

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

2 Running title: Effect of wing position on moth hearing

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10 Key Words: Neurophysiology, Tympanum, Biomechanics, Hearing, Wing Position, *Heliothis virescens*

11 Summary Statement (15-30 words): Noctuid moth wing position affects neural hearing sensitivity.

12 No significant differences in eardrum movement were observed; differences are therefore
13 hypothesized to be due to internal factors such as muscle tension.

14

15 Abstract

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

28 Introduction

29 Hearing is a fundamental tool used by animals to identify danger in their surroundings. Insects
30 are no exception, having evolved tympanal hearing 19 independent times (Hoy et al., 1989; Strauß
31 Stumpner, 2015; Yager, 2012) as well as other forms of particle displacement hearing, e.g., antennae
32 (Gopfert and Hennig, 2016). However, what makes insects unique is that the location of their ears is
33 not always on the outermost appendage (e.g. the head), to capture incoming sound. Furthermore,
34 the range of tympanal hearing mechanisms varies greatly within insects, from a lever system with up
35 to 2000 auditory receptor neurons in cicadas (Sueur et al., 2006), to a frequency-dependent
36 traveling wave triggering just 70 neurons in locusts (Windmill et al., 2005), and only two auditory
37 receptors in noctuid moths (Agee, 1967). Complicating this even further, the position of the ears on
38 the animal's body, such as under movable parts like the wings, could mechanically impede the
39 animal's hearing. Additionally, insect body position and slow movement from respiration and
40 walking have been shown to affect hearing sensitivity (Meyer and Elsner, 1995; Zorovic & Hedwig,
41 2011).

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

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

56 The ears of moths are among the simplest in construction with only 3 neurons per ear—two
57 auditory neurons, A1 and A2, and a third neuron, the B cell. The auditory neurons directly attach to
58 the inside of the tympanal membrane (Fig 1A-B) and then join with the B cell in the adjoining air sac,
59 creating the auditory nerve (Treat and Roeder, 1959; Yack and Fullard, 1990). The auditory nerve
60 then travels through muscle tissue before eventually reaching the pterothoracic ganglion (Fig 1C-D).
61 The auditory neurons have different thresholds, with A1 being approximately 20 dB SPL more
62 sensitive than A2 (Boyan and Fullard, 1986). The third neuron's role is unclear; this neuron is a
63 homolog to that of atympanate moths that is responsible for proprioception of the wing position
64 (Hasenfuss, 1997; Yack and Fullard, 1990; Yack et al., 1999). Previous work has shown that with free
65 flying atympanate moths, in the up position the B cell fires rapidly while in the down position it fires
66 more slowly (Yack and Fullard, 1993). However, the response of the B cell in noctuid moths appears
67 to be mechanically isolated from the wing and so does not respond to wing position (Treat and
68 Roeder, 1959). It is, however, still conceivable that wing position could influence the moth's hearing
69 sensitivity. A downward wing position would physically block the ear from receiving sound while an
70 upward position would leave it more exposed. Furthermore, the physical placement of the wing in
71 these two positions could affect the tension of the tympanal membrane, or that of the internal
72 muscles that reshape the thorax when controlling wing position; these muscles are located directly
73 against the air chamber that backs the tympanum (MacFarlan and Eaton, 1973). This study tests the
74 hypothesis that wing position affects the hearing sensitivity of noctuid moths. A combined
75 neurophysiological and biomechanical approach was used to identify the moth's auditory response.

76

77 Methods

78 Animals

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

84 Neurophysiology

85 Animals were mounted with wax to a glass rod ventral side up. The left meso- and metathorax
86 were dissected to reveal the auditory nerve, leaving the dorsal flight muscles and entire right half
87 intact. Tungsten 0.005" electrodes (Model: 575400, A-M Systems, Carlsborg, WA, USA) were glued
88 together to create parallel hooks that hooked the auditory nerve before it joined the main nerve (Fig
89 1C-D). The wings were waxed into 3 positions (up, flat, and down) and snipped near the base to
90 keep the ear exposed in all instances. In addition, one group was left with freely moving wings,
91 though these were still clipped near the base and the animal's body was constrained. Electrical
92 signals were amplified by a differential amplifier (Model: DP 301, Warner Instruments, Hamden, CT,
93 USA) and further amplified using an UltraSoundGate (Model: 416h200, Avisoft, Glienicke, Germany).
94 Recordings were then manually analyzed for spike timing and count in Avisoft-SASLab Pro (Avisoft,
95 Glienicke, Germany). Data were analyzed using the JMP package as a one-way ANOVA with the F
96 ratio (degrees of freedom and sample size) and p values reported. All trials were conducted in a
97 sound proof room (ETS-Lindgren, Cedar Park, Texas).

98 Sound was generated in Avisoft-SASLab Pro, with 10 ms tone bursts every 10 kHz from 20 to
99 80 kHz, over a 60 dB SPL range with 2 dB SPL step intervals. The order of the frequencies was
100 randomized within the Avisoft-Recorder software. Sound was then amplified via an Avisoft USG
101 Player 216H and played through an Avisoft Ultrasonic speaker at least 0.5 m away from the animal.
102 The maximum sound level, 90 dB SPL, was calibrated with a ¼ inch free-field microphone (Model
103 4939, Brüel & Kjær, Nærum, Denmark) at the position of the moth. Sound was played at a right
104 angle to the animal with no obstructions.

105 Laser Doppler Vibrometry

106 Animals were mounted with their anterior portion immobilized facing down on a glass slide.
107 The wings were snipped near the base after being set with wax in one of three positions: up, flat, or
108 down, with the abdomen gently moved to the side to expose the tympanum. Each animal was then
109 placed on a microscope-based scanning laser vibrometer system (Model: MSA100-3D, Polytec,
110 Waldbronn, Germany), measuring at the point of neural attachment (Fig 1 A-B). A signal generator

111 (Model 33220A, Agilent/Keysight, Santa Rosa, USA) was used to create 10ms pulses for 20-80 kHz,
112 every 10 kHz. The sound was amplified (TA-FE370, Sony, Tokyo, Japan) and played through a
113 speaker (Model: ST50, Tannoy, Coatbridge, Scotland) and calibrated in real time with a 1/8 inch
114 microphone (Model: 4138, Bruel & Kjaer, Naerum, Denmark). The Root Mean Square (RMS) values
115 were then analyzed in R (R Core Team, 2014) for a 1 ms window beginning 0.5 ms after the sound
116 started. Data were then analyzed in JMP as a one way ANOVA with the F ratio and p values
117 reported.

118 Results and Discussion

119 The hearing of *H. virescens* varied based on the frequency, regardless of wing position, with
120 threshold responses of A1 approximately 20 dB SPL more sensitive than A2 (Fig 2A), similar to other
121 noctuid moths (Agee, 1967). Wing position did not play a significant role in A1 sensitivity, except at
122 the highest frequency tested, i.e. 80 kHz ($F_{3,18} = 4.3$, $p = .024$) (Fig 2A); moths with unconstrained
123 wing movement had a lower threshold for A1, but this result was not significant. There was a
124 significant effect of wing position for A2 threshold in the 40-60 kHz range (40 kHz $F_{3,18} = 3.8$, $p = .035$;
125 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
126 position always responded more sensitively than flat wing; this trend continued for the higher
127 frequencies but was not significantly different. However, the down position did not show a
128 consistent trend for its A2 threshold response. Again, animals with unconstrained wing movement
129 had a lower A2 threshold. Position also affected the maximum number of A1 action potentials
130 measured before A2 began firing: the down position always had fewer, averaging around 6-7, while
131 the flat and up positions averaged 8-9, and animals unconstrained from wing movement had 7-8 A1
132 action potentials fire before A2 started (Fig 2B). However, this was only significantly different at 50
133 kHz ($F_{2,18} = 4.5$, $p = .022$).

134 Overall these results suggest the downward wing position is significantly less sensitive to low
135 sound levels compared to the flat or up position, though may require fewer action potentials for A2
136 to begin firing. A similar result was found in the underwing noctuid moth, where ears were found to
137 be less sensitive with the wings folded over in the resting position compared to an exposed up
138 position (Faure et al., 1993). However, the results of our experiment eliminate the wing blocking the
139 tympanal membrane as a possible explanation for this discrepancy, as the wing was surgically
140 removed and the ears were equally exposed. Therefore, the strong result found in Faure et al.
141 (1993) may be a factor of 1) blocking the tympanum with the wing, and 2) wing position itself
142 altering the mechanics of the ear. In the down position, the dorsoventral muscles are relaxed and
143 the dorsolongitudinal muscles are contracted (MacFarlan and Eaton, 1973). The muscles switch

144 activation to get to the up position, transitioning through the flat position. This thoracic
145 deformation could change the tension and movement of the tympanal membrane. Therefore, the
146 next step into understanding how wing position affects hearing was measuring the mechanical
147 response of the tympanal membrane to sound. Sound ranges that should affect A2 were the focus
148 of the second part of the study.

149 The amplitude of displacement of the tympanal membrane significantly increased with higher
150 sound levels, but not evenly across frequencies, regardless of wing position (Fig 3A). The tympanum
151 was most sensitive to 30-60 kHz, which corresponds to the frequency of the bat calls the moths may
152 be avoiding. When data were divided by wing position, fewer significant differences were seen for
153 the sound levels below 80 dB SPL stimuli (Supplemental Fig 1). This result is notable as at 80 dB SPL
154 the A2 neuron has already begun firing. Interestingly, from 40-60 kHz the tested sound levels were
155 not low enough to identify movement differences below 50-70 dB SPL even when wing position was
156 not considered (Fig 3A); despite seeing no differences, something triggers the A2 to begin firing as
157 threshold is at approximately 60-70 dB SPL. When wing position, frequency, and sound level were
158 considered together, the wing position resulted in no significant differences at any frequency/sound
159 level combination (Fig 3B).

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

168 One explanation for the disconnection between tympanal deflection and neural response
169 could be internal muscle tensions. The auditory nerve lays next to the flight muscles and
170 dorsolongitudinal muscles (Fig 1); distinct muscle groups are contracted/relaxed during the up/down
171 strokes of flight (MacFarlan and Eaton, 2005). Tension variation in these muscles may therefore
172 change the tension acting on the nerve, which may in turn affect its sensitivity to the same
173 movements of the tympanal membrane.

174 As the auditory nerve goes directly to the pterothoracic ganglion, there should be no other
175 afferent information influencing the auditory neurons. However, the B cell also connects to that

176 nerve, and its role is as of yet unknown (Yack and Fullard, 1993). Testing the firing rate of the B cell
177 identified no significant difference based on wing position (averages: up 3.0 ± 1 , flat 4.7 ± 1 , down:
178 2.3 ± 1 , $F_{2,8} = 1.25$, $p = .35$). Treat and Roeder (1959) also found no effect of wing position on the B
179 cell, but did find that artificially changing the tension of the B cell changed its firing rate, and that
180 changing the tension by thorax depressions altered both the firing rate of both the B cell and the A
181 cells. Due to the number of experimental approaches they used and the unclear results those
182 yielded, they did not draw any strong conclusions as to what the role of the B cell might be. Perhaps
183 the firing rate is not due to a static wing position, but fires more dynamically based on the
184 transitional movement of the wing. Therefore, the static mounting of the wing would miss this
185 differing response. If the firing rate of the B cell dynamically indicates to the moth a change from
186 down-to-up and up-to-down, this information converging with that coming from the A cells at the
187 ganglion may facilitate the dynamic problem of hearing while flying.

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

196

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202 Competing Interests

203 There are no competing interests declared.

204

205 Author Contributions

206 S. D. Gordon proposed this research, ran and analyzed the neurophysiology and biomechanical
207 experiments. E. Klenschi designed software to compile data for the biomechanical studies and ran
208 some trials. J. F. C. Windmill helped design the biomechanical trials. All authors contributed to the
209 writing of this text.

210

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217

218 Data Availability

219 Pending acceptance, all data created during this research are openly available from the University of
220 Strathclyde Pure/KnowledgeBase at <http://dx.doi.org/xxx.xxx>.

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223

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270

271

272 Figure Legends

273 Fig 1. An outside (A) and inside (B) view of a tympanal membrane of the moth *Heliothis virescens*.
274 The membrane has a cut window in (A) exposing the point of neural attachment of A1 and A2,
275 indicated by the hollow white arrow. The light purple outline indicates the perimeter of the thin
276 tympanal membrane. (C) Internal view of the auditory nerve connecting to the IIN1b nerve and then
277 into the ganglion. The black object is an insect pin holding down the dorsolateral muscles just under
278 the auditory nerve/IIN1b junction. D) the same as C but outlined to identify internal structures:
279 yellow dotted line is nerve/ganglion, blue dashed line is muscle, green double line is tracheal pieces.

280 Figure 2. A) Neurophysiological threshold response of the A1 and A2 cells. B) The maximum
281 number of A1 action potentials fired just before A2 began firing. Yellow regions represent
282 significance of at least $p = .05$, $n = 18$.

283 Figure 3. A) Displacement of the tympanal membrane due to sound, averaged for all three wing
284 positions. Color indicates significant differences according to Tukey-Kramer, for significance of each
285 wing position see supplemental data. B) Displacement of individual wing positions at each
286 frequency for 80 dB SPL, data were not significantly different. $n = 21$.

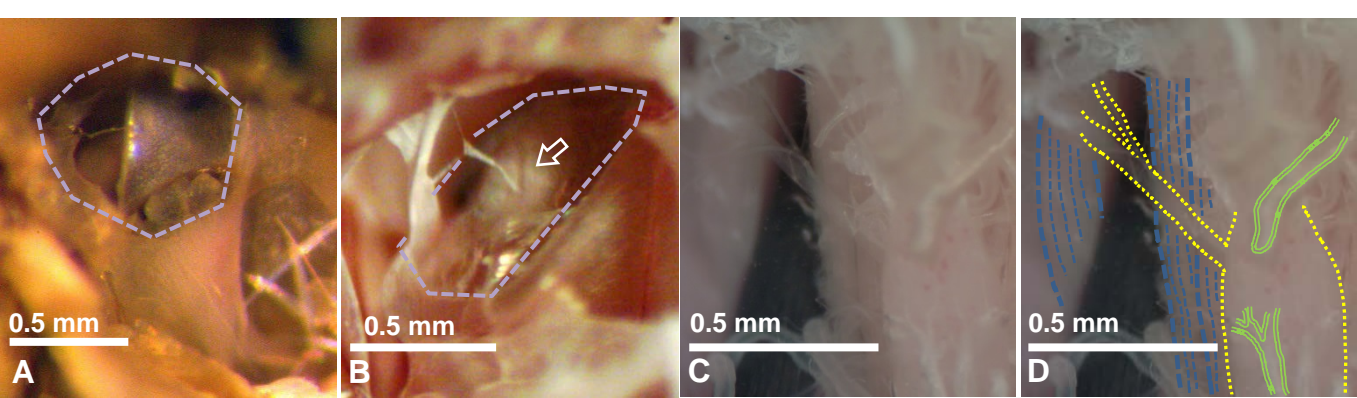


Figure 1

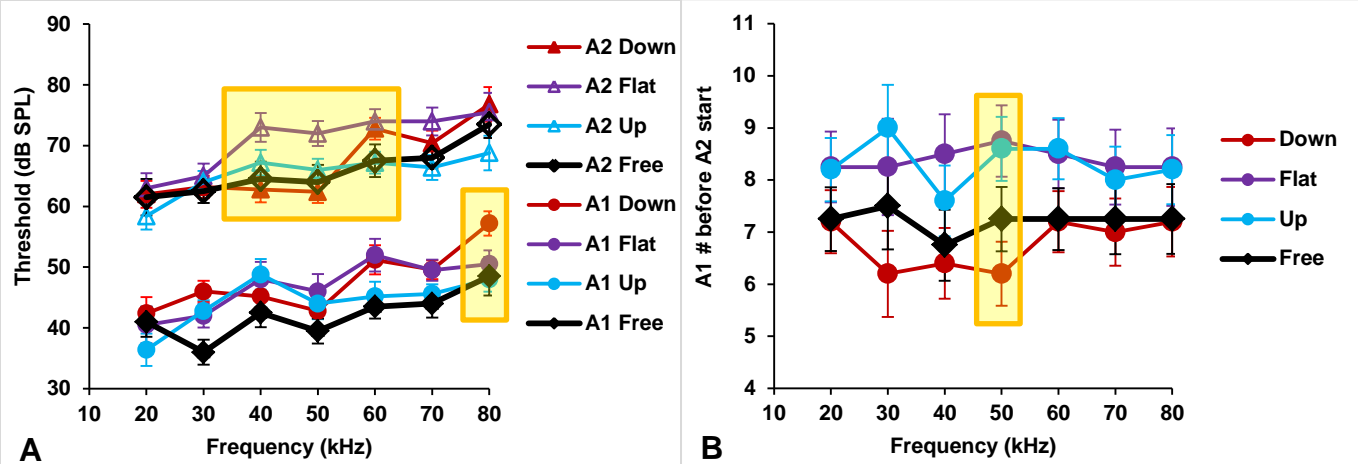


Figure 2

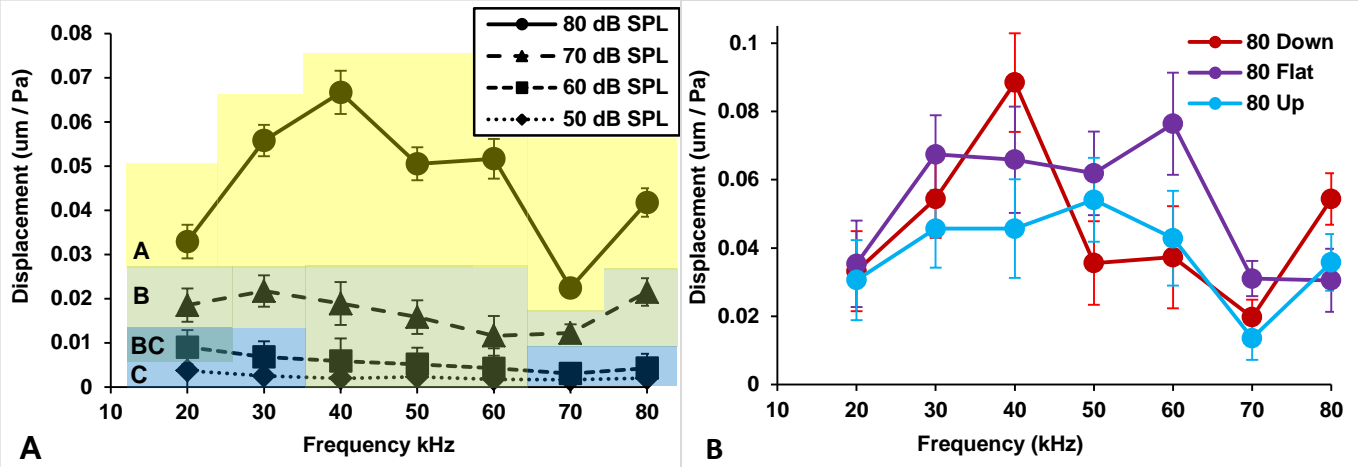


Figure 3