how the separate organs are firing because of our use of whole nerve recordings. It is indeed possible that some units in the N.II branch responding to higher frequencies were missed with this technique. A similar situation occurred in the cicada *T. josei*, where extracellular nerve recordings suggested these insects were most sensitive to 3–6 kHz, which doesn't match the spectral energy of their communication calls (Fonseca et al., 2000). Intracellular recordings of interneurons showed that these cells were sharply tuned to both higher and lower frequencies, indicating that *T. josei* had the ability to discriminate between frequencies (Fonseca et al., 2000). Further research using intracellular techniques will be performed to address this.

Function of hearing in butterflies

Research into butterfly hearing is relatively new to the field of insect bioacoustics. An increasing number of morphological (e.g. Vogel, 1912; Otero, 1990; Yack et al., 2000; Mahony, 2006; Yack et al., 2007; Lane et al., 2008) and physiological and behavioural studies (e.g. Ribaric and Gogala, 1996; Rydell et al., 2003; Yack et al., 2000; Yack and Fullard, 2000; Yack et al., 2007; Lane et al., 2008) demonstrate that hearing is widespread among the butterfly taxa Hedyloidea and Nymphalidae. However, little is still known about the function of hearing in most butterflies. Hearing in nocturnal species such as *Macrosoma heliconaria* and *Manataria maculata* is understood to be for predator detection, as these butterflies engage in evasive flight manoeuvres in response to high frequency bat calls (Yack and Fullard, 2000; Yack et al., 2007; Rydell et al., 2003). In

sound producing butterflies, such as *Hamadryas feronia*, hearing is probably for conspecific communication (Yack et al., 2000). For the many other butterflies that possess VOs, the function remains unknown. Currently, it is hypothesized that these butterflies, such as *M. peleides*, are listening to avian predators (Lane et al., 2008). Birds are a major predator and strong selective pressure against butterflies, and are considered one of the primary reasons why selection has resulted in the high variability in the morphology, physiology and behaviour of butterflies [see Chai (Chai, 1996) and references therein]. The low-frequency ears, described here in *M. peleides* and in other satyrine species such as *Pararge aegeria* (Mahony, 2006) and *Erebia* spp. (Ribaric and Gogala, 1996), overlap with the broadband, low-frequency flight sounds that approaching avian predators produce (most energy between 1 and 15 kHz; J.E.Y., unpublished). It is worth noting here that the detection of frequencies around 1–2 kHz is unusually low when compared with the majority of tympanate insects. It may be reasonable to hypothesize that low frequency sensitivity using tympanal ears serves the detection of avian predators. Given their frequency sensitivity and capability to detect a broad range of frequencies, *M. peleides* may use its VO to detect both singing and flying birds.

We have shown that the vibrational response of the *M. peleides* tympanal membrane is not that of a simple eardrum; it comprises two distinct modes. The inner and outer membranes show different frequency-dependent patterns of vibration in support of our hypothesis. The sensitivity thresholds of the neural branches correspond to the vibrational response and suggest a possible method of frequency discrimination in these butterflies. Future research will focus on assessing whether and to what extent frequency partitioning is occurring at the neural level. Also, behavioural experiments will be necessary to assess the function of hearing in many butterfly species. With a vast diversity of hearing organs (Otero, 1990; Mahony, 2006) (J.E.Y., unpublished), butterflies constitute an exciting source of wonders that is likely to greatly enrich our understanding of hearing and its evolution.

APPENDIX

Statistical comparison of the auditory thresholds of N.II and N.III

The difference between the auditory thresholds of the two nerve branches N.II and N.III was not initially compared statistically because of low sample size. To do this, we would compare whether the auditory threshold recorded was consistent between nerve branch and frequency across individuals (a single nerve branch was recorded per individual). Based on the experimental set-up, a non-parametric Friedman test would have to be used. If both the left and right ear of an individual had been measured, only one audiogram was used, leaving N=6 for N.II and N=13 for N.III. Because of these small sample sizes, the Friedman test could not be performed.

Instead, we performed Mann–Whitney U-tests at each frequency point, since our samples are independent (i.e. different animals in each ‘nerve branch’ group). In all, 12 frequency points were tested, between 1 and 13 kHz, which had a measured auditory threshold from each individual. If there was one measurement missing within a frequency, the average (per nerve branch) was calculated and used to fill the gaps. If any more than two threshold measurements were missing for a specific frequency, the frequency was not tested. The dB SPL threshold values were converted to mPa for calculation. The results of the Mann–Whitney tests are reported in Table A1, comparing the auditory threshold of N.II (N=6) *versus* N.III (N=13) at each frequency point. At some frequency points there are significant z-values, denoting a significant difference in the auditory thresholds of N.II *versus* N.III at 4–8 kHz, and at 11 and 13 kHz. While this suggests that the nerve branches may have different auditory thresholds, there is insufficient statistical power to make this conclusion because of a risk of false positives (type 1 error) (see Moran, 2003).

Table A1. Statistical comparison of the auditory threshold of the two nerve branches N.II and N.III in *Morpho peleides*

Frequency (kHz)	z-statistic	P-value
1	–1.579	0.114
2	–0.175	0.861
3	–0.877	0.380
4	–2.195	0.028*
5	–2.369	0.018*
6	–2.280	0.023*
7	–2.720	0.007*
8	–2.281	0.023*
9	–1.141	0.254
10	–1.843	0.065
11	–3.071	0.002*
13	–2.282	0.022*

Analysis conducted in SPSS v.16.0.1.
*Significant P-values, based on alpha=0.05.

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