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ORIGINAL PAPER

Neuroethology of ultrasonic hearing in nocturnal butterflies (Hedyloidea)

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Abstract Nocturnal Hedyloidea butterflies possess ultrasound-sensitive ears that mediate evasive flight maneuvers. Tympanal ear morphology, auditory physiology and behavioural responses to ultrasound are described for *Macrosoma heliconiaria*, and evidence for hearing is described for eight other hedylid species. The ear is formed by modifications of the cubital and subcostal veins at the forewing base, where the thin (1–3 μm), ovoid (520 × 220 μm) tympanal membrane occurs in a cavity. The ear is innervated by nerve IIN1c, with three chordotonal organs attaching to separate regions of the tympanal membrane. Extracellular recordings from IIN1c reveal sensory responses to ultrasonic (>20 kHz), but not low frequency (<10 kHz) sounds. Hearing is broadly tuned to frequencies between 40 and 80 kHz, with best thresholds around 60 dB SPL. Free flying butterflies exposed to ultrasound exhibit a variety of evasive maneuvers, characterized by sudden and unpredictable changes in direction, increased velocity, and durations of ~500 ms. Hedylid hearing is compared to that of several other insects that have independently evolved ears for the same purpose-bat detection. Hedylid hearing may also represent an interesting example of evolutionary divergence, since we demonstrate that the ears are homologous to low frequency ears in some diurnal Nymphalidae butterflies.

Keywords Hedylidae · Ultrasound · Hearing · Butterfly · Bat avoidance

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Introduction

Sensory organs connect animals in a unique way to their physical environment and provide them with cues relevant for their survival. For butterflies, the best-studied sensory modalities are vision, taste and smell. Vision is widely used for orientation, mate detection and selection of host plants for feeding and oviposition. Consequently, the well developed compound eyes and visual centers of the brain have received much research attention in butterflies (Silberglied 1984; Warrant et al. 2003). Chemical senses are commonly used in mate choice and host plant selection. Hence, the receptor organs and brain centers are also well characterized (Boppré 1984; Hallberg and Poppy 2003; Hallberg et al. 2003). In contrast, a sense of hearing in butterflies has received little research attention, despite evidence that this sensory modality may be prominent in some groups.

Butterflies comprise 3 of the 46 superfamilies forming the order Lepidoptera (Ackery et al. 1999; Kristensen and Skalski 1999). These include the well-known and cosmopolitan Papilionoidea (true butterflies) and Hesperioidea (skippers), and a smaller, less known neotropical group, the Hedyloidea (Scoble 1986, 1990, 1996) (Fig. 1). The remaining 43 superfamilies are commonly known as moths. Hearing in moths has evolved independently at least six

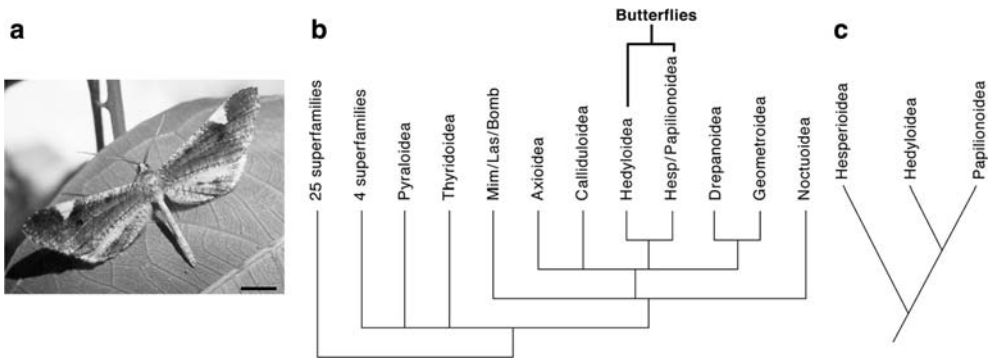

Fig. 1 **a** A male *Macrosoma heliconiaria*. Scale bar: 5 mm. **b** Phylogeny of the Lepidoptera adapted from Kristensen and Skalski (1999) depicting the proposed relationship between the three butterfly superfamilies (Hedyloidea, Papilionoidea, and Hesperoidea) and moths. **c** An alternative hypothesis proposed by Scoble (1986) for the relationships between butterflies

times (Minet and Surlykke 2003). It functions primarily for detecting the ultrasonic calls of insectivorous bats, which echolocate for orientation in space and for detection, classification and localization of prey (Schnitzler and Kalko 2001). The neuroethology of moth hearing has been extensively researched (reviewed in Spangler 1988; Fullard 1998; Conner 1999; Minet and Surlykke 2003; Waters 2003) in contrast to butterflies, where little research has been conducted on acoustic communication. To date, there have been no reports on an acoustic sense in Hesperoidea, but a growing body of evidence (see Discussion) suggests that hearing may be widespread in both Nymphalidae (Papilionoidea) and Hedyloidea. The present study focuses on Hedyloidea, combining aspects of morphology, physiology and behaviours associated with hearing.

The Hedyloidea are an obscure group of nocturnal butterflies occurring in Central and South America. The superfamily comprises an estimated 40 species (Scoble 1986), all currently assigned to a single family, Hedylidae, and a single genus, *Macrosoma*. Hedylids are of particular interest because they share morphological and behavioural traits with both moths and day-flying butterflies. They have been dubbed the 'living ancestors' of butterflies (Aiello 1992) and are thought to represent the evolutionary 'missing link' between moths and other butterflies. Currently, hedylids are placed as a sister group to the Papilionoidea + Hesperoidea, although the exact relationship between the three superfamilies remains unresolved (Scoble 1996; Wahlberg et al. 2005). Considering the interest in their taxonomy with respect to butterfly evolution, surprisingly little is known about the behaviour or life history traits of the Hedyloidea.

In an earlier study it was demonstrated that one hedylid species, *Macrosoma heliconiaria*, possesses a tympanal hearing organ on the forewing, and that flying butterflies respond to ultrasound with evasive flight maneuvers (Yack and Fullard 2000). Some external morphological characteristics of hedylid ears have been noted by Scoble (1986) (although not identified as a hearing organ in this publication), and Minet and Surlykke (2003). The purpose of our study was to investigate the neuromorphological and neurophysiological characteristics of the ear in *M. heliconiaria*, and to characterize behavioural responses to ultrasound in flying and resting individuals. In addition, evidence for hearing is described for eight other hedylid species. We discuss how hedylid hearing compares to that of other insects, and address its relevance with regard to the evolution of hearing in other butterflies.

Materials and methods

Animals and study site

Live butterflies used for physiological, behavioural or histological investigations included *Macrosoma heliconiaria*, *M. conifera*, *M. rubidinaria* and *M. semiermis*. All specimens were collected at ultraviolet, mercury vapour, or fluorescent lights in October 1999 and November 2000 on Barro Colorado Island (BCI), a field station of the Smithsonian Tropical Research Institute in Panamá. The 15.6 km^2 island next to the Panama Canal is covered with moist, semi-deciduous tropical lowland forest. Climate is seasonal with a dry season from the end of December until the middle of April and a wet season where most (90%) of the rainfall (2,600 mm) falls (Leigh 1999). Dried specimens of *M. subornata*, *M. semiermis*, *M. hedylaria*, *M. nigrimaculata*, *M. satellitiata*, *M. bahiata*, *M. rubedinaria*, and *M. conifera*, used for comparative morphology were obtained from the American Museum of Natural History (New York, USA), and Agriculture and Agrifood Canada (Ottawa, Canada).

Morphology

Nerve branches innervating the ear were described by following whole nerves from the thoracic ganglia in 8 live and 14 fixed specimens (Chauthani and Callahan 1966), using

Janus Green B (Yack 1993). Nomenclature used to describe the thoracic and wing nerve branches, muscles and skeletal structures follows Nüesch (1953, 1957) and Vogel (1911, 1912). For histology, three male *M. heliconiaria* were fixed by injection with 2.5% glutaraldehyde in phosphate buffer. Dissected tissues were washed in buffer, dehydrated in ethanol, and embedded in Spurr's low viscosity epoxy. Specimen blocks were sectioned at 1–5 μm with glass knives on a LKB 8800 ultramicrotome, and sections stained with toluidine blue. Slides were photographed using a Zeiss Axio Imager M1 compound microscope equipped with a Zeiss AxioCam MRm camera.

The external and internal characteristics of the forewing base and its specialized tympanal structures, as well as some putative sound producing structures, were measured and imaged using scanning electron microscopy. Dissected specimens were air-dried, mounted on aluminum stubs, sputter-coated with gold–palladium and examined with a JSM-6400 (JEOL) microscope.

Physiology

Prior to physiology experiments we examined behavioural responses of all prospective experimental animals by stimulating with intense ultrasonic pulses using an electronic dog whistle (Pet Trainer™) emitting a pure 26 kHz tone with a sound pressure of 110 dB SPL rms at 1 m. Of all 28 animals tested, 26 showed clear behavioural responses to sound and were used for physiology. All 26 subjects (24 *M. heliconiaria* including 22 males and 2 females and 2 female *M. conifera*) were tested in the electrophysiology set-up on the evening of capture at our field site.

Initially we verified reactions to sound stimuli by recording activity in the dorsal longitudinal flight muscle in three animals using an extracellular tungsten electrode inserted in the muscle. The animal was tethered to a wooden rod that was glued to the dorsal thorax such that the wings, legs and head could move freely. In the remaining 23 animals we recorded neural activity directly from the tympanal nerve. Animals were secured to a block of modelling clay, with the ventral surface of the wing positioned upward and the hindwing placed behind the forewing to ensure that the sound path to the ear was not obstructed. An extracellular tungsten electrode was placed on the nerve by carefully pushing it through the counter-tympanal membrane. Nerve activity was amplified with a custom built battery operated AC preamplifier and displayed on a portable digital oscilloscope (Tektronix, TekScope model THS710A) and through an audiomonitor. The oscilloscope and audiomonitor were used for on-line establishment of auditory thresholds at different frequencies. For off-line analysis we recorded traces of electrophysiological responses digitally (sampling rate 100 kHz) using a Wavebook (IoTech) A/D with 128 MB circulating memory stored onto an IBM notebook computer, which was also used to control the Wavebook and to check the recordings on-line.

For both the muscle and auditory nerve recordings, animals were initially stimulated by intense stimuli delivered by the Pet Trainer™ in order to recruit maximum electrophysiological activity to determine if the preparation was responding. Some of the response traces were recorded digitally using the Wavebook for later off-line analyses.

If the preparation was stable and responded well to the PetTrainer™ we continued by stimulating with sound from a loudspeaker in order to control frequency and intensity for determining audiograms. Sound stimuli were generated in one of two ways. In 1999 ultrasonic sound pulses were generated using a custom-built ultrasound pulse generator ("Portabat") equipped with a custom built electrostatic loudspeaker. Sound pressure levels were set by adjusting the output voltage to the loudspeaker and later converting to dB SPL (rms) by measuring the sound pressure at the same voltage levels with a Brüel and Kjaer ¼″ microphone (model 4135 without protecting grid) and Brüel and Kjaer amplifier (Type 2804) calibrated by a Brüel and Kjaer Calibrator. In 2000, sound pulses were generated by a battery-operated function generator (ISO-Tech) connected to a custom built amplifier, adjusted by a dB attenuator (Hatfield) and projected through a Technics Tweeter. The same Brüel and Kjaer measuring chain as in 1999 was used to calibrate the sound generating system. Sound pulses were 10 ms long repeated at 10 Hz. Frequencies between 10 and 120 kHz were tested in random order in 10 kHz steps. Auditory thresholds were determined as the sound pressure level that elicited rhythmic neural activity that was clearly detectable on both the oscilloscope and audiomonitor. After the entire frequency range was tested, threshold determination was repeated at the first three test frequencies of that run. The audiogram was only included in the data base if these thresholds were within ±2 dB of the original thresholds. Sensitivity to audible frequencies was tested for two individuals where the recordings had been stable enough to determine the full audiogram from 10 to 110 kHz. Sound pulses from 500 Hz to 5 kHz were generated digitally, D/A converted through the sound board of the computer and projected using a computer speaker (Sony Active speaker, SRS-88) calibrated using the same Brüel and Kjaer equipment as above.

Behaviour

In a previous study it was reported that flying *M. heliconiaria* exhibited short latency (~45 ms) evasive maneuvers when exposed to ultrasound (Yack and Fullard 2000). The objectives of the current study were to extend these findings by (a) characterizing flight evasive responses of *M. heliconiaria*

with respect to speed, duration, distance traveled and direction of flight path; (b) testing the responses of resting (non-flying) hedylids to ultrasound; and (c) examining the evasive flight responses of three other species (*M. conifera, M. rubidinaria* and *M. semiermis*). Trials were conducted on individuals that had been captured on the same evening, between 2000 and 0300 hours, when the butterflies were naturally active. A total of 210 *M. heliconiaria* flight trials (number of individuals = 128), and 80 resting trials (number of individuals = 80) were recorded onto video. If an individual was used twice, trials were separated by a minimum of 2 min. Two different sets of experimental conditions were used to conduct behavioural trials, and each is described below.

In one condition trials were conducted inside a tall concrete utility building (6 × 3 × 3 m) of the field station located in the rainforest. After dusk, several *M. heliconiaria* would enter the building through openings in the bricks near the ceiling, and gather on the ceiling or near the fluorescent lights inside the building. When a butterfly took flight it was presented with a 200–500 ms ultrasonic stimulus produced by a hand-held transducer (K-II Enterprises, Camilla, NY, USA) at a distance of 1.5–3 m. The stimulus consisted of an intense (116 dB SPL at 1 m) sinusoidal fundamental frequency of 24.8 kHz with considerable energy at second and third harmonics, which is an adequate representation of the sound emitted from an aerial insectivorous bat searching for prey. Pre- and post-stimulus flight was recorded with a Panasonic (PV-320-K) VHS camcorder while the sound stimulus was monitored simultaneously using a Mini-2 bat-detector (Ultrasound Advice, London, UK) connected to the camera's audio input. These sounds were used to determine the onset of the stimulus during video analysis. In order to analyze the VHS tapes at slow speed, they were transferred to Hi-8, and replayed using a Hi-8 Sony EVO-9800 deck and Sony RM-450 controller. Responses of 80 resting butterflies were also recorded in this room, by exposing the butterfly to the same ultrasonic sound at a distance of 2 m. Videotapes were subsequently analyzed to note the presence or absence of a response to sound.

In the second condition trials were conducted in a flight room (4.5 × 4.5 × 2 m), located in the rainforest, that is routinely used to study bat behaviour. This room was darkened with black velvet or cloth, with no lights other than a single hand-held ultraviolet light suspended at a height of about 2 m at one end of the room to entice the butterfly to fly across the room. A video camera (Sony TR7000 Digital 8), set on night-shot™, was focused on one of the walls. At the beginning of each trial a butterfly was released at one end of the wall upon which the camera was focused, and would fly to the other end of that wall, attracted by the small UV lamp. When the butterfly was in the camera's field of view, one of us would deliver the sound stimulus at distances between 1.5 and 3 m from the butterfly. The stimulus was recorded with a Mini-2 bat-detector (Ultrasound Advice, London, UK) connected to the camera's audio input. Videotapes were analyzed using iMovie (5.0.2) on a Mac G4 computer (see details below).

Videotapes of flying hedylids were analyzed to determine the percentage of individuals reacting to sound, changes in flight direction and speed following stimulation, and distances traveled before the butterfly resumed normal flight. Since we used only one video camera, responses to sound could only be monitored in two dimensions, and any movements toward or away from the camera could not be assessed. However, our methods gave us a good estimation of the types and characteristics of flight responses. The percentage of trials where individuals responded to sound was assessed from all 210 trials recorded onto video (137 from the utility room and 73 from the bat flight room). Videotapes were observed initially with the sound turned off to determine if there was a marked change in flight speed or direction. Trials were categorized into three groups: (a) an evasive response (b) no detectable response, where the butterfly did not exhibit a marked behavioural response; and (c) those whose flight path was not sufficiently straight near the beginning of the trial to enable us to assess whether or not a change in direction had occurred. We also conducted 16 controls without sound. Once the behavioural responses were categorized, the clips were reviewed at reduced speed with the sound turned on to confirm the occurrence and timing of the stimulus.

Sixty trials were selected to study the changes in initial flight direction (upward or downward and forward or backward) during the first ten video frames following stimulation, and the general type of response overall (e.g. spiral, upward climb and steep dive). The trials were chosen based on the butterflies exhibiting a clear response to sound, and being in the camera's view for a minimum of 30 frames following stimulation, which typically covered the entire evasive maneuver.

Pre- and post-stimulus flight speeds were estimated from 25 trials in the bat flight room. Flight speeds were determined by noting the total distance (in meters) the butterfly traveled over ten consecutive video frames prior to stimulus onset, and ten frames following stimulus onset. We then divided each distance by 0.33 s (the time period covered by ten consecutive video frames) to provide estimates of flight speed in m/s. The duration of the entire response and the distance traveled throughout the response were estimated from the same trials, by noting the times and locations from when the butterfly first changed direction to when it resumed normal flight. Composite photos of evasive maneuvers (Fig. 9) were made by importing the digital video clips to iMovie (5.0.2) on a Mac G4 computer, then

exporting them to QuickTime Pro where a sequence of images was created. Composite image sequences were made using Adobe Photoshop (7.0).

Results

Wing base and ear morphology

The tympanal ears are formed by cuticular, tracheal and neural specializations of the subcostal (Sc), radial (R), cubital (Cu) and anal (A) veins (Figs. 2, 3, 4, 5) at the base of the forewing. The tympanal membrane is not immediately obvious from visual inspection of the wing since it resides in a pocket (=tympanal cavity) at the wing base, at the proximal and narrow end of a funnel shaped 'canal'. The canal is bordered rostrally by a prominent ventral fold of Sc, and caudally by the anterior edge of the hind wing. Overhanging the anterior portion of the tympanal membrane is a protective covering formed by an expansion of Sc (Figs. 2, 3, 4). A dense fringe of elongate scales extends from the proximal and posterior edge of this Sc expansion to cover the tympanal cavity and protect the delicate tympanal membrane (Fig. 2b). The tympanal membrane, and a second membrane, the counter tympanal membrane, form two walls of an enlarged air-filled chamber (=tympanal chamber), which comprises proximal expansions of the Sc, Cu and A veins. Two expansions that are particularly noticeable include the anterior chamber (AC) (Scoble 1986), which is most prominent in the ventral plane of the wing, and the posterior chamber (PC) (Scoble 1986), which is formed by an expansion of Cu and is particularly prominent in the dorsal plane of the wing (Figs. 2, 4). The PC is self-contained distally but merges with the tympanal chamber proximally (Fig. 4).

The tympanal membrane is a very thin (1–3 μm) ovoid (~522 ± 59 × 224 ± 16 μm) membrane supported by a heavily sclerotized cuticular frame at the base of Cu. The tympanal membrane is not structurally homogeneous. At its proximal end it is separated from the wing base by a thickened strip of tissue (5–15 μm) that extends downward on the underside of the tympanal membrane (Fig. 3b, c). When viewed under a light microscope, most of the tympanal membrane is transparent, except for this proximal region, which is translucent. Three chordotonal organs attach to the inner surface of the tympanal membrane, and are arranged in a row along the longitudinal midline of the tympanal membrane (Fig. 3b, c). The most proximal chordotonal organ attaches to the tympanal membrane adjacent to the cuticular thickening. When viewed at high magnification with the scanning electron microscope, the tympanal membrane is a smooth, uniform surface devoid of scales or seta (Fig. 3 d).

The counter tympanal membrane is named after an analogous structure in moth ears (e.g. Noctuoidea, Geometroidea, Pyraloidea) (Minet and Surlykke 2003). In hedylids, it is triangular in shape (500 ± 100 × 266 ± 36 μm) and forms the posterior wall of the tympanal chamber (Figs. 3a, 4e). When viewed under the light microscope the surface is whitish, translucent, and has a 'wrinkled' texture. In cross section, the membrane is 2–8 μm thick and as far as we could determine, has no sensory organs attached directly to its inner surface, as does the tympanal membrane. The counter tympanal membrane is devoid of scales or seta, but less smooth than the tympanal membrane (Fig. 3e).

Examinations of the external forewing morphology of eight other hedylid species, including *M. subornata* (male), *M. semiermis* (male and female), *M. hedylaria* (male), *M. nigrimaculata* (male), *M. satellitiata* (male), *M. bahiata* (male), *M. rubedinaria* (male and female), *M. conifera* (male and female) established that each possesses similar ear structures, and the presence of three chordotonal organ attachment sites visible through the tympanal membrane.

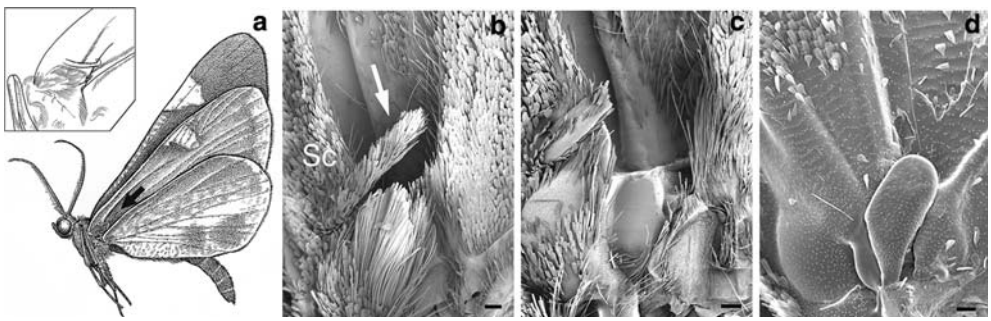

Fig. 2 External features of the tympanal ear in *M. heliconiaria*. **a** *Left lateral view* of the butterfly with an *arrow* pointing toward the tympanal cavity where the tympanal membrane resides. A wing coupling mechanism, portrayed in the inset, joins the fore- and hind-wings. **b** Scanning electron micrograph of the left ventral forewing base, with an *arrow* pointing toward the tympanal cavity. A dense fringe of scales extends from the base of the subcostal (*Sc*) fold to protect the delicate tympanal membrane. Scale bar: 100 μm. **c** The scale fringe has been removed to reveal the tympanal and counter tympanal membranes. The hind wing, and part of the forewing retinaculum have been removed. Scale bar: 100 μm. **d** Dorsal surface of the forewing (anterior is at the left), illustrating the enlarged posterior chamber. Scale bar: 100 μm

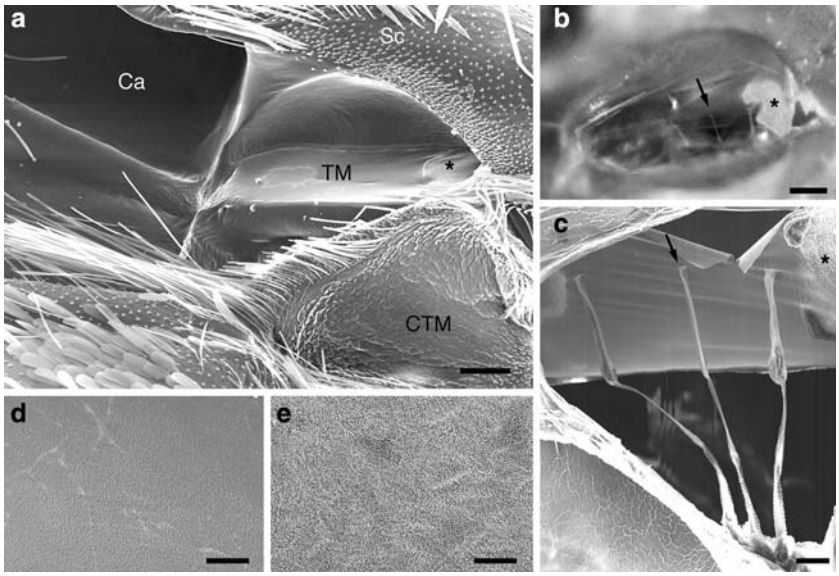

Fig. 3 Micrographs of the tympanal ear of *M. heliconiaria*. **a** Scanning electron micrograph of the tympanal cavity. A canal (*Ca*) formed by a fold of the subcostal vein (*Sc*) leads to the cavity. The dense fringe of scales extending from the bulbous enlargement of the subcostal vein (*Sc*) has been removed to reveal the tympanal membrane (*TM*). An accessory membrane, the counter tympanal membrane (*CTM*) lies perpendicular to the plane of the tympanal membrane. Scale bar: 100 μm. **b** Light micrograph of the tympanal membrane in a similar orientation to that shown in **a.** An *arrow* points to the middle of three chordotonal organs that attach to the inner surface of the transparent tympanal membrane. An *asterisk* marks a thickened proximal region of the tympanal membrane. Scale bar: 100 μm. **c** Scanning electron micrograph of the three chordotonal organs viewed from within the tympanal chamber. The middle chordotonal organ is marked with an *arrow*, and the proximal thickening of the tympanal membrane, with an *asterisk*. Scale bar: 20 μm. **d** Higher magnification of the tympanal membrane surface. Scale bar: 10 μm. **e** Higher magnification of the CTM surface. Scale bar: 10 μm

In males of some species examined in this study (*M. conifera*, *M. satellitiata*, *M. bahiata*, and *M. nigrimaculata*) we noted unusual modifications of the hind wing. In *M. nigrimacula* (Fig. 6a) and *M. bahiata* (Fig. 6b–d), the hind wing appears crinkled or buckled near the base as seen from the dorsal surface, with a knoblike protuberance extending from the ventral surface. In *M. satellitiata*, a transparent oval patch occurs near the wing base (Fig. 6e). These structures have been previously noted by Scoble (1986 in Figs. 10, 16, 44–47, 50) in *M. satellitiata*, *M. leptosiata*, *M. tipulata*, and *M. conifera*. Although we have no evidence that these structures are associated with acoustic communication, we mention them here because they bear resemblance to sound producing structures in some other Lepidoptera (Minet and Surlykke 2003).

Innervation

Janus Green B was used to follow the nerve branches to the tympanal chamber, and histology was used to determine if these nerve branches innervated chordotonal organs. The nerve branches supplying the ear arise from IIN1c of the main mesothoracic wing nerve, IIN1. IIN1c passes around the anterior edge of the mesothoracic dorsoventral flight musculature, and proceeds laterally under the tegular arm, which is continuous with the pleural wing process. Immediately proximal to the tegular arm, a fine nerve branch extends rostrally to innervate the tegula. Distal to the tegular arm the nerve divides into three branches, from anterior to posterior NI, NII, and NIII.

The anterior branch, NI, runs distally up the subcostal wing vein and does not enter the tympanal chamber. The middle and largest of the three branches, NII, innervates the tympanal chamber and the radial wing vein. NII enters the tympanal chamber, where it divides into two branches. One branch innervates three chordotonal organs comprising monodynal, mononematic scolopidia (Fig. 5) that attach to the inner surface of the tympanal membrane. The three chordotonal organs join at their bases to a cuticular ridge of the radial vein at the base of the tympanal chamber (Figs. 3 ,4, 5). We were not able to determine the exact number of scolopidia contained within each chordotonal organ, but our counts ranged between 5 and 15 for each. The distal branch of NII passes through the base of the tympanal chamber and continues up the radial vein. NIII is the smallest and most posterior of the three branches. Proximal to the tympanal chamber NIII splits into two. One branch enters the tympanal chamber where it innervates a chordotonal organ with 12–15 monodynal and mononematic scolopidia. This chordotonal organ appears to lie adjacent to the counter tympanal membrane, but does not attach directly to this membrane. The more distal branch of NIII

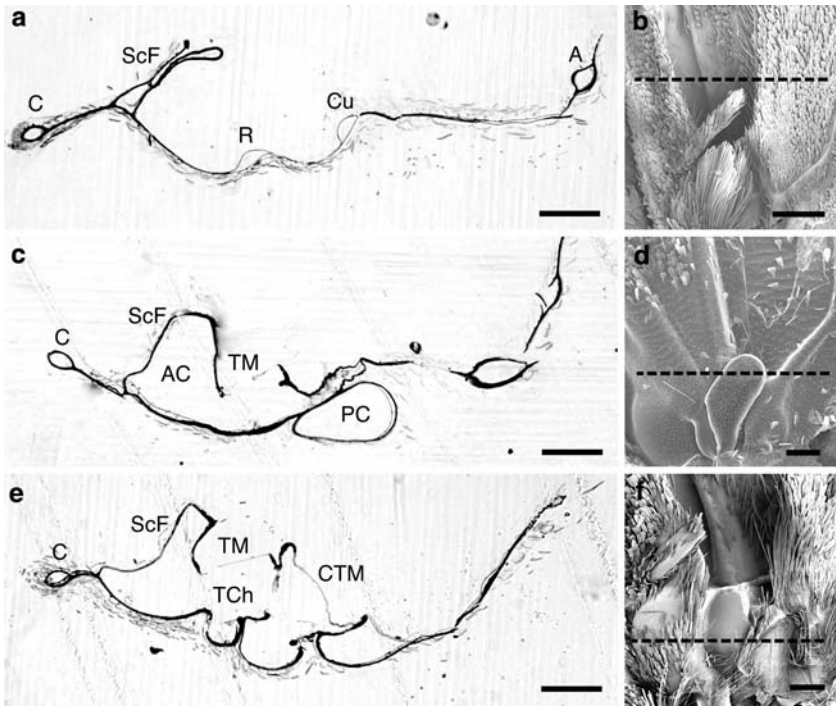

Fig. 4 Histological sections through the forewing base in *M. heliconiaria*. **a**, **c** and **e** are sections through the left forewing, from more distal (**a**) to proximal (**e**), and **b**, **d** and **f** are scanning electron micrographs of the wing base indicating approximate regions where sections were taken. **a** Wing veins distal to the tympanal chamber. The subcostal fold (*ScF*) forms the anterior edge of the canal that leads to the tympanal cavity. Scale bar: 200 μm. **b** Ventral surface of the left forewing. The *dashed line* corresponds to the general region of the forewing sectioned in **a**. Scale bar: 500 μm. **c** The subcostal vein is enlarged to form part of the anterior chamber (*AC*). The distal region of the tympanal membrane (*TM*), which is broken in this section, and the posterior chamber (*PC*) are in view. Scale bar: 200 μm. **d** Dorsal view of the forewing base, indicating the prominent swelling of the posterior chamber. The *dashed line* corresponds to the general region of the forewing sectioned in **c**. Scale bar: 250 μm. **e** The subcostal vein folds over the tympanal membrane to form part of the tympanal cavity. The posterior chamber has now merged with the anterior chamber to form the tympanal chamber (*TCh*). Scale bar: 200 μm. **f** Ventral view of the forewing base with the scales covering the tympanal cavity removed to show the tympanal membrane. The *dashed line* corresponds to the general region of the forewing sectioned in **e**. Scale bar: 200 μm. *A* Anal vein; *AC* Anterior chamber; *C* Costal vein; *CTM* Counter tympanal membrane; *Cu* Cubital vein; *PC* Posterior chamber; *R* Radial vein; *ScF* Subcostal fold; *TCh* Tympanal chamber

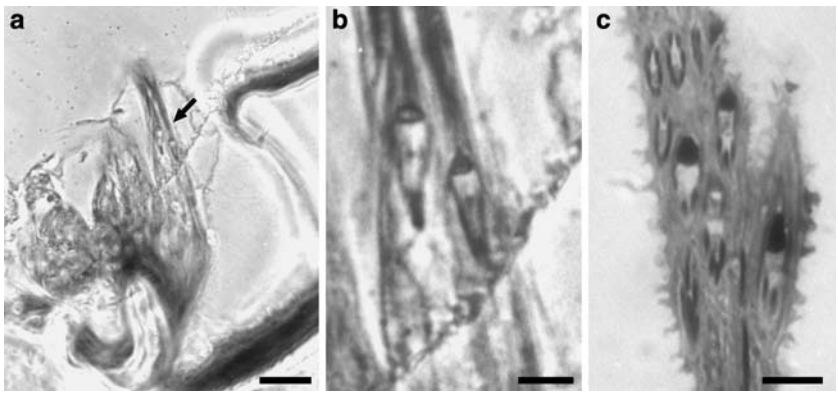

Fig. 5 Histological sections through the tympanal scolopidia. **a** Transverse section through the tympanal chamber, showing the bases of the three chordotonal organs, where they attach to the radial wing vein. The location of the two scolopidia of the middle organ is indicated with an *arrow*, and enlarged in **b**. Scale bar: 15 μm. **b** Longitudinal section through the middle chordotonal organ showing two monodynal, mononematic scolopidia. Scale bar: 5 μm. **c** Longitudinal section through the base of the proximal chordotonal organ, showing evidence of eight scolopidia. Scale bar: 5 μm

continues up the anal vein. Although we could confirm that both NII and NIII innervate chordotonal organs in the tympanal chamber, we were unable, due to the limited number of fixed specimens and the delicate nature of the preparation, to discern the precise location and number of scolopidia in each.

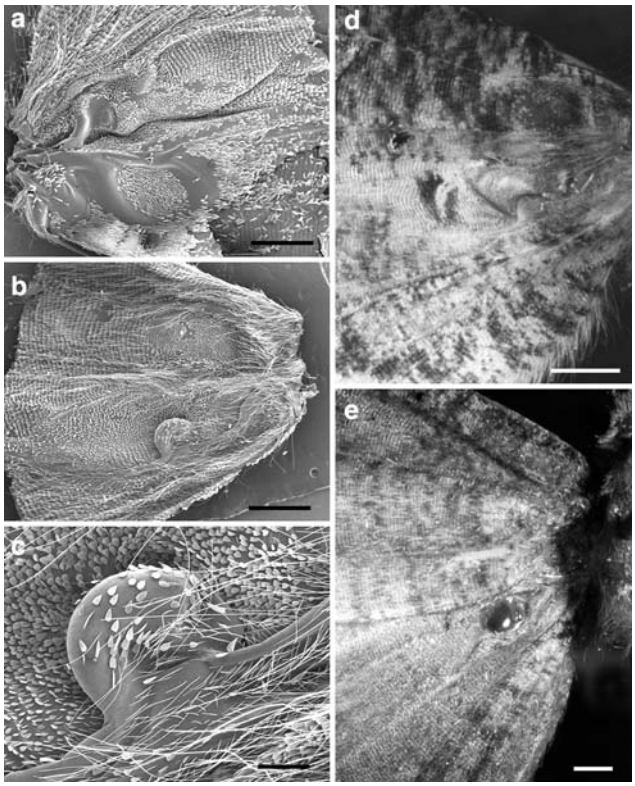

Fig. 6 Specialized hindwing structures in males of certain hedylid species. **a**, **b** *Dorsal* and *ventral views* (scanning electron micrographs) respectively, of the hindwing in *M. nigrimacula*. Scale bars: 1 mm. **c** Detail of knob-like protuberance shown in **b,** scale bar: 200 µm. **d** *Ventral view* (light micrograph) of knob-like protuberance in *M. bahiata*. Scale bar: 1 mm. **e** *Dorsal view* of forewing and hind wing in *M. satellitiata*. Scale bar: 1 mm

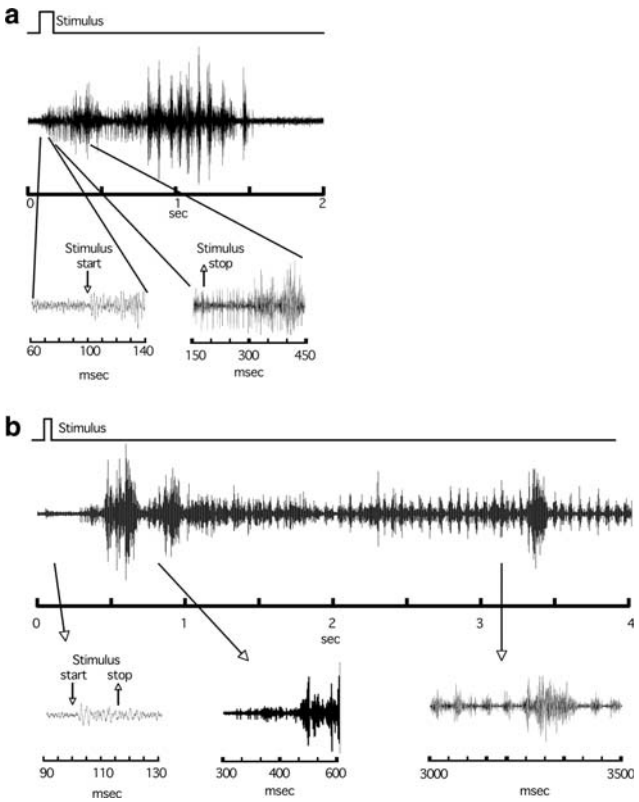

Fig. 7 Extracellular physiological responses of the tympanal nerve, IIN1. **a** Response to an 80 ms 26 kHz ultrasonic stimulus (produced by the PetTrainer™) with a pressure level of around 98 dB SPL at the insect. The sequence of activity is typical: at a short 2.1 ms delay the first sensory cell activity is observed, shown expanded and at a higher amplitude in the lower left part of the figure. Later a train of motor activity (40–80 ms) with higher amplitude begins. Between 0.8 and 1.5 s there is rhythmic (muscle) activity at a rate corresponding to the wing beat frequency. **b** A similar response to a shorter (∼15 ms) stimulus, but showing a more prolonged rhythmic motor activity interspersed with higher amplitude muscle bursts. This demonstrates that flight activity is induced by ultrasound, and suggests that random turns during evasive maneuvers may result from corresponding random bursts of muscle activity

Physiology

Of 26 animals where electrophysiological activity was recorded in response to ultrasound, 3 animals were tested by recording activity in the flight muscles and the remaining 23 individuals were tested by recording directly from the auditory nerve. The animals used for muscle recordings were tethered to a rod so that they could freely move their wings. Behavioural reactions to ultrasound included head rolls, wing and leg twitches, abdominal movements and changes in wing beat patterns. We recorded prominent spikes from the flight muscle from all three preparations in response to sound. The most responsive reacted with prolonged rhythmic activity at approximately 17 Hz.

The remaining 23 animals were examined by recording from the auditory nerve branch, IIN1c. Twenty animals showed neural activity in response to sound stimuli from the PetTrainer™, which elicited a typical sequence of electrical activity (Fig. 7). Thirteen recordings were analyzed off-line. Following the stimulus onset, with a short constant delay of 2.1 ± 0.2 ms ($n = 13$), neural activity was observed. This activity was quite predictable, appearing at the same short delay and with the same spike amplitude every time. It is believed to originate from the auditory receptors due to the short delay and the low relative amplitude, indicative of thin axons in extracellular recordings. The activity following the initial sensory spikes varied much more from trial to trial. At a variable delay ranging from 40 to 140 ms (average 90 ± 60 ms, $n = 13$) post stimulus time, began a train of larger spikes, which we interpret as motor activity. Finally, most preparations exhibited rhythmic activity of high amplitude spikes, with a rate of around 12–20 Hz. Although the insects were pinned down, corresponding wing movements were observed to coincide with this rhythmic activity. This was close to the wing beat frequency of 17 Hz measured from muscle activity of tethered individuals. In Fig. 7a this rhythmic activity begins at ca. 0.8 and ends at 1.5 s, but in many preparations

(e.g. Fig. 7b) the rhythmic activity lasted several seconds. Most preparations showed bouts of high activity at irregular and unpredictable intervals either before or interspersed with the rhythmic activity. This can be seen for example, around 400 ms in Fig. 7a and around 500, 800, and 3,300 ms in Fig. 7b.

Fifteen preparations (including 14 *M. heliconiaria* and 1 *M. conifera*) were responsive enough to the PetTrainer™ to determine audiograms. All audiograms showed broad tuning to frequencies between 20 and 100 kHz. However, the preparations were delicate and only seven were stable enough to fulfill the criteria for inclusion into the database. Best frequencies were between 40 and 80 kHz with a median threshold at 50 kHz of 61 dB SPL (Fig. 8). There was no apparent difference between males and females, but the low number of females makes it difficult to properly assess sexual dimorphism.

After determining the audiograms, the sensitivity to audible frequencies was tested for two individuals. No behavioural or neural response could be elicited for frequencies between 500 Hz and 5 kHz at intensities up to 100 dB SPL, the maximum output of the speaker.

Behaviour

Prior to acoustic stimulation, the flight path of *M. heliconiaria* was straight and unwavering, characteristic of many nocturnal moths (Fig. 9e). Upon exposure to ultrasound flying butterflies responded with an evasive flight maneuver characterized by a sudden change in direction and increased flight speed. Flight paths included steep dives or climbs, upward or downward loops, downward spirals, and horizontal sweeps (Fig. 9a–d). Butterflies were never seen to drop to the ground. Most typically the butterfly recovered its original flight path following stimulation. Of 210 videotaped flight trials 178 (84.8%) exhibited an unequivocal response to sound, 13 (6.2%) showed no detectable response, and 19 (9.0%) could not be assessed because the flight path was already erratic prior to stimulus presentation.

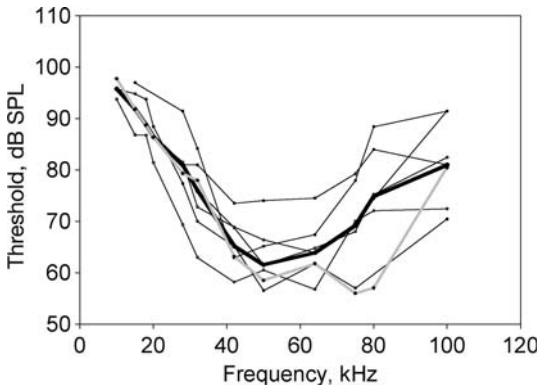

Fig. 8 Auditory threshold curves of 7 *M. heliconaria* (six males and one female). Lowest thresholds are between 40 and 80 kHz with a median threshold (*bold line*) of 61 dB SPL at the best frequency of 50 kHz. Only one female preparation was stable enough to be included in the audiogram (*grey line*)

Pre- and post-stimulus flight speeds, the initial direction of flight upon exposure to sound, and the duration and distance of the evasive response were estimated from video taped trials. Upon stimulation, flight speed increased significantly (paired 2-tailed t test, $P < 0.001$) (Table 1). The initial flight path of the evasive maneuver took a number of directions. More butterflies initially moved downwards than upwards (Chi test $P = 0.038$), and more initially moved forward than backwards, but the latter was not significant (Chi test $P = 0.0630$). The two most common response combinations with respect to initial flight direction were downward and forward (26/60) and upward and backward (14/60). Following the initial change in flight direction, 43% of the butterflies made a loop in the vertical plane and resumed their original flight paths. In the remaining trials the butterflies appeared to rapidly change direction and then continue flying without making an entire loop. Evasive responses lasted on average 0.5 ± 0.2 s, and carried the butterfly an average distance of 0.9 ± 0.2 m before normal flight resumed (Table 1).

Butterflies show a pronounced response to sound only during flight. Resting hedylids responded minimally to ultrasound. Of 80 trials, only 5% responded to the stimulus by taking flight, while the remaining 95% remained stationary. When observed closely, a stimulated butterfly would occasionally flick its wings in response to sound, but it remained in the same location.

The results reported above are for one species, *M. heliconiaria*, since these were most frequently caught at lights at our study site. During our field trials we also tested two males *M. rubidinaria*, two male and one female *M. conifera*, and one male *M. semiermis*, all of which demonstrated similar flight evasive responses.

Discussion

In response to ultrasound, flying hedylids perform a variety of evasive maneuvers that are mediated by a pair of tympanal ears located on the forewings. Our study provides morphological, physiological and behavioural evidence supporting the hypothesis that hedylid hearing functions to detect and avoid aerial insectivorous bats. In the following sections we discuss how the hedylid auditory system compares to other insect tympanal ears, and what implications this research has for understanding the evolution of hearing in butterflies.

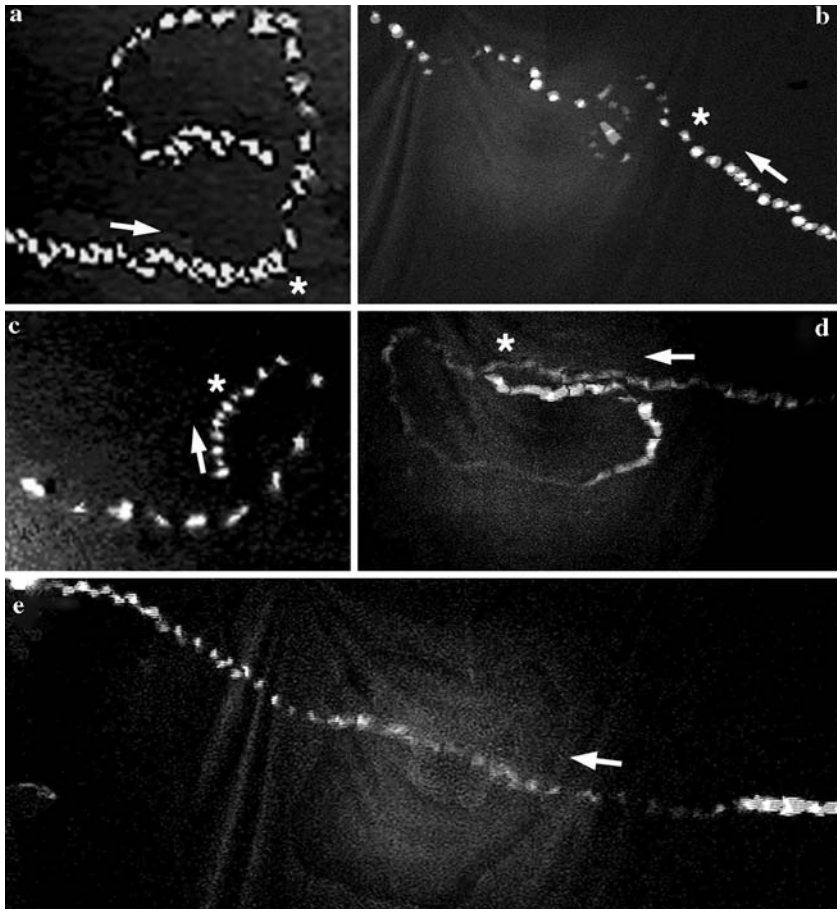

Fig. 9 Consecutive video frames (taken at ~33 ms intervals) of free-flying *M. heliconaria.* **a**–**d** Examples of different evasive flight maneuvers evoked by an ultrasonic stimulus. The direction of flight prior to stimulation is marked with an *arrow*, and the stimulus onset, with an *asterisk*. It can be seen that a response to sound typically occurs within one or two video frames following the onset of the acoustic stimulus. **e** A control flight showing the flight path of an individual not stimulated with ultrasound

Table 1 Flight parameters of evasive flight maneuvers

	Pre-stimulus speed (ms^{-1}) (n = 25)	Post-stimulus speed (ms^{-1}) (n = 25)	Response duration (s) (n = 20)	Response distance (m) (n = 20)
Mean	0.58	2.37	0.57	0.95
SD	0.25	0.68	0.21	0.27
Range	0.28–1.26	.83–3.70	.30–1.30	0.53–1.40

Hedylid ear morphology

Morphologically, hedylid ears are similar to most other insect tympanal ears, which are characterized by a tympanal membrane adjacent to an air filled chamber, and one or more chordotonal organs containing mononematic, monodynal scolopidia that attach either directly or indirectly to the tympanal membrane (Yager 1999; Yack 2004). The location of the hedylid ear, on the wing, is presumably well positioned for mediating evasive flight maneuvers. Insect tympanal ears have evolved in a variety of body locations, due to the fact that it is relatively easy to 'make' an ear from preexisting chordotonal organ proprioceptors (see Hasenfuss 1997; Yager 1999; Yack 2004). Lepidoptera that lack an ear at the base of the forewing have homologous chordotonal organs that are presumed to function as wing proprioceptors (Vogel 1912). Thus, the neural circuitry is already established to communicate directly with wing flight control circuits. Theoretically, the location of hedylid ears could equip the butterfly with the ability to localize sounds in both the vertical and horizontal planes. Placing the ears on the wings could functionally increase the interaural distance to aid in localizing the sound source. In addition, the unique feature of being able to move the ears up and down during flight could provide information about the location of sound in the vertical plane by implementing a sound shadow, when the wings are down, from a bat approaching from above. We surmise however, that hedylid ears are not designed for directional hearing: first, the distance between ears is minor even though they are further apart sitting on the wings than had they been placed on the body, and second, processing signals from 'moving' ears

would require quite complicated neural machinery. We argue that hearing in hedylids functions to detect bats at close range, when the butterfly has little chance of evading a bat by turning and flying away. This hypothesis is also supported by physiological and behavioural evidence presented in this study (see below).

An interesting morphological feature of the hedylid ear is that the chordotonal organs attach to separate regions of the tympanal membrane. This feature is unlike most other bat detectors studied to date, which have a single attachment site: e.g. Noctuoidea, Geometroidea and Pyralidae (Minet and Surlykke 2003), scarab beetles (Forrest et al. 1997), mantids (Yager 1999; Yager and Hoy 1987). In these insects, the single attachment renders the ear 'tone deaf', but multiple scolopidia within the single chordotonal organ extend the range of sound intensities detectable by the insect. In grasshoppers (Acrididae), multiple chordotonal organ attachment sites enable the insect to discriminate frequencies, since different regions of the tympanal membrane to which the receptors attach differ in their resonant properties (Michelsen 1971a, b; Jacobs et al. 1999; Van Staaden et al. 2003). In hedylids, the three chordotonal organs may indeed respond to different frequencies due to the resonant properties of the tympanal membrane. The most proximal chordotonal organ for example, attaches close to a thickening of the tympanal membrane, and therefore may respond to lower frequencies than the other two organs. Even if frequency separation does occur at the periphery however, this does not mean that the insect uses this information directly- it may just be a way of expanding the frequency range of hearing in general. Multiple attachments therefore, may explain the broad tuning curve of the butterfly so that it can detect more bat species. Further studies on the physiological properties of the three chordotonal organs, as well as examining the vibration properties of the tympanal membrane are required to understand the significance of the separate attachments.

Sensitivity to ultrasound

The electrophysiological responses in muscles and sensory nerves unequivocally show that hedylids of the tested species, *M. heliconiaria* and *M. conifera*, have evolved ears that are sensitive to ultrasound. Neurons in IIN1c respond with a sensory response characterized by short latencies to ultrasonic but not low frequency sounds. The audiograms show that hedylids are broadly tuned to frequencies between 40 and 80 kHz. The hearing sensitivity corresponds well to the broad frequency range produced by the guild of insectivorous bats that are likely to feed on hedylids (Schnitzler and Kalko 2001; Siemers et al. 2001; Jung et al. 2007). For instance, small sheath-tailed bats (Emballonuridae), that are common in neotropical lowland forests, produce high intensity, multiharmonic calls, and regularly catch small to medium-sized moths. Other common bats likely to feed on hedylids, and whose calls match the frequency range of their hearing include the Vespertilionidae (e.g. *Myotis nigricans*) and the Molossidae (e.g. *Molossus molossus*).

Best thresholds for hedylid ears were observed to be around 60 dB SPL. Most Noctuoidea moths are more sensitive, with best thresholds of 30–40 dB SPL (e.g. Fullard 1998), but these values refer mostly to large noctuids. There is evidence that thresholds are related to the size of insect, with larger insects in general having lower thresholds, presumably because larger insects are more easily detected by bats, and must detect them from further distances (Forrest et al. 1995; Surlykke et al. 1999). Thus small noctuids with best thresholds of 40–50 dB SPL are not much more sensitive than hedylids. Several other bat detecting insects, including lacewings (Miller 1971), scarab beetles (Forrest et al. 1997), and several mantids (Yager 1999) have best thresholds around 50–60 dB SPL, which are quite similar to the sensitivity of *M. heliconiaria*. High thresholds, together with the apparent absence of negative phonotaxis, provide additional support for our hypothesis that hedylid hearing functions to detect bats at close range rather than at long distances.

In addition to sensory responses, we observed a direct effect of sound on the behaviour of tethered individuals. Similar behavioural responses (changes in wing beat frequency, head rolls, leg extensions and abdominal movements) have been reported for several other insects stimulated with ultrasound in tethered flight: e.g. beetles (Forrest et al. 1995; Yager and Spangler 1997); locusts (Dawson et al. 1997); moths (Skals and Surlykke 2000; Roeder 1962; Göpfert and Wasserthal 1999); mantids (Yager and May 1990), and are presumably involved in mediating the changes in flight speed and direction during evasive maneuvers. In addition to these behavioural responses in tethered hedylids, we recorded rhythmic motor activity interspersed with random bursts of higher amplitude motor activity. These bursts could represent a physiological explanation for such sudden and unpredictable changes in direction seen throughout the evasive maneuvers.

Behaviour

Flying hedylids responded to a loud burst of ultrasound with the classic acoustic startle/escape response used by many other nocturnal insects to avoid bats (reviewed in Hoy et al. 1989; Miller and Surlykke 2001). In hedylids, evasive maneuvers were typically characterized by a sudden and unpredictable change in the flight path and an increase in flight speed. The strategy of responding in an

unpredictable manner, to quote Roeder (1962), "is probably as confusing to the bats as it is to the experimenter, and therefore is of importance to the survival value of the behaviour". Although for the most part the initial flight direction following stimulation in hedylids was unpredictable, we did note a small but significant tendency to move downward. Similar tendencies to dive in response to ultrasound have been noted for other insects: e.g. moths (Roeder 1962), lacewings (Miller and Olesen 1979), scarab beetles (Forrest et al. 1995), mantids (Yager et al. 1990), and one species of nymphalid butterfly (Rydell et al. 2003). The prevalence of dives or downward directed evasive maneuvers may simply reflect the fact that when flight is interrupted, the insect tends to fall.

Of the many evasive responses we have observed in hedylids in the field (while flying toward lights) or during experimental trials, we did not observe an individual dropping directly to the ground. Diving to the ground has been reported to form part of the behavioural 'repertoire' of other insects responding to ultrasound: e.g. lacewings (Miller and Olesen 1979), moths (Roeder 1962), mantids (Yager et al. 1990). One explanation for the absence of this response in hedylids could be that in the tropics, falling to the ground could increase the chances of being captured by terrestrial predators (ants, lizards, spiders and frogs). Another explanation might be that hedylids have very delicate wings, and perhaps falling to the ground would increase the chances of the wings getting wet or damaged. Another feature that appeared to be absent from the behavioural repertoire of hedylids was negative phonotaxis, which has been reported for other insects (reviewed in Miller and Surlykke 2001). Again, given the high thresholds of the ear, we believe that the ear functions at close range, and does not detect bats in sufficient time to warrant a turning response. However, we did not test specifically for negative phonotaxis by systematically varying sound intensities during free flight, and the purported absence of negative phonotaxis in hedylids should be addressed experimentally.

Finally, our observation that a behavioural response is triggered almost exclusively when butterflies are engaged in flight (and consequently, when they are most vulnerable to bats), further substantiates the argument that the ears function primarily for detecting the echolocation cries of aerial hawking bats.

Evolution of hearing in butterflies

Although originally classified as a tribe of Geometridae moths, more recent morphological and molecular evidence places the Hedyloidea taxonomically closer to butterflies (Weller and Pashley 1995; de Jong et al. 1996; Scoble 1996; Wahlberg et al. 2005). The precise relationship among the three butterfly superfamilies however, remains unresolved, and whether or not hedylids are the 'living ancestors' of other butterflies remains to be determined.

An interesting observation we have made, based on comparative morphology of wing venation and nerve branches, is that the hedylid ear appears to be homologous to Vogel's Organ of Nymphalidae (Papilionoidea) butterflies, which at least in some species, has been demonstrated to function as an ear (see below). Like the hedylid ear, Vogel's Organ comprises a tympanal membrane and an air-filled tympanal chamber formed by modifications of Cu, R, and Sc at the base of the forewing. In both groups, the tympanal membrane is formed by Cu. In addition, both ears are innervated by chordotonal organs supplied by the middle (NII) and posterior (NIII) branches of IIN1c. Based on these similarities, we surmise that hedylid and nymphalid ears are homologous.

Vogel's Organ has been shown to be widespread, with varying degrees of development (assessed by examinations of external wing morphology), throughout the Nymphalidae (Otero 1990; Yack, unpublished). Although physiological or behavioural evidence for hearing in nymphalids has only been reported for a few species to date, the function of Vogel's Organ also appears to vary between species. In *Hamadryas feronia* (Eurytelinae), a species that produces audible sounds below 20 kHz, the ear is thought to function in conspecific communication (Yack et al. 2000). In other genera: e.g. *Erebia*, *Pararge*, *Morpho* (Satyrinae), Vogel's Organ is responsive to low frequency sounds and is believed to function in detecting avian predators (Ribaric and Gogala 1996; Yack, unpublished). These functional differences in hearing between Hedylidae and Nymphalidae are reflected in morphological differences between the ears. For example, the hedylid ear is a very thin, transparent membrane well protected within a tympanal cavity. In contrast, the Vogel's Organ in species with demonstrated low frequency hearing, has a thicker, translucent (tympanal) membrane that is less well protected, and more characteristic of low frequency insect ears (see Yack 2004). Interestingly, in one nymphalid species, *Manataria maculata* (Satyrinae), a crepuscular butterfly that exhibits evasive flight maneuvers when exposed the ultrasound, Vogel's Organ is thought to function as a bat detector (Rydell et al. 2003). However, the morphology of this proposed high frequency ear has not yet been investigated. Given that the Nymphalidae and Hedyloidea appear to have homologous hearing organs, we can hypothesize three different scenarios for the evolution of hearing in butterflies.

First, if the Hedyloidea are indeed ancestral to the other two butterfly superfamilies (Fig. 1b), it could be argued that the hedylid ear represents the ancestral condition of Vogel's Organ. When the butterfly ancestor became diurnal, the morphological characteristics and hence functional

attributes of the ear were modified according to the selection pressures of different Nymphalidae species. The problem with this hypothesis is that to date, Vogel's Organ has only been reported for one family of Papilionoidea, the Nymphalidae. Since the Nymphalidae are considered to be a highly derived family of the Papilionoidea (Wahlberg et al. 2005), there would have had to be multiple losses of hearing in the other Papilionoidea, and the Hesperoidea. The second hypothesis is that the Hedyloidea are more closely related to the Nymphalidae, but currently we have no direct evidence for this. A third scenario is that the ears of Hedyloidea and Nymphalidae evolved independently. It could be that this region of the wing is a 'hotspot' for hearing (Yager 1999), and evolved twice in butterflies. Interestingly, an ear has evolved in a similar location (radial vein) in lacewings (Miller 1970, 1971) and possibly, another moth (Thyrididae) (see Minet and Surlykke 2003). A better understanding of the evolution of hearing in butterflies awaits further resolution of the phylogenetic relationships between butterflies, and more research into the structure and function of tympanal hearing in more butterfly species.

Despite their prominent position as the 'missing link' between butterflies and moths, very little is known about the life history and behaviour of Hedyloidea. In this study we have presented morphological, physiological and behavioural evidence that hearing in one species, *M. heliconiaria*, functions for detecting and avoiding bats. We also confirmed physiological and/or behavioural responses to sound in three other species, and that a similar tympanal ear is present in eight other species. Further studies on hedylid hearing should explore the functional significance of the three separate attachment sites, behavioural responses to the natural stimulus intensities of an approaching bat, and the possibility that some species may be using hearing for conspecific communication. We hope that the present study leads to further research on this interesting group of butterflies.

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