



Research

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Physiology

Extremely high frequency sensitivity in a 'simple' ear

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An evolutionary war is being played out between the bat, which uses ultrasonic calls to locate insect prey, and the moth, which uses microscale ears to listen for the approaching bat. While the highest known frequency of bat echolocation calls is 212 kHz, the upper limit of moth hearing is considered much lower. Here, we show that the greater wax moth, *Galleria mellonella*, is capable of hearing ultrasonic frequencies approaching 300 kHz; the highest frequency sensitivity of any animal. With auditory frequency sensitivity that is unprecedented in the animal kingdom, the greater wax moth is ready and armed for any echolocation call adaptations made by the bat in the on-going bat–moth evolutionary war.

1. Introduction

Many species of moth have evolved ultrasound-sensitive ears owing to the predation pressure of echolocating bats—this system is one of the best known examples of an evolutionary 'arms-race' between predator and prey [1–3]. As both bat and moth respond to adaptations in each other, a wide variety of mechanisms have evolved in both animals. In an attempt to avoid the detectability of their ultrasonic signals, some bats are known to emit very high ultrasonic echolocation calls—some species have frequency content up to 212 kHz [4]. In the moth, some species have evolved unique mechanical tuning mechanisms to adjust frequency sensitivity and follow the spectral changes in bat calls during a hunt [5]. However, there is currently no known insect that is capable of detecting the highest frequencies used by bats. The highest known hearing in an insect, to the best of our knowledge, is another species of moth, the North American gypsy moth, *Lymantria dispar*, which can hear frequencies up to 150 kHz [6].

The use of very high frequencies by certain bats led us to ask the question: are any moths keeping up in the evolutionary arms-race? We chose to investigate the hearing of the greater wax moth (*Galleria mellonella*, figure 1*a,b*), which as a worldwide apicultural pest may come into contact with a wide variety of bat calls. Audition in greater wax moths is used for bat detection and also for intra-specific communication, where the males of the species produce trains of ultrasonic pulses for courtship [7]. The tympanal ears of moths are simple auditory mechanoreceptors that have only one to four receptor cells within the hearing organ [8]; the greater wax moth has four auditory receptor cells [9]. We investigated the upper limit of *G. mellonella* hearing using two different experimental methods: laser Doppler vibrometry to record the tympanal membrane mechanics, and electrophysiology to record the neural response of the auditory nerve. Both experiments were done 'separately or simultaneously' to record the mechanical response of the membrane and the neural response of the ear.

2. Material and methods

(a) Animals

Larval greater wax moths were obtained from Livefood UK, and kept in an incubator at 25°C on a 12 h photoperiod before pupating and emerging. Before

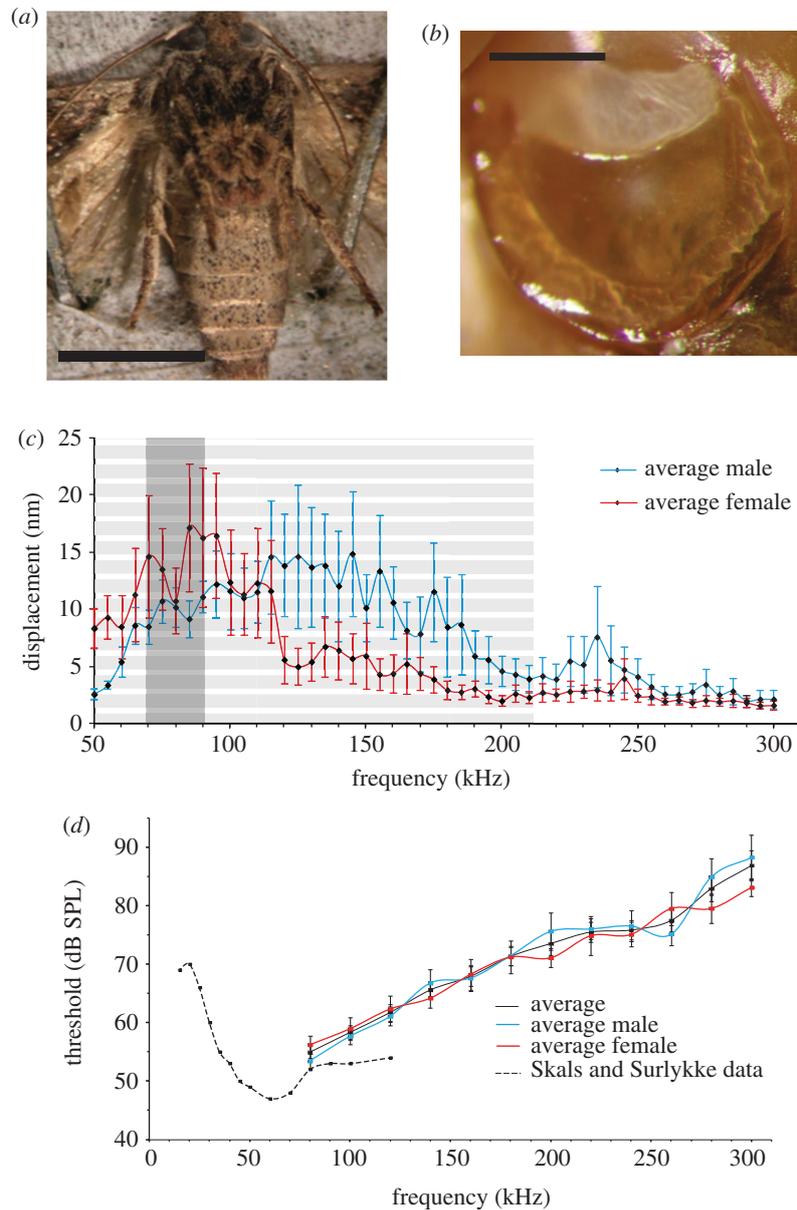


Figure 1. The auditory response of the greater wax moth. (a) Adult greater wax moth, scale bar is 5 mm. (b) Tympanal membrane, scale bar is 0.1 mm. (c) The average membrane displacement recorded using laser Doppler vibrometry over frequencies 50–300 kHz at 90 dB SPL. The solid grey area represents the courtship call frequency range, and the dashed grey area is the frequencies of bat echolocation calls. (d) Average neural threshold curves over frequencies 10–300 kHz, previous neural results from Skals & Surlykke [9] are also shown for comparison, with permission.

experimentation, adult moths were placed in a refrigerator to immobilize them. They were then sexed and pinned to a Plastine block using staples. Sound stimuli were produced using a custom-built air-coupled transducer (see the electronic supplementary material). The sound level was altered using an attenuator (JFW Industries, 50BR-009) which allowed the sound to be increased or decreased in steps of 5 dB SPL (sound pressure level, re $20 \mu\text{Pa}$). All frequencies and sound levels of auditory stimuli were randomized.

(b) Laser vibrometry

The vibration displacements of the tympanal membrane were recorded using a scanning laser Doppler vibrometer (Polytec PSV-300-F), using a close-up attachment on a OFV-056 scanning head. Animals were placed onto a metal holder on top of a tripod, and the mesothoracic segment was held back to expose the tympanal membranes. The right tympanal membrane was used for all moths tested as it was positioned nearer to the sound source owing to the layout of the equipment. The SPL

was measured using a calibrated microphone coupled to a pre-amplifier (Bruel and Kjael, Microphone: 4138, Pre-amplifier: Nexus 2690). The microphone secured in the holder could be adjusted so that it was as close to the tympanal organ as possible. Single-frequency sine waves were created using a function generator (Tektronix, Dual Channel AFG 3102), which were passed to the transducer, powered by a high voltage power supply (Brandenburg, 475R). SPL was altered in steps of 10 dB SPL using an attenuator (JFW Industries, 50BR-009). The displacement of the membrane surface was recorded, and using the PSV software, an animation of the membrane vibration could be created.

(c) Electrophysiology

The moths were dissected to expose the auditory nerves which run parallel to the abdominal connective of the metathoracic ganglion [9]. One of the auditory nerves was hooked with a tungsten wire electrode, and a reference electrode was placed in the abdomen. The ultrasound transducer was set in place directly above the moth. Sound pulse signals were produced by a

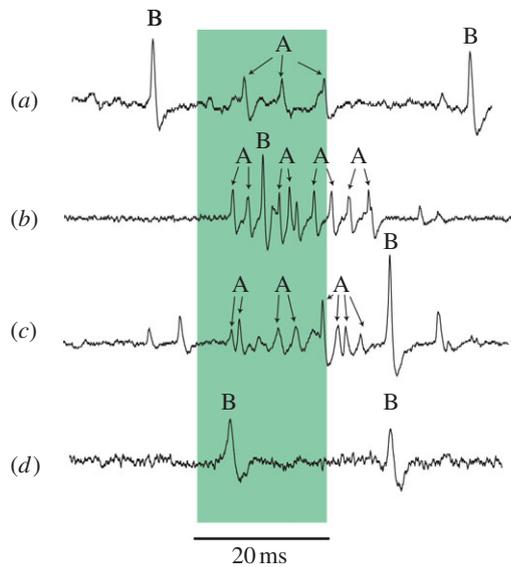


Figure 2. Examples of electrophysiological recordings from an individual female moth, the 20 ms sound pulse stimuli are highlighted in green. The four auditory receptor A cells have different sensitivity levels with A1 being the most sensitive and appearing first at the lowest sound levels. (a) Nerve response at 80 kHz at threshold level 60 dB SPL. (b) Nerve recording at 80 kHz and 90 dB SPL, showing all A cells firing. (c) Nerve response at 300 kHz and 90 dB SPL with spikes present. (d) Nerve response from another individual female moth at 280 kHz and 90 dB SPL, where no relevant spikes are found during sound stimulus. The A cell and non-acoustic B cell responses are labelled.

function generator (TTI Instruments, TGA12102), with each sound pulse lasting 20 ms; all sounds used were a continuous single sine wave; frequencies ranged from 50 to 300 kHz. Auditory nerve action potentials were measured via the electrodes and passed through an amplifier (WPI, DAM 50), and then to an oscilloscope (Tektronix, DPO 1014), the oscilloscope recording was transferred to a LABVIEW program (National Instruments, 8.6.1) which saved the data as a text file from the oscilloscope, raw data have been deposited in Dryad [10]. Using another custom-built LABVIEW program, the data were analysed after the electrophysiology data were filtered to remove noise. The threshold of the A1 cell was then calculated from the recordings using criteria from previous electrophysiological studies of greater wax moths [9], i.e. two or more auditory spikes are present in eight out of 10 sound stimuli, to overcome potential false positives owing to the auditory cell's spontaneous firing coinciding with the sound stimulus; see figure 2 for examples of neural traces. Only the neural threshold of the A1 receptor cell was analysed. Experiments recording simultaneously the mechanical and neural responses at high frequencies were also carried out; see the electronic supplementary material.

3. Results

Using a micro-scanning laser Doppler vibrometer, a highly sensitive (10 pm amplitude resolution) non-contact optical technique, we found that the membrane moved maximally at the point of the receptor cell's attachment [11], this was noted for each frequency tested, 50–300 kHz in steps of 5 kHz for each moth tested, $n = 20$. *Galleria mellonella* membranes had oscillation amplitudes over 1 nm at all frequencies tested (50–300 kHz), at a sound level of 90 dB SPL (figure 1c). As the auditory sensory cells are mechanoreceptors, it is thought that a minimum displacement of the membrane is required to

activate a neural response; previous studies in noctuid moths have shown that the minimum displacement required to generate a neural response is approximately 100 pm [12]. The largest membrane displacement took place at 90–95 kHz (16.4 ± 3.3 nm; mean \pm s.e., $n = 8$, 90 dB SPL), occurring in the females of the individuals tested. This tuning matches the male moth's calling frequency ([7]; figure 1c), suggesting that the female moth hearing is mechanically most sensitive to the intra-specific communication sounds. The greatest mechanical sensitivity in males occurred at a higher frequency, 125 kHz (14.9 ± 5.5 nm, $n = 8$, 90 dB SPL), although this difference in the displacement curves was not statistically significant (one-way ANOVA, $p > 0.1$, $n = 8$ for each sex).

Neural auditory-threshold responses were calculated for 20 individuals, 10 of each sex (figure 1d). The greatest neural sensitivity of our measurements was at 80 kHz (54.9 ± 1.0 dB SPL; mean \pm s.e., $n = 20$), matching earlier studies [9]. The results of our electrophysiological experiments demonstrate that the majority of moths tested would produce a neural response for stimuli up to 300 kHz. However, not all moths were found to react to tones of 280 and 300 kHz: at 280 kHz, two out of 20 individuals did not respond at 90 dB SPL (figure 2d), and in five out of 20 individuals tested, 300 kHz sound pulses at 90 dB SPL did not elicit nerve spikes. The auditory-threshold level for the moths that did respond to these high frequencies matched the trend: at 280 kHz the auditory threshold was (82.9 ± 2.2 dB SPL; mean \pm s.e., $n = 18$), and at 300 kHz, it was (86.8 ± 2.6 dB SPL, $n = 15$). It is conceivable that at higher SPLs not examined in this study, neural responses would be elicited at these frequencies by moths that did not hear at 90 dB SPL.

4. Discussion

Auditory sensitivity up to 300 kHz is unprecedented. Physical constraints on sound propagation suggest that above 200 kHz, attenuation (on the order of 10 dB per metre per atmosphere) renders such signals suboptimal for bat echolocation [13]. Furthermore, there is no known bat capable of producing such high frequency calls or indeed listening to them. Therefore, the reasons for the exceptional frequency sensitivity in this moth are unclear. We suggest two possible explanations. It is possible that the range of spectral content in bat calls is underestimated—the extreme acoustic beam directionality at these frequencies and technical difficulties in detecting them could mean that this spectral content may have been missed. It is known that multiple harmonics of bat chirps exist—for the moth, enhanced sensitivity to these harmonics could be beneficial by increasing the energy capture from the signal, thus improving the moth's ability to detect the bat.

The second, and in our opinion more probable, conjecture is that this frequency sensitivity is a by-product of a need for greater mechanical temporal acuity [11]. The response time of a mechanical oscillator to a force is inversely proportional to the bandwidth—so this preternatural mechanical sensitivity provides the moth with a faster temporal response than a sharply tuned tympanum, which would aid the moth in both predator avoidance and possibly courtship. We calculated the temporal acuity from the average displacement curves measured with the laser vibrometer (figure 1c). The normalized data were fitted to a Lorentzian model to calculate the effective

mechanical resonant frequency (ω_0) and dissipation (γ) of the greater wax moth ear. From this the quality factors (ω_0/γ) were calculated as 1.35 ± 0.13 and 0.94 ± 0.13 (mean \pm s.e.) for males and females, respectively ($n = 8$). The quality factor can be interpreted as the number of cycles needed to reach maximal displacement (or the time taken to dissipate 99% of its energy), and so the temporal acuity can be calculated as 10.21 ± 0.95 and $8.96 \pm 1.19 \mu\text{s}$ (mean \pm s.e.) for males and females, respectively. These values are faster than the values of temporal acuity previously recorded in other moths such as the lesser wax moth between (20–50 μs [11]) and noctuid moths (60 μs [14]).

In the future, behavioural studies would aid the understanding of the purpose of this high frequency hearing and

establish if the moths respond to these very high frequencies. In any case, the greater wax moth's auditory capability mean that, in the context of the on-going bat–moth evolutionary war, should the calls of bats adapt to even higher frequencies, the wax moth is pre-armed. Such extreme auditory frequency sensitivity is unmatched in the animal kingdom.

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