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SHORT COMMUNICATION

Hearing on the fly: the effects of wing position on noctuid moth hearing

Shira D. Gordon[1,*,‡], Elizabeth Klenschi[2] and James F. C. Windmill[2]

ABSTRACT

The ear of the noctuid moth has only two auditory neurons, A1 and A2, which function in detecting predatory bats. However, the noctuid's ears are located on the thorax behind the wings. Therefore, as these moths need to hear during flight, it was hypothesized that wing position may affect their hearing. The wing was fixed in three different positions: up, flat and down. An additional subset of animals was measured with freely moving wings. In order to negate any possible acoustic shadowing or diffractive effects, all wings were snipped, leaving the proximal-most portion and the wing hinge intact. Results revealed that wing position plays a factor in threshold sensitivity of the less sensitive auditory neuron A2, but not in the more sensitive neuron A1. Furthermore, when the wing was set in the down position, fewer A1 action potentials were generated prior to the initiation of A2 activity. Analyzing the motion of the tympanal membrane did not reveal differences in movement due to wing position. Therefore, these neural differences arising from wing position are proposed to be due to other factors within the animal such as different muscle tensions.

KEY WORDS: Neurophysiology, Tympanum, Biomechanics, Insect, Flight, Bat defense, *Heliothis virescens*

INTRODUCTION

Hearing is a fundamental tool used by animals to identify danger in their surroundings. Insects are no exception, having evolved tympanal hearing 19 independent times (Hoy et al., 1989; Strauß Stumpner, 2015; Yager, 2012) as well as other forms of particle displacement hearing, e.g. antennae (Gopfert and Hennig, 2016). However, what makes insects unique is that the location of their ears is not always on the outermost appendage (e.g. the head), to capture incoming sound. Furthermore, the range of tympanal hearing mechanisms varies greatly within insects, from a lever system with up to 2000 auditory receptor neurons in cicadas (Sueur et al., 2006), to a frequency-dependent traveling wave triggering just 70 neurons in locusts (Windmill et al., 2005), and only two auditory receptors in noctuid moths (Agee, 1967). Complicating this even further, the position of the ears on the animal's body, such as under movable parts like the wings, could mechanically impede the animal's hearing. Additionally, insect body position and slow movement from respiration and walking have been shown to affect hearing sensitivity (Meyer and Elsner, 1995; Zorovic and Hedwig, 2011).

[1]Department of Biological Sciences, Dartmouth College, Hanover, NH 03755, USA. [2]Centre for Ultrasonic Engineering, Department of Electronic & Electrical Engineering, University of Strathclyde, Glasgow G1 1XW, UK.
*Present address: United States Department of Agriculture-Agriculture Research Service, Parlier, CA 93648, USA.

‡Author for correspondence (shira.gordon@ars.usda.gov)

S.D.G., 0000-0002-5795-2614

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Many insects need to hear in order to avoid their predators while they are actively flying (Roeder, 1967). Elegant long-exposure photos of insects flying at night and steering away from a normal trajectory exemplify how well these animals respond to such threats (Agee, 1969). However, if their ears are obstructed by their wings in positions such as a down-stroke versus an upstroke, then how does the animal perceive the looming threat?

Noctuid moths are a useful group with which to study insect auditory systems because of their simple ear morphology. With only two auditory receptor neurons, they exhibit two behaviors: a negative phonotaxis of flying away from distant bats, and a more erratic looping and falling to the ground in response to a more immediate threat (Waters, 2003). Noctuid moths have their ears located on their metathorax, and these are therefore directly blocked by their folded wings during resting. During flight, muscles contract the whole thorax (Tu and Daniel, 2007), with the dorsoventral muscles indirectly raising the wings and the dorsolongitudinal muscles indirectly controlling the down stroke (Macfarlane and Eaton, 1973). Therefore, flight itself may interfere with the motion of the ear's tympanum by contorting it or tightening the membrane components.

The ears of these moths are among the simplest in construction with only three neurons per ear – two auditory neurons, A1 and A2, and a third neuron, the B cell. The auditory neurons directly attach to the inside of the tympanal membrane (Fig. 1A,B) and then join with the B cell in the adjoining air sac, creating the auditory nerve (Treat and Roeder, 1959; Yack and Fullard, 1990). The auditory nerve then travels through muscle tissue before eventually reaching the pterothoracic ganglion (Fig. 1C,D). The auditory neurons have different thresholds, with A1 being approximately 20 dB SPL more sensitive than A2 (Boyan and Fullard, 1986). The third neuron's role is unclear; this neuron is a homolog to a neuron in atympanate moths that is responsible for proprioception of the wing position (Hasenfuss, 1997; Yack and Fullard, 1990; Yack et al., 1999). Previous work has shown that with free-flying atympanate moths, in the wing up position the B cell fires rapidly while in the wing down position it fires more slowly (Yack and Fullard, 1993). However, the response of the B cell in noctuid moths appears to be mechanically isolated from the wing and so does not respond to wing position (Treat and Roeder, 1959). It is, however, still conceivable that wing position could influence the moth's hearing sensitivity. A downward wing position would physically block the ear from receiving sound while an upward position would leave it more exposed. Furthermore, the physical placement of the wing in these two positions could affect the tension of the tympanal membrane, or that of the internal muscles that reshape the thorax when controlling wing position; these muscles are located directly against the air chamber that backs the tympanum (Macfarlane and Eaton, 1973). This study tested the hypothesis that wing position affects the hearing sensitivity of noctuid moths. A combined neurophysiological and biomechanical approach was used to identify the moth's auditory response.

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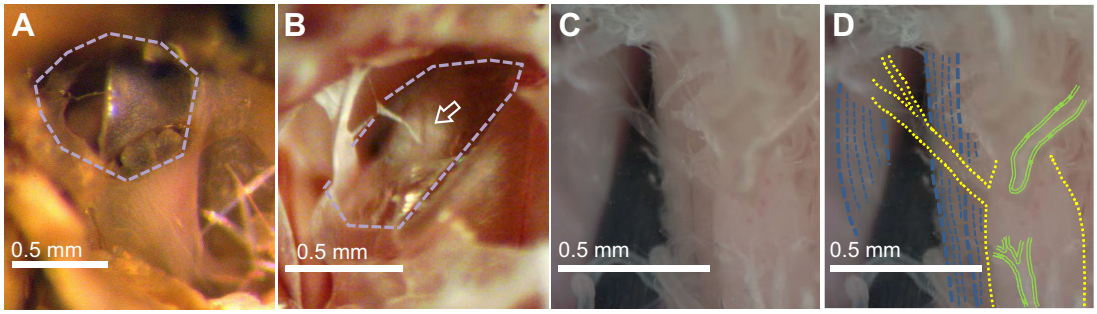

Fig. 1. A tympanal membrane of the moth *Heliothis virescens*. (A) Outside view. (B) Inside view. The membrane has a window cut in A, exposing the point of neural attachment of A1 and A2, indicated by the arrow in B. The purple outline indicates the perimeter of the thin tympanal membrane. (C) Internal view of the auditory nerve connecting to the IIIN1b nerve and then into the ganglion. The black object is an insect pin holding down the dorsolateral muscles just under the auditory nerve/IIIN1b junction. (D) Same as C but outlined to identify internal structures: yellow dotted line is nerve/ganglion, blue dashed line is muscle, green double line is tracheal pieces.

MATERIALS AND METHODS

Animals

Neurophysiology trials were conducted with *Heliothis virescens* (Fabricius 1777) moths (n=18) (Benzon Research, Carlisle, PA, USA). Laser Doppler vibrometry trials were conducted with a reared supply of *H. virescens* moths (n=21) from A. T. Groot's laboratory (University of Amsterdam). All animals were used within 2–25 days of emergence and stored at 20–24°C with an *ad libitum* supply of 10% sugar water.

Neurophysiology

Animals were mounted with wax to a glass rod ventral side up. The left mesothorax and metathorax were dissected to reveal the auditory nerve, leaving the dorsal flight muscles and entire right half intact. Tungsten 0.005 in electrodes (model 575400, A-M Systems, Carlsborg, WA, USA) were glued together to create parallel hooks that hooked the auditory nerve before it joined the main nerve (Fig. 1C,D). The wings were waxed into three positions (up, flat and down) and snipped near the base to keep the ear exposed in all instances. In addition, one group was left with freely moving wings, though these were still clipped near the base and the animal's body was constrained. Electrical signals were amplified by a differential amplifier (model DP 301, Warner Instruments, Hamden, CT, USA) and further amplified using an UltraSoundGate (model 416h200, Avisoft, Glienicke, Germany). Recordings were then manually analyzed for spike timing and number in Avisoft-SASLab Pro (Avisoft). Data were analyzed using the JMP package as a one-way ANOVA with the F-ratio (degrees of freedom and sample size) and P-values reported. All trials were conducted in a soundproof room (ETS-Lindgren, Cedar Park, TX, USA).

Sound was generated in Avisoft-SASLab Pro, with 10 ms tone bursts every 10 kHz from 20 to 80 kHz, over a 60 dB SPL range with 2 dB SPL step intervals. The order of the frequencies was randomized within the Avisoft-Recorder software. Sound was then amplified via an Avisoft USG Player 216H and played through an Avisoft Ultrasonic speaker at least 0.5 m away from the animal. The maximum sound level, 90 dB SPL, was calibrated with a ¼ inch free-field microphone (model 4939, Brüel & Kjær, Nærum, Denmark) at the position of the moth. Sound was played at a right angle to the animal with no obstructions.

Laser Doppler vibrometry

Animals were mounted with their anterior portion immobilized facing down on a glass slide. The wings were snipped near the base after being set with wax in one of three positions – up, flat or down – with the abdomen gently moved to the side to expose the tympanum. Each animal was then placed on a microscope-based scanning laser vibrometer system (model MSA100-3D, Polytec, Waldbronn, Germany), measuring at the point of neural attachment (Fig. 1A,B). A signal generator (model 33220A, Agilent/Keysight, Santa Rosa, CA, USA) was used to create 10 ms pulses for 20–80 kHz, every 10 kHz. The sound was amplified (TA-FE370, Sony, Tokyo, Japan) and played through a speaker (model ST50, Tannoy, Coatbridge, UK) and calibrated in real time with a ⅛ inch microphone (model 4138, Bruel & Kjaer). The root mean square (RMS) values were then analyzed in R (http://www.R-project.org/) for a 1 ms window beginning 0.5 ms after the sound started. Data were then analyzed in JMP as a one way ANOVA with the F-ratio and P-values reported.

RESULTS AND DISCUSSION

The hearing of *H. virescens* varied based on the frequency, regardless of wing position, with threshold responses of A1 approximately 20 dB SPL more sensitive than those of A2 (Fig. 2A), similar to other noctuid moths (Agee, 1967). Wing position did not play a significant role in A1 sensitivity, except at the highest frequency tested, i.e. 80 kHz ($F_{3,18}$=4.3, P=0.024; Fig. 2A); moths with unconstrained wing movement had a lower threshold for A1, but this result was not significant. There was a significant effect of wing position for A2 threshold in the 40–60 kHz range (40 kHz, $F_{3,18}$=3.8, P=0.035; 50 kHz, $F_{3,18}$=4.4, P=0.022; 60 kHz, $F_{3,18}$=3.5, P=0.044; Fig. 2A). For these frequencies, the up position always responded more sensitively than the flat wing; this trend continued for the higher frequencies but was not significantly different. However, the down position did not show a consistent trend for its A2 threshold response. Again, animals with unconstrained wing movement had a lower A2 threshold. Position also affected the maximum number of A1 action potentials measured before A2 began firing: the down position always had fewer action potentials, averaging around 6–7, while the flat and up positions averaged 8–9, and animals unconstrained from wing movement had 7–8 A1 action potentials fire before A2 started (Fig. 2B). However, this was only significantly different at 50 kHz ($F_{2,18}$=4.5, P=0.022).

Overall, these results suggest the downward wing position is significantly less sensitive to low sound levels than the flat or up position, though it may require fewer action potentials for A2 to begin firing. A similar result was found in the underwing noctuid moth, where ears were found to be less sensitive with the wings folded over in the resting position compared with an exposed up

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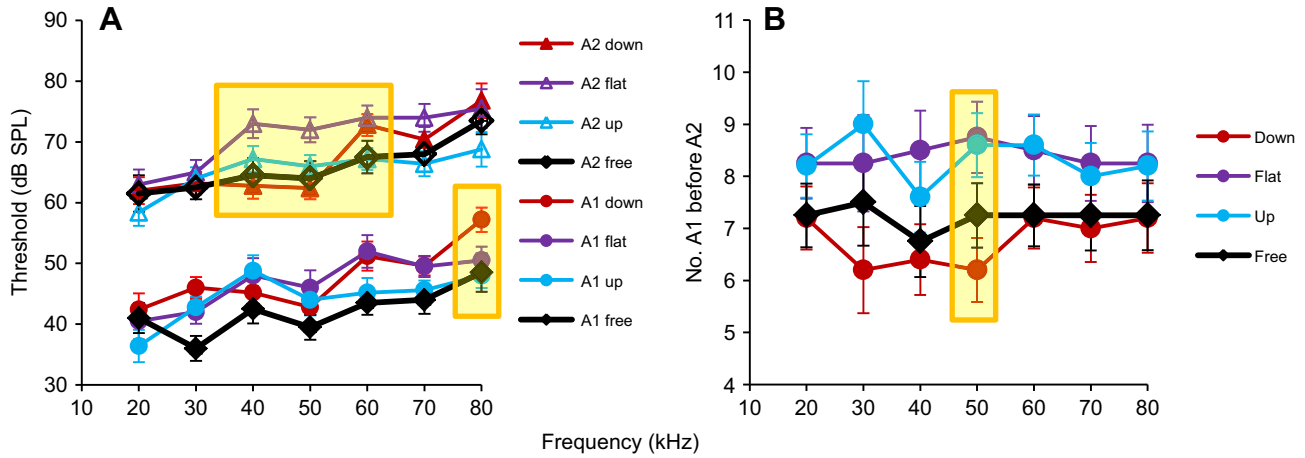

Fig. 2. A1 and A2 threshold neural response. (A) Neurophysiological threshold response of the A1 and A2 cells according to wing position. (B) The maximum number of A1 action potentials fired just before A2 began firing, with wings in the up, flat, down and unconstrained position. Yellow regions represent significance of at least P=0.05, n=18.

position (Faure et al., 1993). However, the results of our experiment eliminate the wing blocking the tympanal membrane as a possible explanation for this discrepancy, as the wing was surgically removed and the ears were equally exposed. Therefore, the strong result found in Faure et al. (1993) may be a factor of (1) blocking the tympanum with the wing, and (2) wing position itself altering the mechanics of the ear. In the down position, the dorsoventral muscles are relaxed and the dorsolongitudinal muscles are contracted (Macfarlane and Eaton, 1973). The muscles switch activation to get to the up position, transitioning through the flat position. This thoracic deformation could change the tension and movement of the tympanal membrane. Therefore, the next step in understanding how wing position affects hearing was measuring the mechanical response of the tympanal membrane to sound. Sound ranges that should affect A2 were the focus of the second part of the study.

The amplitude of displacement of the tympanal membrane significantly increased with higher sound levels, but not evenly across frequencies, regardless of wing position (Fig. 3A). The tympanum was most sensitive to 30–60 kHz, which corresponds to the frequency of the bat calls the moths may be avoiding. When data were divided according to wing position, fewer significant differences were seen for the sound levels below 80 dB SPL stimuli (Fig. S1). This result is notable as at 80 dB SPL the A2 neuron has already begun firing. Interestingly, from 40 to 60 kHz, the tested sound levels were not low enough to identify movement differences below 50–70 dB SPL even when wing position was not considered (Fig. 3A); despite the lack of differences, something triggers the A2 neuron to begin firing as threshold is at approximately 60–70 dB SPL. When wing position, frequency and sound level were considered together, the wing position resulted in no significant differences at any frequency/sound level combination (Fig. 3B).

Focusing on the 80 dB SPL results, as these were significantly different within each frequency level, the tympanal membrane was displaced more when the wing was in the flat rather than up position, albeit not significantly (Fig. 3B). These data oppose what could be expected based on the neural data (Fig. 2A), as more movement should amplify the deflection of the attached sensory neurons, which should in turn increase firing of the mechanosensors. Thus, it is likely that internal muscle and/or air chamber compression plays a factor in neural sensitivity. Similar to the neural data, membrane displacement in the down wing position did not follow a specific pattern across frequencies at 80 dB SPL (Fig. 3).

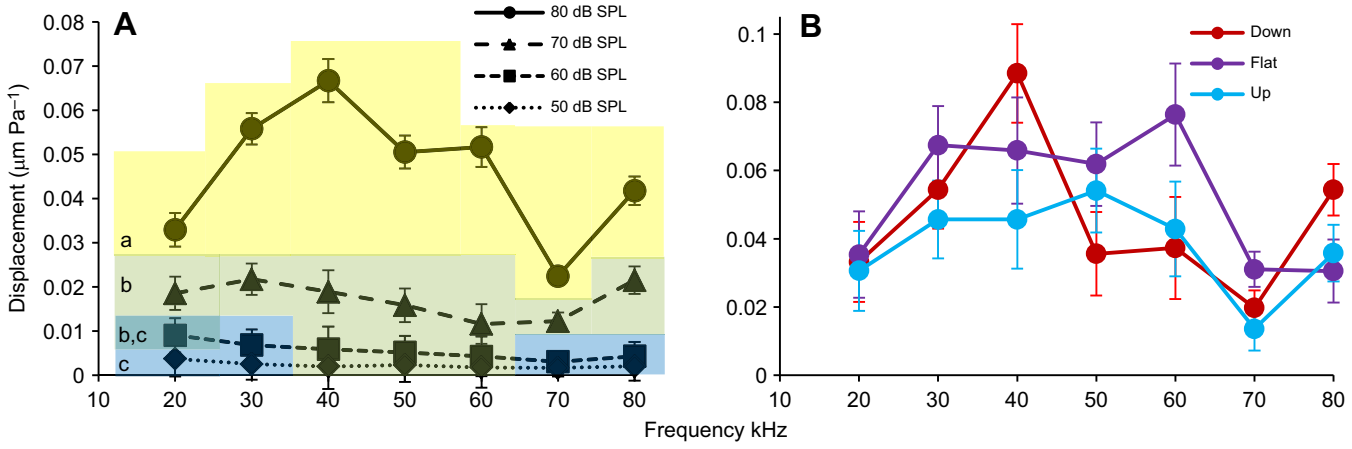

Fig. 3. Displacement of the tympanal membrane due to sound. (A) Average displacement for all three wing positions. Color indicates significant differences according to Tukey–Kramer. For significance of each wing position, see Fig. S1. (B) Displacement of individual wing positions at each frequency for 80 dB SPL. Data were not significantly different. n=21.

One explanation for the disconnection between tympanal deflection and neural response could be internal muscle tensions. The auditory nerve lies next to the flight muscles and dorsolongitudinal muscles (Fig. 1); distinct muscle groups are contracted/relaxed during the up/down strokes of flight (Macfarlane and Eaton, 1973). Tension variation in these muscles may therefore change the tension acting on the nerve, which may in turn affect its sensitivity to the same movements of the tympanal membrane.

As the auditory nerve goes directly to the pterothoracic ganglion, there should be no other afferent information influencing the auditory neurons. However, the B cell also connects to that nerve, and its role is as yet unknown (Yack and Fullard, 1993). Testing the firing rate of the B cell identified no significant difference based on wing position (averages±s.d., action potentials/0.25 s: up 3.0±1, flat 4.7±1, down: 2.3±1, $F_{2,8}$=1.25, P=0.35). Treat and Roeder (1959) also found no effect of wing position on the B cell, but did find that artificially changing the tension of the B cell changed its firing rate, and that changing the tension by thorax depressions altered the firing rate of both the B cell and the A cells. Because of the number of experimental approaches they used and the unclear results those yielded, Treat and Roeder (1959) did not draw any strong conclusions as to what the role of the B cell might be. Perhaps the firing rate is not due to a static wing position, and instead the B cell fires more dynamically based on the transitional movement of the wing. Therefore, the static mounting of the wing would miss this differing response. If the firing rate of the B cell dynamically indicates to the moth a change from down-to-up and up-to-down, this information converging with that coming from the A cells at the ganglion may facilitate the dynamic problem of hearing while flying.

Sensitivity to wing position is more obvious in the neural response than in tympanal membrane movement. While the sensory neurons are mechnoreceptors reliant on deflections of the tympanum, other factors such as muscle configuration or compression of the internal air chamber backing the tympanum may play a role. As the methods used for this study are less invasive than previous lepidopteran neural physiological analyses, this research opens possibilities to understanding responses of the animal from a more organismal approach. Future studies should examine questions of noctuid hearing sensitivity considering wing position during mounting, and could perhaps examine wing muscle activity at the same time.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: S.D.G., J.F.C.W.; Methodology: S.D.G., J.F.C.W., E.K.; Software: J.F.C.W., E.K.; Validation: J.F.C.W., E.K.; Formal analysis: S.D.G., J.F.C.W., E.K.; Investigation: S.D.G.; Resources: J.F.C.W.; Data curation: J.F.C.W., E.K.; Writing - original draft: S.D.G.; Writing - review & editing: S.D.G., J.F.C.W., E.K.; Visualization: S.D.G.; Supervision: J.F.W.; Project administration: J.F.W.; Funding acquisition: S.D.G.

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Data availability
All data created during this research are openly available from the University of Strathclyde Pure/KnowledgeBase at http://dx.doi.org/10.15129/1af642b7-f467-46d8-a43f-2259472a0286.

Supplementary information
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References
Agee, H. R. (1967). Response of acoustic sense cell of the bollworm and tobacco budworm to ultrasound. *J. Econ. Entomol.* **60**, 366-369.
Agee, H. R. (1969). Response of flying bollworm moths and other tympanate moths to pulsed ultrasound. *Ann. Entomol. Soc. Am.* **62**, 801-807.
Boyan, G. S. and Fullard, J. H. (1986). Interneurones responding to sound in the tobacco budworm moth Heliothis virescens (Noctuidae): morphological and physiological characteristics. *J. Comp. Physiol. A* **158**, 391-404.
Faure, P. A., Fullard, J. H. and Dawson, J. W. (1993). The gleaning attacks of the northern long-eared bat, *Myotis septentrionalis*, are relatively inaudible to moths. *J. Exp. Biol.* **178**, 173-189.
Göpfert, M. C. and Hennig, R. M. (2016). Hearing in Insects. *Annu. Rev. Entomol.* **61**, 257-276.
Hasenfuss, I. (1997). Precursor structures and evolution of tympanal organs in Lepidoptera (Insecta, Pterygota). *Zoomorphology* **117**, 155-164.
Hoy, R. R., Nolen, T. and Brodfuehrer, P. (1989). The neuroethology of acoustic startle and escape in flying insects. *J. Exp. Biol.* **146**, 287-306.
Macfarlane, J. and Eaton, J. L. (1973). Skeleton and Musculature of the Head and Thorax of Trichoplusia ni (Hubner), (Lepidoptera: Noctuidae). *J. Morphol.* **139**, 185-210.
Meyer, J. and Elsner, N. (1995). How respiration affects auditory sensitivity in the grasshopper *Chorthippus biguttulus* (L.). *J. Comp. Physiol. A* **176**, 563-573.
Roeder, K. D. (1967). Turning tendency of moths exposed to ultrasound while in stationary flight. *J. Insect. Physiol.* **13**, 873-888.
Strauß, J. and Stumpner, A. (2015). Selective forces on origin, adaptation and reduction of tympanal ears in insects. *J. Comp. Physiol. A* **201**, 155-169.
Sueur, J., Windmill, J. F. C. and Robert, D. (2006). Tuning the drum: the mechanical basis for frequency discrimination in a Mediterranean cicada. *J. Exp. Biol.* **209**, 4115-4128.
Treat, A. E. and Roeder, K. D. (1959). A nervous element of unknown function in the tympanic organs of moths. *J. Insect. Physiol.* **3**, 262-270.
Tu, M. S. and Daniel, T. L. (2007). Cardiac-like behavior of an insect flight muscle. *J. Exp. Biol.* **207**, 2455-2464.
Waters, D. A. (2003). Bats and moths: what is there left to learn? *Physiol. Entomol.* **28**, 237-250.
Windmill, J. F. C., Göpfert, M. C. and Robert, D. (2005). Tympanal travelling waves in migratory locusts. *J. Exp. Biol.* **208**, 157-168.
Yack, J. E. and Fullard, J. H. (1990). The mechanoreceptive origin of insect tympanal organs: a comparative study of similar nerves in tympanate and atympanate moths. *J. Comp. Neurol.* **300**, 523-534.
Yack, J. E. and Fullard, J. H. (1993). Proprioceptive activity of the wing-hinge stretch receptor in Manduca sexta and other atympanate moths: a study of the noctuoid moth ear B cell homologue. *J. Comp. Physiol. A* **173**, 301-307.
Yack, J. E., Scudder, G. G. E. and Fullard, J. H. (1999). Evolution of the metathoracic tympanal ear and its mesothoracic homologue in the Macrolepidoptera (Insecta). *Zoomorphology* **119**, 93-103.
Yager, D. D. (2012). Predator detection and evasion by flying insects. *Curr. Opin. Neurobiol.* **22**, 201-207.
Zorovic, M. and Hedwig, B. (2011). Processing of species-specific auditory patterns in the cricket brain by ascending, local, and descending neurons during standing and walking. *J. Neurophysiol.* **105**, 2181-2194.