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Hearing in hooktip moths (Drepanidae: Lepidoptera)

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Summary

This study presents anatomical and physiological evidence for a sense of hearing in hooktip moths (Drepanoidea). Two example species, *Drepana arcuata* and *Watsonalla uncinula*, were examined. The abdominal ears of drepanids are structurally unique compared to those of other Lepidoptera and other insects, by having an internal tympanal membrane, and auditory sensilla embedded within the membrane. The tympanum is formed by two thin tracheal walls that stretch across a teardrop-shaped opening between dorsal and ventral air chambers in the first abdominal segment. There are four sensory organs (scolopidia) embedded separately between the tympanal membrane layers: two larger lateral scolopidia within the tympanal area, and two smaller scolopidia at the medial margin of the tympanal frame. Sound is thought to reach the tympanal membrane through two external membranes that connect indirectly to the dorsal chamber.

The ear is tuned to ultrasonic frequencies between 30 and 65 kHz, with a best threshold of around 52 dB SPL at 40 kHz, and no apparent difference between genders. Thus, drepanid hearing resembles that of other moths, indicating that the main function is bat detection. Two sensory cells are excited by sound stimuli. Those two cells differ in threshold by approximately 19 dB. The morphology of the ear suggests that the two larger scolopidia function as auditory sensilla; the two smaller scolopidia, located near the tympanal frame, were not excited by sound. We present a biophysical model to explain the possible functional organization of this unique tympanal ear.

Key words: *Drepana arcuata*, *Watsonalla uncinula*, moth, Drepanidae, Lepidoptera, hearing physiology, chordotonal organ, neuroanatomy.

Introduction

Insect tympanal ears come in a great variety of forms. They can occur on virtually any part of an insect's body, and may have as few as one auditory cell, or as many as two thousand. Despite their anatomical diversity, insect tympanal ears typically follow a similar 'morphological plan', having a tympanal membrane exposed to the body's exterior, an enlarged tracheal air chamber associated with the tympanal membrane allowing it to vibrate, and a chordotonal sensory organ. In the majority of ears studied to date, one end of the chordotonal organ attaches directly to the inner surface of the tympanal membrane where, presumably, the scolopidia are stretched longitudinally by sound-induced vibrations of the membrane (e.g. Field and Matheson, 1998). In other cases (e.g. Ensifera: crickets and katydids) the chordotonal organ scolopidia are indirectly associated with the external tympanal membranes, through attachments to tracheal air sacs (for reviews of insect hearing organs, see Hoy and Robert, 1996; Michelsen and Larsen, 1985; Römer and Tautz, 1992; Yager, 1999a).

Tympanal ears have evolved more times in the Lepidoptera (moths and butterflies) than in any other insect order. Ears are located on the thorax (Noctuoidea), abdomen (Pyraloidea, Geometroidea, Drepanoidea, Tineoidea), mouthparts (Sphingoidea) and wings (Hedyloidea, Nymphalidae, Thyridoidea) (for reviews, see Hasenfuss, 2000; Scoble, 1995; Yack et al., 2000). Ears of the nocturnal moths Noctuoidea, Pyraloidea, Sphingoidea and Geometroidea have been investigated physiologically, and shown to be sensitive to ultrasound (Göpfert and Wasserthal, 1999; Roeder, 1974, 1975; Skals and Surlykke, 2000; Surlykke and Filskov, 1997). Moth ears have evolved primarily to detect the ultrasonic echolocation cries of insectivorous bats, but in some cases have become secondarily adapted for conspecific communication (Conner, 1999; Fullard, 1998). Despite their independent origins, the body ears of all moths, except for the Drepanoidea, are structurally similar: they have a very thin (approximately 1 μm) iridescent tympanal membrane covering an air-filled chamber, and a simple chordotonal organ (with one to four scolopidia) that attaches directly, in a perpendicular or slightly oblique orientation, to the membrane's inner surface. The

tympanal membrane's outer surface is located within a protective chamber, which in turn is exposed to the moth's exterior.

The ears of hooktip moths are structurally unique compared to those of other Lepidoptera, and even those of other insects, by having, presumably, an internal tympanal membrane, and scolopidia embedded within this membrane. The superfamily Drepanoidea comprises two families: the Epicopeiidae (approximately 25 described species) that lack ears, and the Drepanidae (the 'hook tip moths') (approximately 650 described species), with ears. The Drepanidae are widely distributed throughout the world except for the New World tropics, and range in size from small to large. There are three subfamilies, the Thyatirinae (=Cymatophorinae), Drepaninae and Cyclidiinae, and one unassigned genus, *Hypsidia* (Minet and Scoble, 1999). General descriptions of the gross morphology and/or the scolopidia innervating the proposed hearing organ have been provided by Gohrbandt (1937), Kennel and Eggers (1933), Minet (1985) and Scoble and Edwards (1988). The proposed tympanal membrane of the drepanid ear is not exposed to the moth's exterior, but rather is located internally, appearing as a partition wall between two air-filled chambers. This characteristic structure is consistent throughout the drepanid family (Minet and Scoble, 1999). Early postmortal Methylene Blue studies described four 'inverted' scolopidia (however, see our results with vital staining) between the tympanal membrane layers (Kennel and Eggers, 1933). Preliminary behavioural observations suggest that drepanids react to high-pitched sounds (Gohrbandt, 1937; Treat, 1962) indicating that they have ears that function as bat detectors. However, a sense of hearing in drepanids has not yet been validated experimentally. In the present study we test the hypothesis that hooktip moths possess ultrasound-sensitive hearing organs, using neuroanatomical and neurophysiological techniques.

Materials and methods

Animals

Drepana arcuata Walker (Fig. 1A) were reared from eggs laid by specimens collected at ultraviolet lights from two locations: Queen's University Biology Station in eastern Ontario, Canada, and Ringwood Preserve (Cornell University Plantations) in central New York, USA, between May and August, 2000 and 2001. Larvae were raised on paper birch *Betula papyrifera*, and pupae were stored in soil and leaf litter at 5–8°C until adults were needed. Pupae were then transferred to an environmental chamber maintained at 26–28°C and a 12 h:12 h light:dark cycle, where adults emerged within a few days. Some moths were shipped as pupae to Denmark and Germany, where additional electrophysiological and anatomical studies were performed. Here they were kept at room temperature until they emerged. Then they were kept cool (6°C) until used (for a maximum of 1 week). 44 moths (21 males and 23 females) were used for the morphological studies and 17 moths (6 males and 11 females) for the physiological studies. 11 specimens (8 males and 3 females) of *Watsonalla uncinula* (Borkhausen) (Drepanidae) were examined for comparative studies of tympanal innervation patterns. Larvae were reared from eggs laid by specimens collected with an ultraviolet light trap in Bolou (France, Pyrenees) by Dr H. Beck at the beginning of June 2001. The larvae were reared on foliage of *Quercus robur*.

Morphology

The peripheral nerve branches and tympanal nerve were identified using Janus Green B (Yack, 1993). The thoracic and abdominal connectives were exposed using a dorsal approach (cf. Roeder, 1966), which involved removing the mesothoracic scutum and scutellum along with the attached flight musculature. Following this dissection, the body was cut in half in a sagittal plane (leaving the ganglia intact on one side) and pinned to Sylgard (Dow Corning Corporation, Midland, MI, USA) in a Petri dish. A few drops of 0.02% Janus Green B were applied to the peripheral nerve branches of the fresh preparation, and replaced after 1–3 min with fresh saline (Paul, 1974). The stained nerve branches were drawn with a Wild M5 (Leica Microsystems Inc., Bannockburn, IL, USA) dissection microscope and drawing tube attachment. This procedure was repeated until the nerve branches of interest were traced to their peripheral targets. Tympanal membranes were imaged with a compound microscope (Leitz Aristoplan with fiber optic illumination; Leica Microsystems Inc.) and a digital image acquisition system [KAPPA Image Base software and DX30 CCD camera (C)]. Tympanal chambers were sputter-coated with gold–palladium and examined with a JSM-6400 scanning electron microscope (JEOL, Peabody, MA, USA). Anatomical nomenclature used for muscles, nerve branches and tympanal structures follows earlier conventions (Gohrbandt, 1937; Hasenfuss, 1997, 2000; Nüesch, 1957).

Staining of tympanal sensilla was executed by injecting a Leuco-methylene Blue solution into the living animal. A freshly prepared Methylene Blue solution in water (3% w/v) was boiled briefly to destroy colloidal complexes and then reduced with sodium formaldehyde sulfoxylate (Rongalit BASF; Mount Olive, NJ, USA). The slightly yellowish stain solution was diluted with an aqueous solution of glucose (10% w/v) to which a 0.2% thionine (w/v) was added, and then injected. After 5–15 min incubation, the specimens were prepared by pinning the parts in position on cork plates and then fixed with a solution of ammonium molybdate in water (10% w/v) overnight. Further details are described in Hasenfuss (1973).

A complete series of cross sections 72 μm thick were made from the abdominal base of a female *D. arcuata* fixed in Carnoy, embedded in celloidine, stained with Mallory's Phosphotungstic Acid–Hematoxylin, and mounted in Canada Balsam.

Electrophysiology and sound stimuli

The physiological response of the sensory cells was studied using conventional extracellular techniques. Recordings were

done in Odense, Denmark and at Cornell University, New York. The moths were dissected using a dorsal approach that exposes the nerve 1N1, which contains the tympanic axons (Fig. 2A). The nerve was hooked onto an extracellular tungsten electrode. A silver reference wire was placed in the abdomen.

In Denmark the action potentials were filtered (0.1–5 kHz band pass and 50 Hz notch filter), amplified (custom-built amplifier) and broadcast through an audio monitor, displayed on an oscilloscope and recorded onto a Sony TCD-D8 Dat tape recorder (Sony Corporation, New York, NY, USA; 48 kHz sampling rate). A Zephiro board (Zephiro Acoustics, USA; company no longer exists) was used to transfer the digital data from the Sony Dat to a computer for analysis. The stimuli were ultrasonic sound pulses, produced by multiplying a trapezoidal gating pulse with the continuous sinusoidal output from a Hewlett Packard 3314A digital function generator (HP, Palo Alto, CA, USA). The resulting pulses were of 10 ms duration and 0.5 ms rise/fall time and were repeated at 1 Hz. The pulses were amplified (Xelex Power amplifier, Stockholm, Sweden) and broadcast from a Technics Tweeter (EAS10TH400B; Secaucus, NJ, USA) 40 cm from the preparation. The system was calibrated using G.R.A.S. 1/4″ microphones (Type 40BF without grid) and a G.R.A.S 12AA amplifier (Vedbaek, Denmark).

At Cornell University the signal from the hook electrode was amplified and band-pass filtered (0.3–5 kHz, 40 dB/decade falloff) using an A & M Systems Model 1800 amplifier (Carlsborg, WA, USA). The amplified signal was sent to a PC for digitization (10–50 kHz). Stimulus waveform generation, data acquisition and data analysis were done using Matlab software (Mathworks, Inc., Natick, MA, USA) in conjunction with a multi-function I/O board (National Instruments PCI-MIO 16E; Austin, TX, USA). Stimulus waveforms were attenuated using a programmable attenuator (Tucker-Davis System II PA4 module; Alachua, FL, USA), and then amplified (Harmon Kardon HK6100; Woodbury, NY, USA), before being sent to a speaker (Technics EAS10TH400B) 30 cm from the animal. Pulses were 5 or 30 ms in duration, with 100 μs rise/fall times. The system was calibrated from 5–100 kHz using a Bruel & Kjaer Type 4135 (Norcross, GA, USA) 1/4″ microphone and accompanying amplifier.

Stimulus intensities were measured at the moth's position in both set-ups, and given in dB SPL relative to 20 μPa (rms).

The threshold was defined as the sound pressure level (dB SPL) sufficient to elicit 1–2 spikes within the first 5–10 ms after stimulus onset in at least 9 of 10 stimulations. For audiograms, thresholds were determined in 5 kHz steps from 10 to 60 kHz and 10 kHz steps from 70 to 100 kHz. The frequencies were presented in random order and the whole sequence was always followed by two controls at the first two presentation frequencies. If they were more than 2 dB from the original values, the data for that moth were discarded.

The dynamic range of the ear was determined by stimulating at one frequency with intensities ranging from ca. 10 dB below threshold to maximum output of the speaker, usually corresponding to around +50 dB above threshold. The response was recorded to 10 stimuli at each intensity. The minimum step of the dB-attenuator was 1 dB. Rasters of spike firing times were calculated from recorded post-stimulus responses. Spikes were detected using an appropriate threshold. Repeated presentations at each amplitude were superposed. Color rasters were prepared to depict post-stimulus response *versus* time and stimulus amplitude, with color representing the response amplitude.

Results

External morphology and 'map' of tympanic nerve

The paired ears are located at the anterior end of the first abdominal segment, near the base of the sternum (Fig. 1B,C). The tympanal membrane of each ear is not exposed to the moth's exterior, but located internally between two cuticular oval chambers: a smaller dorsal chamber (0.50±0.045 mm long, 0.24±0.03 mm wide, N=7, and 0.38±0.07 mm deep, N=3), and larger ventral chamber (0.62±0.11 mm long, 0.40±0.05 mm wide, N=8, and 0.36±0.12 mm deep, N=3) (Fig. 1C). The tympanum (0.20±0 mm wide × 0.30±0.03 mm long, N=6, and approximately 1 μm thick, except in the area where the scolopidia are enclosed, where it is 3–5 μm thick) is a smooth, transparent membrane formed by two layers of tracheal epithelial tissue stretched across a rigid, teardrop-shaped opening between the two chambers (Fig. 1D). In an ear like that of drepanids, with external and internal membranes, it is not obvious which is the tympanal membrane. We have decided to call the internal membrane the 'tympanum', since it resembles the tympanal membranes of other insects, being a thin, taut membrane that is associated with chordotonal organ scolopidia and enlarged tracheal air sacs, and supported by a clearly defined chitinous ring. In addition, earlier authors (Gohrbandt, 1937; Kennel and Eggers, 1933; Scoble, 1995) labeled the internal membrane the 'tympanum' and, in order to reduce confusion, we have chosen to do the same. Thus in the following, when we write 'tympanum' we always mean the internal membrane. The tympanum is not flat, but curves somewhat, with the center of the membrane protruding slightly into the dorsal chamber. A third chamber, the pleural chamber, is formed by a prominent fold that extends from the first abdominal segment, and is continuous with and dorsal to the dorsal chamber. The convex lateral wall of the fold is slightly sclerotized, and the median wall forms the posterior external membrane (approximately 0.90 mm long × 0.60 mm wide, 1 μm thick). The broad anterior side is occupied by the anterior external membrane (approximately 0.60 mm long × 0.40 mm wide, 5 μm thick). The shallow spaces outside the external membranes are continuous with the surrounding air through two slit-like openings (Fig. 1B). The border of the anterior membrane is not clearly defined, as it merges with surrounding soft membranous tissue (Fig. 1C). Both the posterior and anterior external membranes are opaque and without tension. The posterior external membrane is smoother than the anterior external membrane, which has a 'wrinkled' texture, resembling

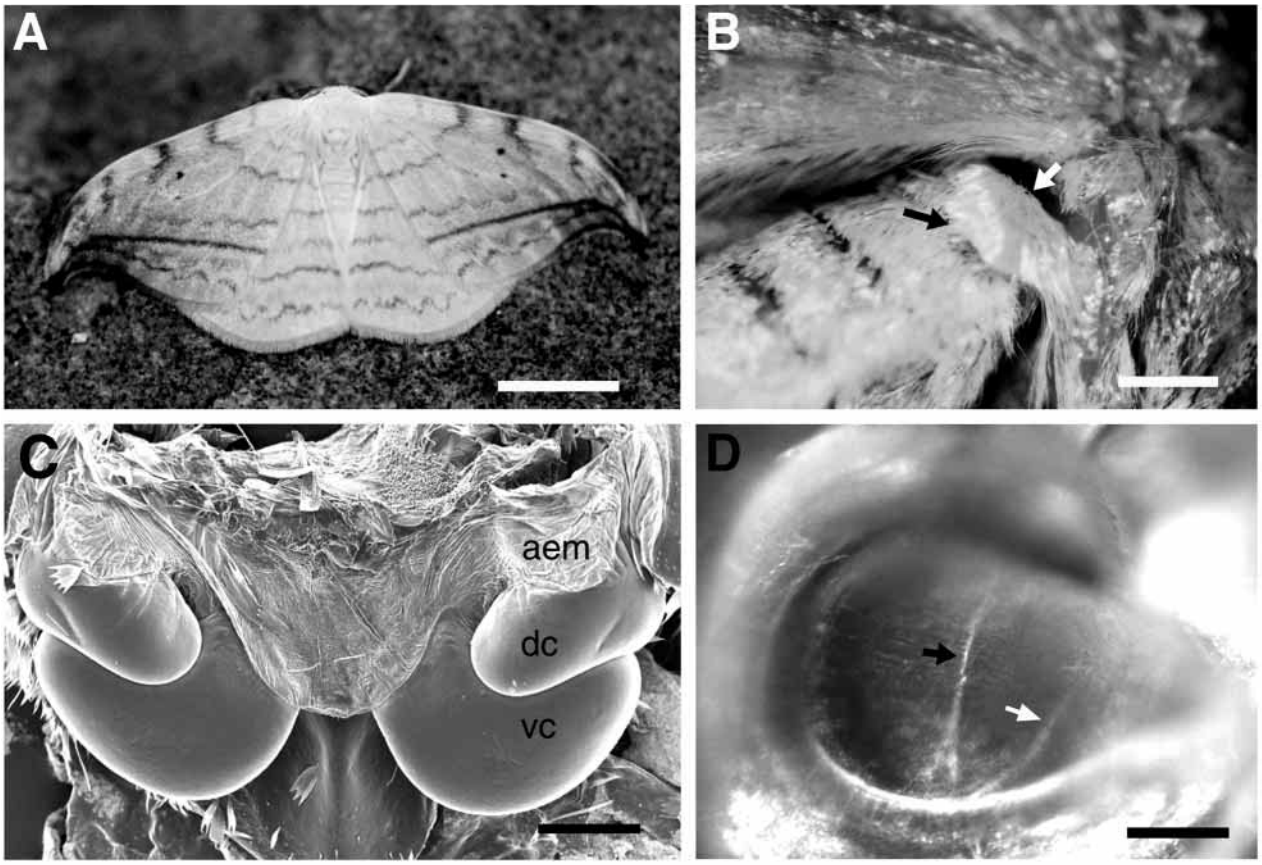

Fig. 1. Tympanal hearing organs in *Drepana arcuata*. (A) A resting female *D. arcuata*. Scale bar, 6 mm. (B) Right lateral view of the thorax and anterior abdomen, showing the general location of the abdominal ear. White arrow, location of the anterior external membrane; black arrow, location of the posterior external membrane. Scale bar, 0.75 mm. (C) Scanning electron micrograph of the anterior and ventral portion of the first abdominal segment in a male *D. arcuata*. Each ear comprises a dorsal (dc) and ventral (vc) air chamber, between which the tympanal membrane is located. Sound is thought to enter the dorsal chamber by means of the anterior external membrane (aem) and posterior external membrane (not shown). Scale bar, 0.25 mm. (D) The tympanal membrane of the left ear as seen following removal of the dorsal chamber. Median is on the right. The four scolopidia are suspended between the two tracheal walls forming the tympanal membrane. The black and white arrows mark the locations of scolopidia 4 and 3, respectively. Scale bar, 0.08 mm.

the counter-tympanal membranes of other lepidopteran ears (Scoble, 1995). There were no apparent differences between males and females with respect to the ear morphology.

The tympanal nerve arises from 1N1 (nerve 1Na of Hasenfuss, 1997), the anterior branch of the first abdominal ganglion (Fig. 2A), which in the Lepidoptera has been incorporated into the pterothoracic ganglion (Nüesch, 1957). In *D. arcuata*, 1N1 is the first nerve branch arising from the thoracic–abdominal connective, 0.62±0.19 mm (N=7) from the posterior end of the pterothoracic ganglion (Fig. 2A). The first branch of 1N1 innervates two ventral muscles, and the second innervates the tympanal cavity and the lateral region of the first abdominal segment. The main branch of 1N1 (containing both afferent and efferent units) continues to the periphery, where it innervates the dorsal and lateral parts of the segment.

The tympanal nerve enters the tympanal cavity medially, and runs along the ventral margin of the tympanal frame, where it terminates in four bipolar sense cells (Figs 2B,C, 3). Each sensory neuron belongs to a scolopidial unit, which includes a bipolar sense cell with one or more perineurium cells at its proximal region, a scolopale cell surrounding the sensory dendrite, and a distal attachment cell (Figs 2B, 3). The scolopidia (numbered 1–4 from medial to lateral, after Gohrbandt, 1937) are separated from one another between the two compressed epithelial layers of the tympanum (Figs 1D, 2B,C, 3). Scolopidia 3 and 4 are the largest and span the midregion of the curved tympanum, while the smaller scolopidia 1 and 2 occur at the median end of the sclerotized tympanal frame. As a measure of how much the tympanum curves we measured the distance, D, directly across the frame, and the distance along the curved membrane, arcMem, to get the radius, R, of the circle that fits the curved membrane at the level of the sensory cells (Fig. 2C,D). At the level of scolopidium 4, D was 0.197±0.031 mm and R was 0.125±0.019 mm on average (N=4). At the level of scolopidium 1, D was 0.114±0.021 mm and R was 0.077±0.019mm (N=4). At scolopidium 3, D was 0.148±0.016 mm (N=5). R was not estimated.

The scolopidia are compressed to a thickness of 2–4 μm between the two tympanal layers. This enabled detection of

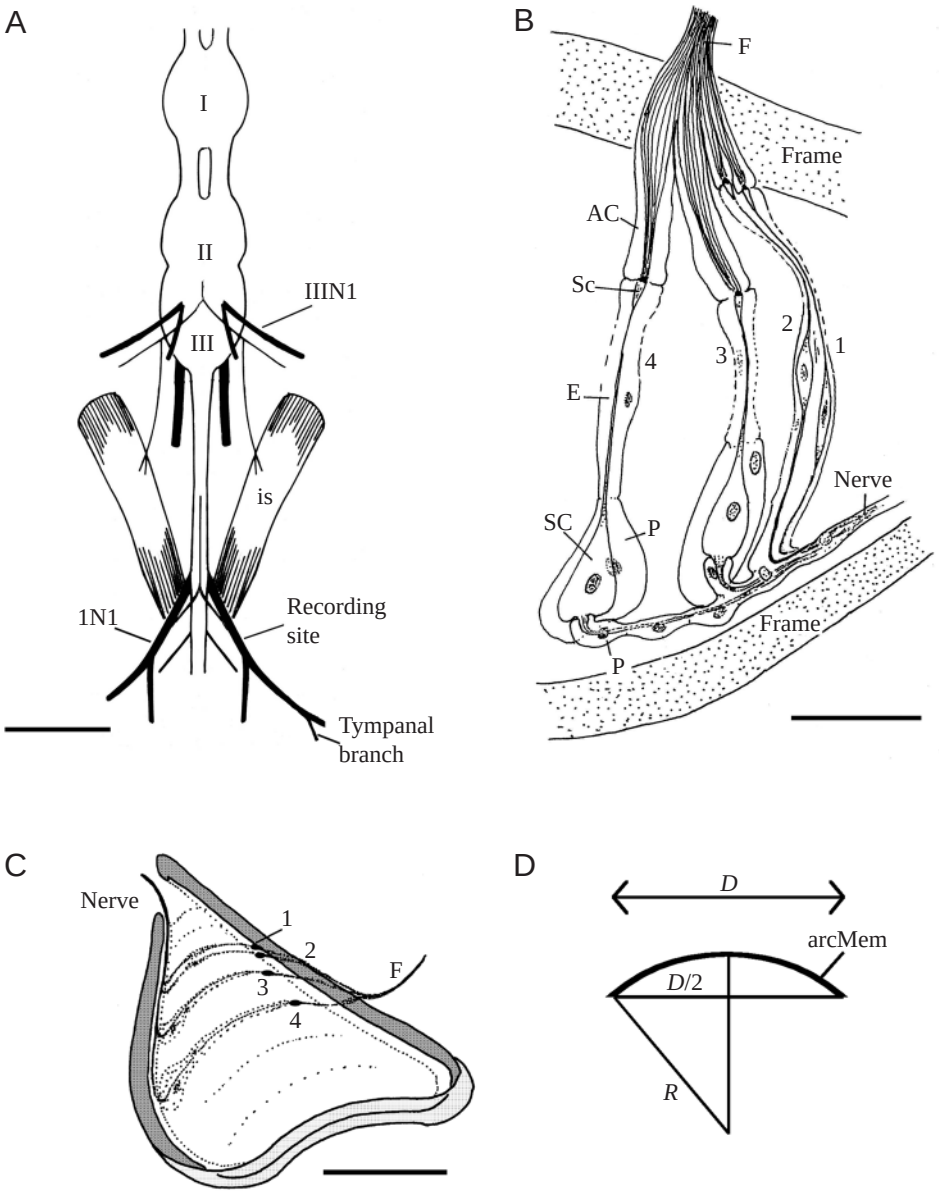

Fig. 2. Innervation of the abdominal tympanal ear in *D. arcuata*. (A) Dorsal view of the thoracic ganglia and the thoracic–abdominal connective. The main nerve roots, identified according to the Nüesch (1957) nomenclature, are illustrated. I, first thoracic ganglion; II, second and III, third thoracic ganglia (= pterothoracic ganglion); the tympanal nerve arises from 1N1 (= anterior branch of first abdominal ganglion), which is the first nerve root from the connective posterior to the pterothoracic ganglion. The second branch of 1N1 innervates the tympanal membrane. is, intersegmental muscle. Scale bar, 0.4 mm. (B) Tympanal scolopidia of a male, viewed from the dorsal chamber. Median is on the right, as in (D). The tympanal nerve departs at the ventral and median edge of the tympanal frame. Scale bar, 0.050 mm. AC, attachment cell; E, enveloping cell (= scolopale cell); F, system of longitudinally oriented fibrils; P, perineurium cell; Sc, 'Scolopale' region, with scolopale rods, cap and dendritic cilium; SC, sensory cell. (C) Diagrammatic representation of the curved tympanic membrane in its frame with scolopidia 1–4 viewed from the dorsal chamber. Median is upwards and to the left. Scolopale regions are represented as dark swellings. Scale bar, 0.1 mm. (D) Schematic illustrating *D*, the distance from frame to frame, arcMem, the distance along the membrane, and *R*, the radius of the circle that follows the membrane.

many structural details without sectioning (Figs 2B, 3). The scolopidia exhibit all the characteristics of both mononematic and monodynal chordotonal organs (Field and Matheson, 1998), having one sensory neuron per scolopidium, and the distal tip of the dendrite inserting into a scolopale cap (Figs 2B, 3). The attachment cells join together distally, pass the tympanal frame, continue within the concave wall of the dorsal chamber, and attach to the integument at a point near the posterior end of the pleural chamber. These cells are densely packed with longitudinally oriented fibrils (probably microtubules) (Figs 2B,C, 3B). Specific attachments to the tympanal frame were not identified. We did not observe differences between the sexes in either the peripheral nerve topography or the innervation of the tympanum.

In *W. uncinula* we observed nerve branching patterns and tympanal receptors similar to those observed in *D. arcuata*. However, in addition to the four bipolar cells, a multipolar unit was observed at the medial and posterior edge of the tympanal frame. Whether this unit is innervated by the tympanal branch, or a branch of the posterior nerve of the first abdominal ganglion (nerve 1Np of Hasenfuss, 1997), is unclear. We did not detect a similar cell in *D. arcuata*.

Audiograms

Full audiograms were determined for 12 *D. arcuata* (8 females and 4 males). All moths were tested between 5 and 100 kHz. The ears were broadly tuned to a frequency range from 30 to 65 kHz (Fig. 4). The mean audiogram showed a best frequency at 40 kHz with a threshold at 52±3.6 dB SPL (N=12). There were no differences between males and females. The audiograms were determined by finding the threshold for the most sensitive auditory cell. We follow the convention of other studies on lepidopteran hearing physiology (e.g. Roeder, 1966, 1974; Surlykke and Filskov,

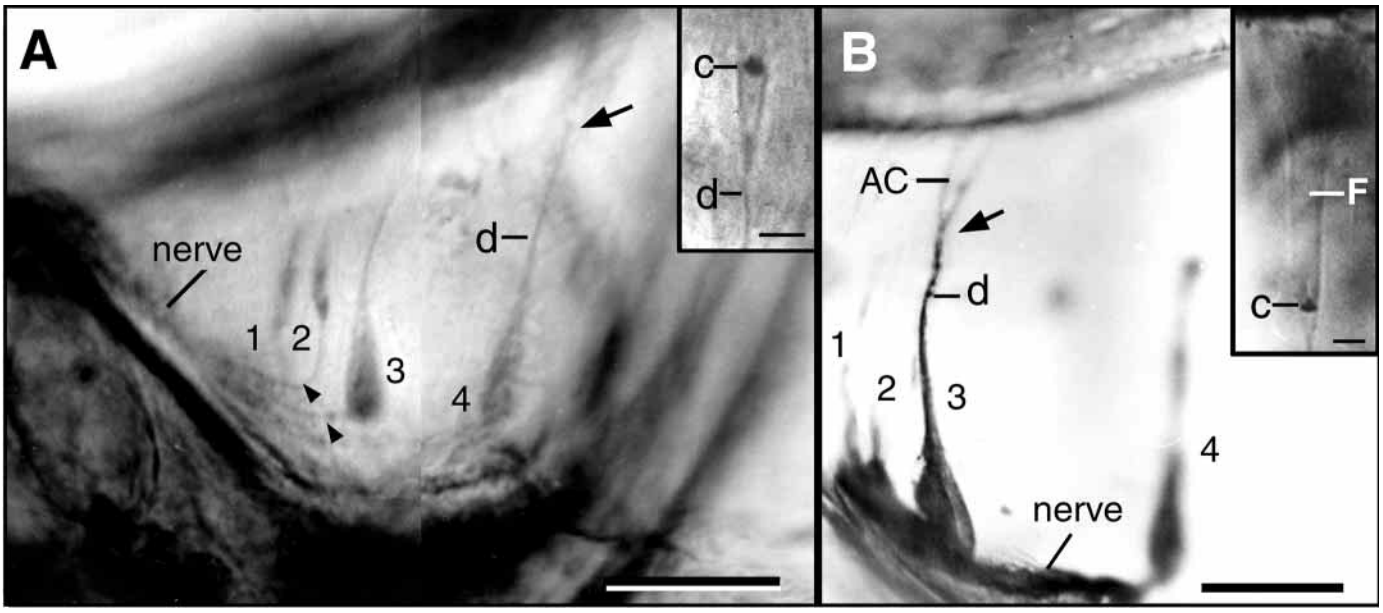

Fig. 3. Methylene Blue preparations of the right side tympanal scolopidia viewed from the dorsal chamber in two species of Drepanidae. Median is on the left. (A) *Drepana arcuata*. Composite image created from two micrographs taken at different focal planes. Sensory cell bodies of scolopidia 1–4 are marked, as well as the axons of cells 2 and 3 (arrowheads), and the location of the dendrite (d) and scolopale cell (arrow) of scolopidium 4. Scale bar, 0.05 mm. Inset: Scolopale rods and cap (c) and distal dendrite (d) of scolopidium 4. Scale bar, 0.005 mm. (B) *Watsonalla uncinula*. Scolopidia 1–4, marking the attachment cell (AC) and the location of the scolopale rods (arrow) of scolopidium 3. Scale bar, 0.05 mm. Inset: Scolopale cap (c) and distal fibrils (F) of the attachment cell in scolopidium 4. Scale bar, 0.01 mm.

1997) and name the sensory cells A-cells, $A_{1–4}$ in order of decreasing sensitivity. Thus, the audiogram depicts the threshold of A_1 (Fig. 4).

Usually spike amplitude was the same for A_1 and A_2 and recruitment of A_2 was indicated by spikes with double height or double peaks (Fig. 5). The preparations were delicate, but in the best the threshold difference between A_1 and A_2 was determined at 2–3 frequencies above and below the best frequency. None of these indicated that the threshold difference changed with frequency. In a single preparation the recorded A_2 spike amplitude was approximately four times that of A_1 (see Fig. 6B), allowing for determination of the whole A_2 threshold curve (Fig. 4, inset). These results indicate that the threshold curve of A_2 is broadly tuned with lowest thresholds in the frequency range 30–60 kHz.

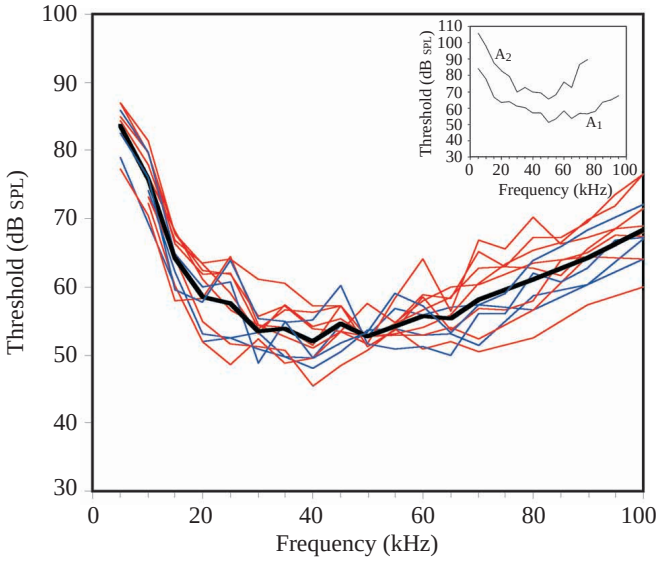

Fig. 4. *D. arcuata* audiograms. Individual audiograms of 8 females (red) and 4 males (blue). Mean audiogram of all is shown by the thick black line. The inset shows threshold curves for both A_1 and A_2 for one female where A_2 spikes were clearly higher than A_1.

Response characteristics of sensory cells and dynamic range

The threshold of the most sensitive cell, A_1, was rather stereotypic from moth to moth. The standard deviation of the threshold at 40 kHz was only 3.7 dB, and the full range of thresholds of all preparations at this frequency, recorded in two different set-ups, was 48–57 dB SPL. However, thresholds of A_2 were more variable, ranging from +12 to +30 dB, mean +19±5 dB (N=14), relative to the threshold of A_1. Activity of other cells, the putative A_3 and A_4, was difficult to detect. In most preparations there was no further increase in spike amplitude or spikes of double height with extra peaks, which would have indicated recruitment of a third cell. In a few preparations, renewed jitter in the rasters at high intensities (90–100 dB SPL) suggested that a third cell was recruited. However, at present we cannot unequivocally say that we had excited A_3 or A_4. A_2 starts saturating around 15 dB above its threshold. Hence, the total dynamic range of the ear is around 30–40 dB (Figs 5, 6).

The dynamic response characteristics of the sensory cells

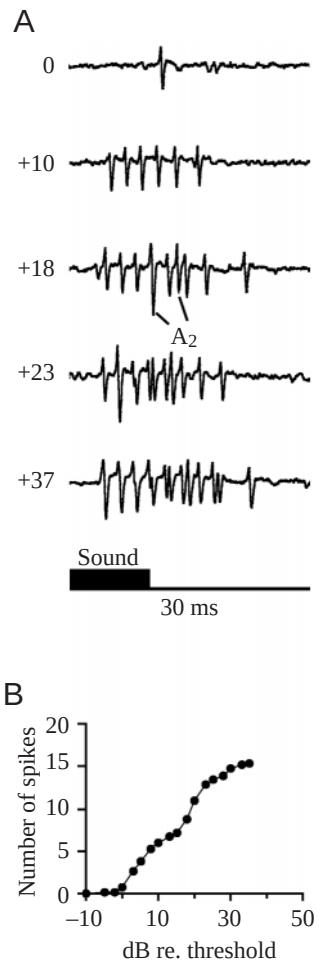

Fig. 5. Spike-traces at different stimulus intensities illustrating response characteristics of the two functional auditory sensory cells A_1 and A_2. (A) The recruitment of A_2 is revealed by spikes with double height or double peaks. In this moth, A_2 threshold was +17 dB re A_1 threshold. Stimulus pulses: 10 ms, 30 kHz. (B) The mean number of spikes per stimulus (10 stimulations) as a function of intensity relative to A_1 threshold (0 dB) from the same moth as in A. The number of A_1 spikes per stimulus increases steeply from threshold to approximately +10 dB, where A_1 starts saturating before the A_2 threshold is exceeded at approximately +15 dB in this preparation. A_2 is saturating at intensities greater than +30 dB above threshold.

were studied by increasing the intensity from 10 dB below threshold to maximum output of the speaker (around +50 dB re threshold) at a constant frequency, which was not the same for all preparations but always within the range of best frequencies (30–65 kHz). In six preparations we examined spike traces (Fig. 5) in 2 or 3 dB steps and in nine other preparations we examined color rasters (Fig. 6) in 1 dB steps. In all cases, the response increased in both amplitude and duration, reflecting the recruitment of more cells and the lengthening of response spike trains of individual cells (e.g. Figs 5, 6). Some characteristics, like A_1 threshold (see above) and response latency, were rather constant throughout the preparations. In all preparations, the latency around threshold was 8–9 ms, decreasing gradually to a minimum of around 2.5 ms at +20 dB re. threshold (e.g. Figs 5, 6). In contrast, substantial variation was seen in A_2 threshold (see above) and in response duration of both A_1 and A_2. The mean response duration determined at 50 dB above threshold from rasters of preparations stimulated with 5 ms pulses was 16 ms (N=8) with a considerable S.D. of 5 ms. Of the eight preparations, four showed response durations lasting no more than approximately 11–13 ms through the entire intensity range tested (e.g. Figs 5, 6A). In one it was 16 ms, and in three the response duration increased greatly to 21–22 ms at intensities above approximately +30 dB relative to the threshold of A_1 (e.g. Fig. 6B). In one preparation there was a large enough difference between A_2 and A_1 spike amplitudes to permit identification of individual spikes, and in this case it was clear that both A_1 and A_2 spikes contributed to the long response duration at high intensities (Fig. 6B).

In a few of the preparations we noted spikes from a cell that was apparently not affected by sound, resembling the activity of a tonically active cell (the so-called B-cell), that is prominent in recordings from noctuoid, geometroid (Roeder, 1974) and pyraloid (Skals and Surlykke, 2000) tympanic nerves. Where this cell was most conspicuous the spike amplitude was comparable to the amplitude of the A-cells. In other preparations we recorded no activity from such a cell.

Discussion

We have identified the nerve branch innervating the proposed abdominal tympanal ear in two species of hooktip moths, and recorded physiological responses to ultrasound from this branch in one species, *D. arcuata*. Thus, our results support the long-standing but untested hypothesis that hooktip moths possess ultrasound-sensitive abdominal tympanal ears. We confirm that drepanid ears are structurally distinct from the tympanal hearing organs of other Lepidoptera and, as far as we know, other insects, in having the auditory scolopidia embedded within an internal (i.e. not directly exposed to the animal's exterior) tympanal membrane. Further, the drepanid ear is different from other lepidopteran ears (with the exception of hedylid butterflies; Yack and Fullard, 2000) in having the scolopidia separated from one another, rather than clustered together. In spite of their unique morphology however, the overall hearing physiology of *D. arcuata* (and presumably other hooktip moths) resembles that of other moths of similar size with respect to frequency range and hearing sensitivity, hence supporting the hypothesis that the ears function as bat detectors.

Morphology

The general morphology of the external ear structures in both *D. arcuata* and *W. uncinula* resembles that reported for other Drepanidae (Gohrbandt, 1937; Kennel and Eggers, 1933; Minet and Scoble, 1999). We noted no variation between the sexes in either *D. arcuata* or *W. uncinula*. This is in accordance

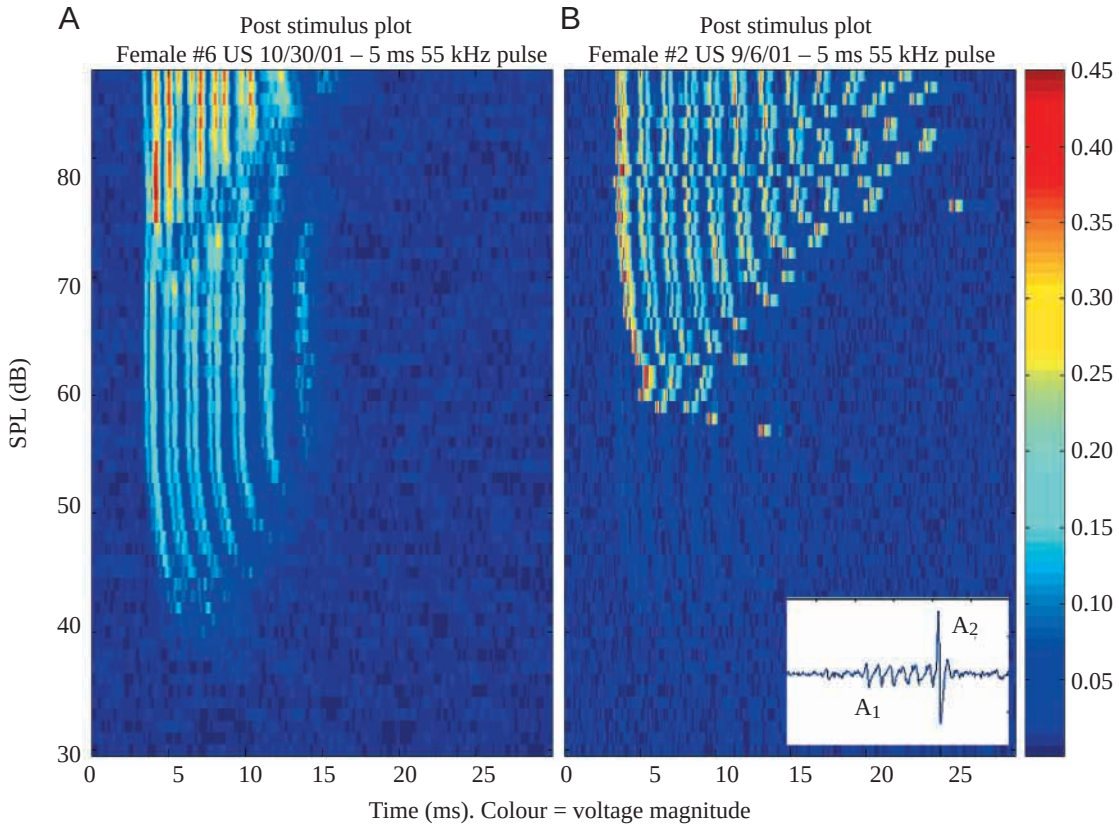

Fig. 6. Color rasters of two moths (A and B) showing dynamics of spike trains as a function of intensity and post-stimulus time. Stimulus intensities were changed in 1 dB steps. In (A) the moth was stimulated 10 times at each intensity level, whereas in (B) only two presentations were possible. Spike amplitudes are represented in color, and the color bar scale is in mV. The moth in B was chosen to illustrate the maximum response duration at high stimulus intensities. In this moth there was a clear difference in spike amplitude between A_1 and A_2 (inset) and both cells contributed to the long response train.

with observations made by Gohrbandt (1937) for several other drepanid species.

Our results show that the tympanal ears of both *D. arcuata* and *W. uncinula* are innervated by the first abdominal nerve branch 1N1, and the chordotonal organ Sc1 (Hasenfuss, 1997). Interestingly, the nervous supply to the ear in drepanids is homologous to that of the abdominal ears of pyraloids and geometroids, despite the fact that all three ears are completely different anatomically, and are believed to have evolved independently (Hasenfuss, 2000). Thus, the chordotonal organ Sc1 has been 'recruited' as an auditory organ several times, supporting the hypothesis that some chordotonal organs are better 'ear-candidates' than others. There may be several reasons why some chordotonal organs in insects are better pre-adapted to become hearing organs than others (see Yack and Roots, 1992; Yager, 1999a). In the Lepidoptera, the first abdominal segment is especially densely packed with tracheal sacs, which are, in combination with a chordotonal organ, a precondition for the evolution of tympanate hearing organs (Hasenfuss, 2000). There are two chordotonal organs in the first abdominal segment, but it is always the lateral organ that became auditory, probably because only this one is surrounded by structures favouring transformation to a hearing organ (Hasenfuss, 1997).

Contrary to previous opinion (Gohrbandt, 1937), the tympanal nerve in drepanids departs from the proximal rather than the distal region of the scolopidia, thus refuting the previously held idea that the scolopidia were of the 'inverted' type found in Geometroidea and Pyraloidea. The attachment cells are densely packed with longitudinally oriented fibrils (probably microtubules) (Figs 2B,C, 3B), and probably these attachment cells were mistaken in the earlier study for the departing nerve.

Sensory physiology

The audiograms show that drepanids are broadly tuned to frequencies between 30 and 65 kHz, with best thresholds around 52 dB SPL. At first, *D. arcuata* may appear to be less sensitive than most Noctuoidea, which have best thresholds of 30–40 dB SPL (e.g. Fullard, 1998). However, thresholds seem to be related to size, with larger moths having lower thresholds (Surlykke et al., 1999), and most noctuoids studied have been larger than the species of Drepanidae studied here. When comparing with extracellular recordings from the tympanal nerves of other 'bat detecting' ears, including those of smaller noctuoids (Surlykke et al., 1999), pyraloids (Skals and Surlykke, 2000), geometroids (Surlykke and Filskov, 1997),

sphingoids (Göpfert and Wasserthal, 1999) and other insects, e.g. some beetles (Yager and Spangler, 1995) and most mantids (Yager, 1999b), the overall sensitivity of *D. arcuata* is similar. Whether the sensitivity of *D. arcuata* reflects that of other Drepanoidea remains to be determined.

There were no apparent differences between audiograms of males and females, reflecting the similar ear morphology of the genders. Hence, our results indicate that ears in drepanids have evolved for the same purpose as in other moths: bat detection.

Based on the ear anatomy, it would appear that sound waves reach the internal tympanal membrane by way of two external membranes (Fig. 1B,C). To test this we performed a preliminary experiment, by covering the two external membranes with vaseline. It was difficult to get both membranes covered entirely and the effect of the vaseline varied. The specimen was inspected after each experiment however, and in the insect where complete coverage was achieved the auditory threshold increased by more than 40 dB. These preliminary occlusion results corroborate earlier proposals (e.g. Hasenfuss, 2000) that sound waves reach the internal (= tympanal) membrane by way of the two external membranes. However, we cannot exclude that covering and thus loading membranes might also have an effect even if sound enters through other putative entrances (e.g. spiracles). Further studies of the vibrations of external membranes, and of the tympanal response with and without removal or covering of the external membranes, are needed to understand the biophysics of this system.

In physiological recordings of other moth ears, a spontaneously firing multipolar cell (the B cell) is evident. In the Noctuoidea this cell is associated with the ear, but its function is unclear, since it does not appear to respond to sound (see Treat and Roeder, 1959; Yack, 1992). Although we also observed a similar unit in recordings of the 1N1 nerve of *D. arcuata*, we do not believe that this activity arose from the tympanal nerve, since we did not observe a multi-terminal cell in our anatomical studies. It is more likely that this activity arises from a multi-terminal cell located in a distal branch of 1N1, which supplies the lateral body wall. Although a multiterminal unit was found in *W. uncinula*, it did not appear to be supplied by the 1N1 branch, but rather the posterior branch (= 1N2).

Relating physiology to morphology, and the role of the curved tympanal membrane

The anatomical results show that there are two larger scolopidia embedded in the tympanum and two smaller scolopidia more closely associated with the margin of the sclerotized tympanal frame. In the morphology section we numbered the scolopidia 1–4 from medial to lateral, after Gohrbandt (1937), i.e. 1–2 for the two small cells and 3–4 for the two large cells. In the physiology section we followed the nomenclature of previous studies (e.g. Roeder, 1966, 1974; Surlykke and Filskov, 1997) and numbered the cells $A_{1–4}$, in order of decreasing sensitivity. The position of the large cells (3 and 4 in morphology) within the tympanic area suggests that they should sense tympanal vibrations much more effectively than the two small cells (1 and 2), indicating that A_1 and A_2 (following Roeder), the two most sensitive cells, correspond to the two large cells: 3 and 4 in morphology.

In the tympanal ears of other Lepidoptera, the scolopidia are clustered together into a single chordotonal organ that attaches directly to the inner surface of a taut, flat tympanic membrane. Displacements of the membrane are believed to be directly translated into elongation and shortening of the scolopidia (Hasenfuss, 2000), particularly in the region of the scolopale cell and dendritic cilium, where sensory transduction is thought to occur (Field and Matheson, 1998). In drepanids, the relationship between the scolopidia and the tympanal membrane is completely different, since the scolopidia are embedded between the two closely compressed layers of the tympanal membrane. If the membrane were flat, vibrations would only result in minor length changes of the embedded scolopidia. Therefore, we suggest that the curvature of the tympanic membrane in Drepanidae is a biomechanical adaptation to sensory transduction, required by the encapsulation of the scolopidia within the membrane. We use Laplace's law modified for a cylindrical membrane (for details, see Hasenfuss, 1999) to predict how pressure changes affect the curvature of the membrane, and thus the length of the embedded scolopidia (Fig. 7). The curvature of the membrane is correlated to the inverse of the radius, R, of the circle that follows the curvature of the membrane. If the membrane is flat, $R=\infty$ and $1/R=0$. If the membrane is a half circle then the distance across the frame, D, is the diameter of that circle, and R is equal to $D/2$ (Fig. 2D). For a given change in pressure, length changes of the scolopidia will increase with increasing curvature of the membrane. The curvature of the drepanid tympanic membrane (mean $D/2R=0.79$ and 0.74 at the levels of scolopidium 4 and 1, respectively) would cause small pressure changes to be translated to substantial length changes of the scolopidia, according to the model (Fig. 7). From the model it also follows that for small pressure variations $\Delta p D/2\gamma = \Delta arcMem/D \Leftrightarrow \Delta arcMem = \Delta p D^2/2\gamma$. Thus, changes in arcMem are proportional to D^2, and therefore length changes are not only proportional to the curvature, but also to the actual diameter of the membrane at the level of the scolopidia. Using our results for D and curvature, the model predicts that approximately twofold more pressure change should be required to give the same length change at the level of scolopidium 3 compared to scolopidium 4. However, the measured threshold difference between A_1 and A_2, was around 19 dB, corresponding approximately to a tenfold pressure difference. Thus, while the model offers a working hypothesis that relates sensory physiology to two of the distinct morphological characteristics of the drepanid ear, the encapsulation of the sensory cells and the curvature of the tympanum, there are obviously other factors involved in addition to dimensions and curvature. These may include differences in tension across the membrane, or resonance and

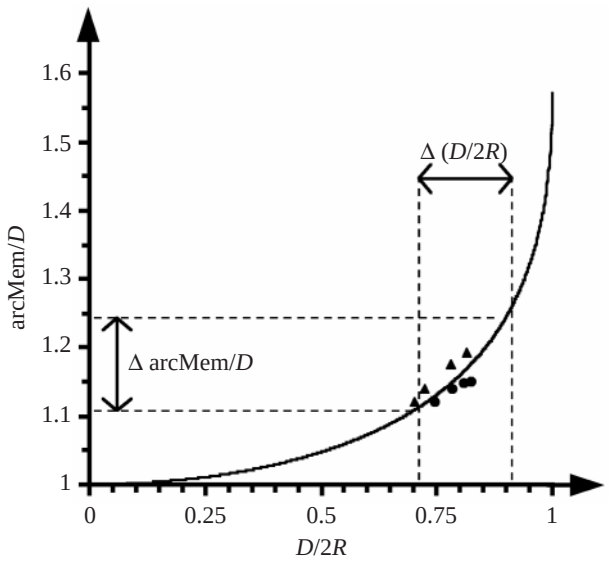

Fig. 7. The relation between pressure changes ($D/2R$) and the curvature of a cylindrical membrane (arcMem/D). Pressure changes can be expressed as $D/2R$ according to Laplace's law modified for a cylindrical membrane: $D/2R=(P–P_0)\times(D/2\gamma)$, where γ is the tension of the membrane. The curvature of the membrane is related to pressure changes by the inverse sine function: arcMem/D=($2R/D$)×arcsine($D/2R$), and varies between 1 (a flat membrane) and $\pi/2$ (a half circle). For constant D the curvature of the membrane will change with arcMem, and the same change in pressure will induce larger arcMem changes with increasing curvature of the membrane. Curvature of the drepanid tympanic membrane is indicated at the level of scolopidium 4 (circles) and at the level of scolopidium 1 (diamonds). Changes in arcMem of the tympanic membrane translates to length changes of the embedded sensory cells.

other effects of the air volume above and below the membrane. Confirmation of the physiological characteristics of individual units in the ear must await further studies involving intracellular recording and staining techniques.

Evolution of moth ears

Moth ears exemplify that even the simplest of insect ears provide sufficient adaptational value as bat detectors. They have one (Notodontidae, some Sphingidae) two [Noctuoidea (other than Notodontidae), Uraniidae], or four (Geometroidea, Pyraloidea, Drepanidae) scolopidia (for reviews, see Miller and Surlykke, 2001; Scoble, 1995; Yager, 1999a). In those with two or more sensory cells, one cell (A_1) is always the most sensitive, and the others are decreasingly sensitive in steps of approximately 10–20 dB. Since all moth ears studied to date are incapable of frequency discrimination, the function of having multiple cells is unknown. Although it has been speculated that in the Noctuoidea, A_2 triggers the switch from a negative phonotaxis behaviour to an evasive flight maneuver, there is no direct evidence for this, and it is possible that A_1 alone is responsible for triggering bat avoidance behaviours (e.g. Miller and Surlykke, 2001). This argument is supported by the fact that notodontids, with only one auditory cell, appear to exhibit the same bimodal evasive behaviours as moths with 2–4 cells (Surlykke, 1984).

Since in drepanid ears only two of the four sensory cells appear to respond to sound at all, this may support the hypothesis that, in ears that function primarily as 'bat detectors', there may be a cost, either functionally or metabolically, to having extra sensory cells. Comparative anatomical and developmental studies indicate that in ears that function as 'bat detectors', there is a trend from having a higher number of cells in the primitively atympanate state, to a lower number of cells in the tympanate state. In contrast, insect ears that function primarily to identify and localize calling songs of other insects are generally more complicated, with an increasing number of cells from the atympanate to tympanate condition (for discussion, see Yack et al., 1999).

In the Drepanidae we recorded acoustic activity from two cells (A_1 and A_2), and, based on morphology, we assume that these represent scolopidia 3 and 4. What then might be the significance, if any, of scolopidia 1 and 2? We propose that they may have retained their functional role as proprioceptors. In drepanids the four scolopidia are separated spatially within the tympanum, which may have allowed scolopidia 1 and 2, close to the frame, to maintain their original function. In accordance with this hypothesis are the facts that: (i) morphologically 1 and 2 do not appear degenerate, and (ii) the very long and conspicuous attachment cells extend far outside the tympanum and are attached to the integument at a point postero-ventrally to the spiracle, not far away from the attachment site of the homologous organs atympanate moths (Hasenfuss, 1997). The presumed sensitive neuronal zone (i.e. the dendritic cilium) of scolopidia 1 and 2 are so near the margin of the frame that it seems possible that these neurones are stimulated by pulling movements of the attachment cell fibrils, rather than by movements of the tympanum. In contrast, the sensitive zone of scolopidia 3 and 4 are within the tympanal area and are thus possibly not affected by pulls of these fibrils, but rather by vibrations of the tympanum. Physiological recordings from individual units are required to answer these questions.

The Drepanidae provide yet another unique solution to the way insects have come up with an ear. Our study supports the hypothesis that drepanid ears, like those of other nocturnal Lepidoptera, function primarily to detect bats, since the ears are sensitive to ultrasound, and are equally well developed in both sexes. Further, our results suggest that the most important physiological characteristic of all moth ears is the threshold of the most sensitive sensory cell, A_1, which is remarkably similar physiologically in all ears, despite their unique morphologies and independent origins. This appears to be where the selection pressure exerts its effect, while other characteristics such as external morphology, details of physiology, and threshold of less sensitive sensory cells, are subject to less selection pressure. The fact that Drepanidae have functional ears with internal tympanal membranes also opens the door to finding ears in other insects that are not so obvious from their external anatomy.

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