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

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Summary Statement: Noctuid moth wing position affects neural hearing sensitivity. No significant differences in eardrum movement were observed; differences are therefore hypothesized to be due to internal factors such as muscle tension.

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, since 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 due to wing position are proposed to be due to other factors within the animal such as different muscle tensions.

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; Strauβ 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 & Hedwig, 2011).

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 so 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 due to 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 (MacFarlan 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 moths are among the simplest in construction with only 3 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 that of 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 up position the B cell fires rapidly while in the 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 (MacFarlan and Eaton, 1973). This study tests 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.

Methods

Animals

Neurophysiology trials were conducted with *Heliothis virescens* moths (n = 18) (Benzon Research, Carlisle, PA). Laser Doppler vibrometry trials were conducted with a reared supply of *H. virescens* moths (n = 21) from A. T. Groot's laboratory (U. of Amsterdam). All animals were used within 2—25 days after 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 meso- and metathorax were dissected to reveal the auditory nerve, leaving the dorsal flight muscles and entire right half intact. Tungsten 0.005" 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 3 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 count in Avisoft-SASLab Pro (Avisoft, Glienicke, Germany). 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 sound proof room (ETS-Lindgren, Cedar Park, Texas).

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 1 A-B). A signal generator (Model 33220A, Agilent/Keysight, Santa Rosa, USA) was used to create 10ms 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, Scotland) and calibrated in real time with a ⅛ inch microphone (Model: 4138, Bruel & Kjaer, Naerum, Denmark). The Root Mean Square (RMS) values were then analyzed in R (R Core Team, 2014) 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 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 = .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 = .035; 50 kHz $F_{3,18}$ = 4.4, p = .022; 60 kHz $F_{3,18}$ = 3.5, p = .044) (Fig 2A). For these frequencies, the up position always responded more sensitively than 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, 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 = .022).

Overall these results suggest the downward wing position is significantly less sensitive to low sound levels compared to the flat or up position, though 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 to an exposed up 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 (MacFarlan 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 into 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 by wing position, fewer significant differences were seen for the sound levels below 80 dB SPL stimuli (Supplemental Fig 1). This result is notable as at 80 dB SPL the A2 neuron has already begun firing. Interestingly, from 40-60 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 seeing no differences, something triggers the A2 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 a 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 play a factor in neural sensitivity. Similar to the neural data, membrane displacement in the down wing position does not follow a specific pattern across frequencies at 80 dB SPL (Fig 3).

One explanation for the disconnection between tympanal deflection and neural response could be internal muscle tensions. The auditory nerve lays next to the flight muscles and dorsolongitudinal muscles (Fig 1); distinct muscle groups are contracted/relaxed during the up/down strokes of flight (MacFarlan and Eaton, 2005). 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 of yet unknown (Yack and Fullard, 1993). Testing the firing rate of the B cell identified no significant difference based on wing position (averages: up 3.0 ±1, flat 4.7 ±1, down: 2.3 ±1, $F_{2,8} = 1.25$, p = .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 both the firing rate of both the B cell and the A cells. Due to the number of experimental approaches they used and the unclear results those yielded, they 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, but 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 factor. 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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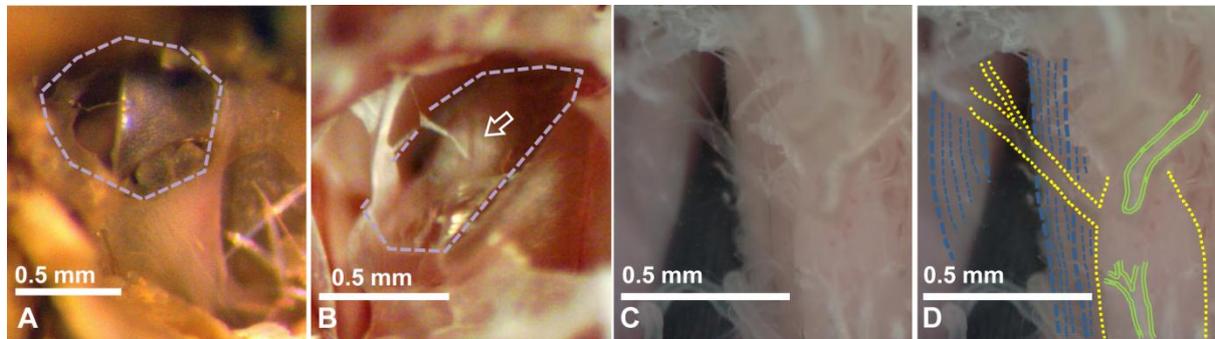
Figures

Fig 1. An outside (A) and inside (B) view of a tympanal membrane of the moth *Heliothis virescens*. The membrane has a cut window in (A) exposing the point of neural attachment of A1 and A2, indicated by the hollow white arrow. The light 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) the 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.
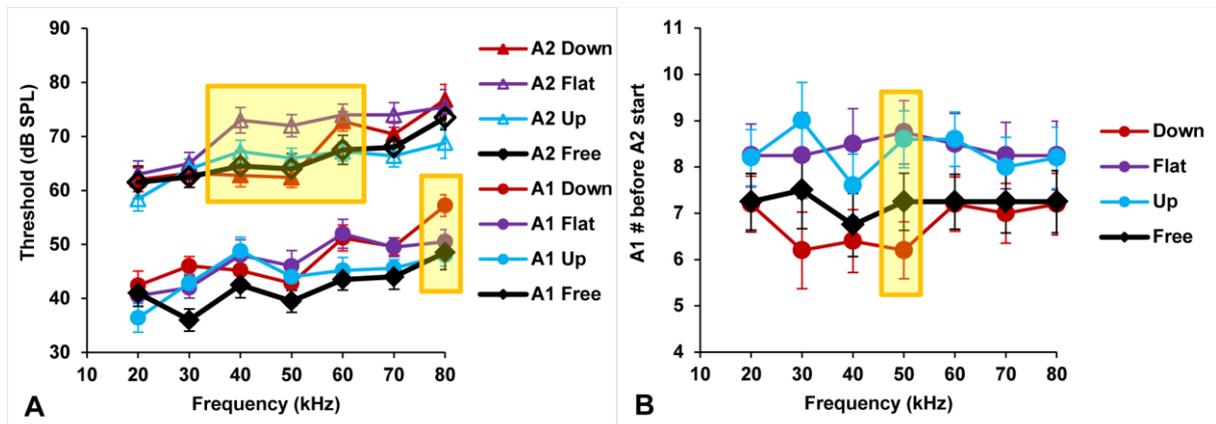
Figure 2. A) Neurophyisiological threshold response of the A1 and A2 cells. B) The maximum number of A1 action potentials fired just before A2 began firing. Yellow regions represent significance of at least p = .05, n = 18.
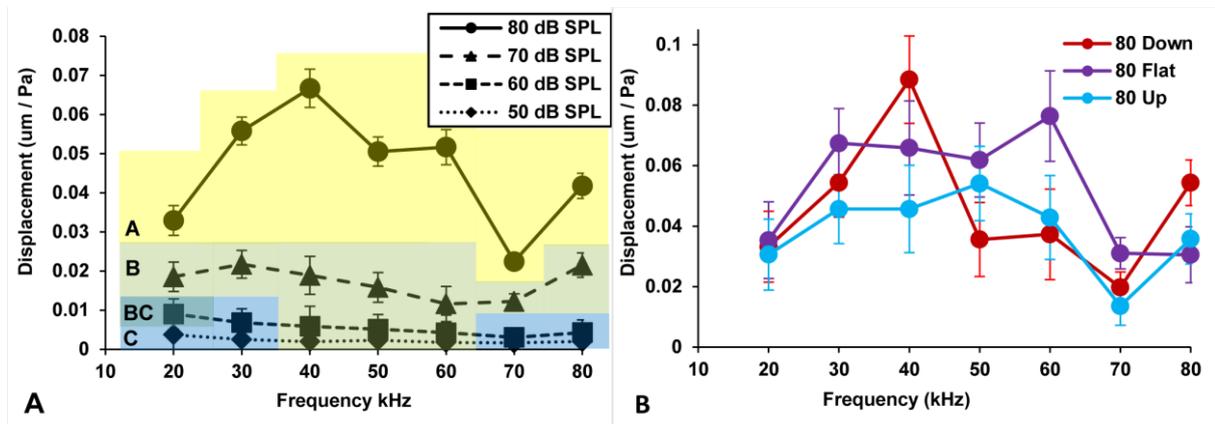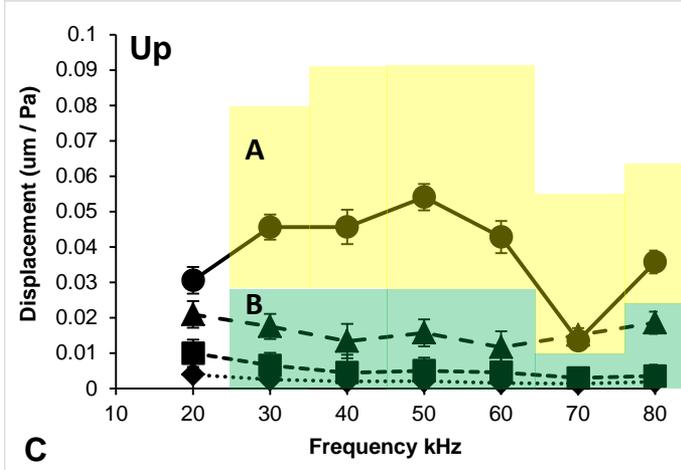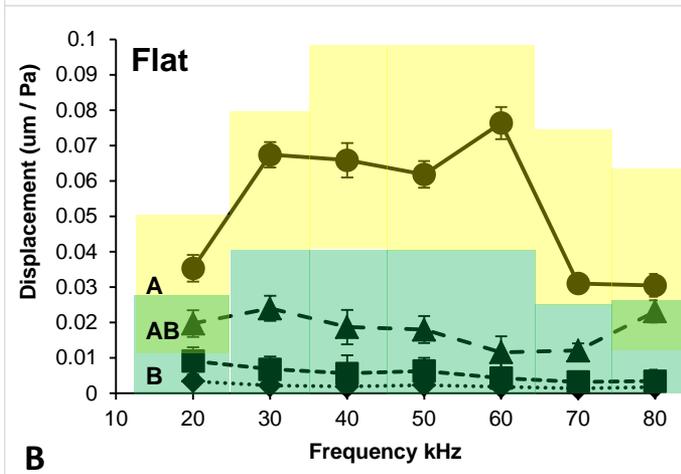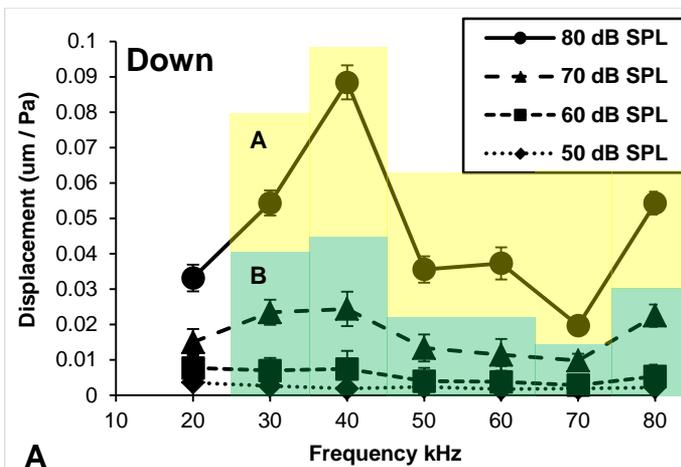
Figure 3. A) Displacement of the tympanal membrane due to sound, averaged for all three wing positions. Color indicates significant differences according to Tukey-Kramer, for significance of each wing position see supplemental data. B) Displacement of individual wing positions at each frequency for 80 dB SPL, data were not significantly different. n = 21.

Supplemental Figure 1

Displacement of the tympanal membrane due to sound in the down (A), flat (B), and up (C) positions. Color indicates significant differences according to Tukey-Kramer.