

1 Title: Hearing Ability Decreases in Aging Locusts

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16 **Abstract**

17 Insects display signs of aging, despite their short lifespan. We focus on the aging of adult locusts and  
18 their subsequent hearing ability. Our results indicate that younger adults (two weeks after maturity)  
19 have a greater neurophysiological response to sound, especially for low frequencies (<10kHz), as well as  
20 shorter latency to this neural response. Interestingly, when measuring displacement of the locust  
21 tympanal membrane, which the receptor neurons are directly attached to, we found the movement is  
22 not directly correlated with the neural response. Therefore we suggest the enhanced response is due to  
23 the condition of their tissues (e.g., elasticity). Secondly, we found that females and males do not have  
24 the same responses, particularly at four weeks post adult moult. We propose that this is due to female  
25 reproductive condition reducing the ability to receive sounds. Our results indicate older animals may  
26 therefore be less sensitive to the sounds of approaching predators.

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28

## 29 **Hearing Ability Decreases in Aging Locusts**

### 30 **Introduction**

31 Many insects have a short life-span, especially during their adult stage. Even so, in this brief, final  
32 stage, insects may experience the effects of aging. For example, some insects show slower locomotion  
33 and lower activity levels with increasing age post maturity (Ridgel and Ritzman, 2005). Older moths,  
34 *Antheraea pernyi*, show a decrease with age in their pheromonal response as well as a decrease in their  
35 living dendrites (Kumar et al., 1998). As adults, most insects will not moult again, and therefore do not  
36 shed the hard outer cuticle used as the scaffold of their body. Indeed, as insects age, aspects of their  
37 cuticle harden and become less elastic, such as the cockroach tarsal pads (Ridgel and Ritzman, 2005).  
38 Adult insects have also been shown to thicken their cuticle by adding new layers in a circadian cycle  
39 (Neville, 1963).

40 To hear, many insects receive sound via a tympanum that is composed of a thin layer of cuticle—  
41 less than a micrometre thick in some regions (Malkin et al, 2014). This tympanum is very flexible and  
42 moves with sound. In locusts, there is a frequency-dependent travelling wave that forms, physically  
43 deflecting the receptor neurons that are directly attached to the membrane (Windmill et al., 2005). The  
44 ears are sensitive to nanometre level movements and such displacements are responsible for differing  
45 frequency sensitivities (Gordon et al., 2014). After emerging as an adult, some insect features continue  
46 to harden and sclerotize (Uvarov, 1966). The locust ear is not fully developed until it reaches its adult  
47 form (Michel and Petersen, 1982). Additionally, female condition changes with time, as female  
48 *Schistocerca gregaria* may lay their first egg pod with as much variation as 33-54 days (Uvarov, 1966).  
49 With these factors in mind, we tested aging adult locusts and their hearing abilities, both through  
50 tympanal membrane deflection and neurophysiology.

### 51 **Results and Discussion**

52 Deflection of the tympanal membrane activates the mechanosensory receptor neurons directly  
53 attached to it. Therefore differences in deflection should result in differing amounts of neural activation.  
54 We measured deflections of the tympanal membrane at two sound levels (60 and 70 dB SPL) finding  
55 similar patterns and so data are displayed for only the higher sound level. Male and female locusts did  
56 not have the same tympanal membrane displacement with sound as they aged (Fig 1). With increasing  
57 female age there was an incrementally reduced amount of tympanal membrane displacement,  
58 significant across all frequencies measured (Fig 1A) (e.g., at 5kHz  $F_{4,37} = 3.92$ ,  $p = .010$ ; at 15kHz  $F_{4,37} =$   
59  $3.33$ ,  $p = .021$ ). As males aged from two to eight weeks, there was no difference in their tympanal  
60 membrane movement for most of the frequencies (Fig 1B). Yet the older age groups, six and eight weeks,  
61 had significantly more displacement for the lower, ~4-5 kHz, frequencies, (e.g., at 4.5kHz  $F_{4,43} = 3.65$ ,  $p =$   
62  $.013$ ). Interestingly, when the displacement data was scaled to approximately the largest peak across all  
63 age groups (9.25-10kHz), the females—like the males—showed the same pattern for the displacement  
64 across the frequency spectrum.

65 At two weeks post adult moult, there were no significant differences between the sexes (Fig 1C). In  
66 contrast, for the remaining age groups the males had greater movement in their tympanal membrane  
67 than the females (Fig 1D-G). The three and four week locusts had an intermediate level of difference,  
68 wherein they were significantly different at low frequencies but not high ones (Fig 1D-E) (e.g., 4 weeks:  
69 at 5kHz  $F_{1,21} = 5.57$ ,  $p = .029$ ; at 15kHz  $F_{1,21} = 2.58$ ,  $p = .124$ ). Old animals were significantly different  
70 across all frequencies (e.g., 8 weeks: at 5kHz  $F_{1,12} = 6.87$ ,  $p = .024$ ; at 15kHz  $F_{1,12} = 8.23$ ,  $p = .015$ ).

71 To understand the neurophysiological response relating to the membrane deflections, we analysed  
72 the neurophysiology of the locusts' hearing in several ways. First, across all frequencies (focusing on 70  
73 dB SPL--the same sound level used for the displacement tests) the youngest, two week post maturity  
74 animals, had the highest neurophysiological response for lower frequencies in both sexes (under 10 kHz)  
75 (Fig 2A & B). This matches with the greater movement of the membrane at lower frequencies; for  
76 females, increased age decreased movement which is mirrored in the neurophysiology, but males show  
77 the same neurophysiology trend as the females (Fig 2) despite similar tympanal membrane movement  
78 among male age groups (Fig 1B). Within age group, there was a trend for the males to have a relatively  
79 higher neurophysiological response than the females especially between 2-5 kHz, though they were not  
80 significantly different at most frequencies (Fig S1).

81 Three frequencies (5, 10, & 15kHz) were examined in detail, across a 50 dB SPL range to determine  
82 any differences in neural activation and saturation with increasing sound levels. At 5kHz, female and  
83 male locusts had significantly higher neural responses for the lower sound levels (Fig 2C, S2 A-B).  
84 Between the sexes within each age group, there were practically no significant differences (Fig S2C-E);  
85 however there was a trend for the males to be more sensitive than the females at a given sound level.  
86 There were almost no significant differences at 10 kHz or at 15 kHz.

87 We then broadened our analysis to include low (50 dB SPL) and high (90 dB SPL) sound levels across  
88 all frequencies (Fig. 2D, S1 F-K, Suppl. Table S1). At low sound levels, there were very few cases of any  
89 significant differences among age groups for either sex or between sexes within age groups. At high  
90 sound levels (90 dB SPL), among females there was a significant difference based on age between 1-5  
91 kHz with a similar trend as at 70 dB SPL (Fig 2D) (e.g., 5kHz  $F_{2,14} = 6.79$ ,  $p = .011$ ; at 15kHz  $F_{2,14} = 1.31$ ,  $p =$   
92  $.31$ ). The males only showed significant differences for the higher sound level between 4.5-5 kHz and  
93 there were few differences between the sexes at each age group (Fig S1 F-K, Suppl. Table S1). However,  
94 evaluating the trend from 50, 70, to 90 dB SPL several patterns are observed (Fig 2D, S1F-K). First, the  
95 neurophysiological response separated into two clear groups with the lower frequencies (2-10 kHz)  
96 having the higher value. In addition, the lower frequencies had a more linear response while the higher  
97 frequencies (11-20 kHz) had a greater increase in their neurophysiological response after 70 dB SPL.  
98 Finally, patterns were similar for each age/sex combination; however, the older animals had a lower high  
99 frequency response for the lower sound level (Fig S1F-K).

100 Finally, we measured the latency for the neurobiological response (Fig 2E-L). Two week post  
101 maturity adults responded significantly faster for the lower frequencies (under 8 kHz) at 70 dB SPL for  
102 both males and females (Fig 2E & F) (e.g., at 5kHz female  $F_{2,14} = 9.28$ ,  $p = .004$ ; male  $F_{2,16} = 5.09$ ,  $p = .022$ ).  
103 The males responded faster than the females, but this was only significant at a few frequencies (Fig 2G &  
104 H). We next expanded the analysis to low and high sound levels (Fig 2 I-L, Table S2). As expected for low  
105 frequencies (<10 kHz) increasing sound levels decreased latency times for two week old animals (red and  
106 orange colours, Fig 2I & K). Surprisingly, in contrast, for high frequencies the two week animals at 70 dB  
107 SPL actually had an increase in latency compared to both the 50 and 90 dB SPL (green and purple  
108 colours, Fig 2I & K). Male locusts of the older age groups followed a similar, yet less dramatic trend (Fig  
109 2J). However, surprisingly, for older female locusts 70dB SPL was the slowest latency for most  
110 frequencies (Fig 2L).

111 Taken together, membrane movement and neurophysiology data show the effects of aging for adult  
112 locusts in their hearing response. At lower frequencies there is an increased neural-response between  
113 two week old adult locusts and the remaining age groups as well as shorter response latencies in the

114 neural activity. Interestingly, these data were not a direct reflection of the tympanal membrane  
115 displacement. Males showed little difference in their tympanal membrane displacements with age, yet  
116 significant differences in their neurobiological response. Females showed a constant decrease in  
117 tympanal movement with age, though it was not directly proportional to the change in their  
118 neurobiological response. We suggest the decrease in the neuro-response initially is likely due to a  
119 change in the cellular physiology of the animals, but also due to animal reproductive status.

120 As two week old locusts of both sexes have greater and faster neurobiological responses despite  
121 equal changes in male tympanal membrane movement; we suggest the two week old locust's tissues are  
122 in prime condition. Their ability to detect sounds at lower sound levels is superior to that of older  
123 animals, at low frequencies. As insects age, their tissues are known to lose elasticity and become harder  
124 (Ridgel and Ritzman, 2005), which consequently could affect the movement of internal air sacs and the  
125 neuronal attachment points involved in sensing tympanal movement. Additionally, some invertebrates  
126 are known to have a decrease in live dendrites with age (Kumar et al., 1998) and an increase in the  
127 threshold for action potential generation (Yeoman and Faragher, 2001) which could explain the observed  
128 results. Perhaps the hormones present in both sexes during the height of their mating time period may  
129 reduce their neurophysiology response.

130 Another component that affects locust hearing is sound travelling through the body creating a  
131 pressure-gradient receiver for lower frequencies (Miller, 1977). As adult female locusts age they develop  
132 eggs that increase their weight and decrease the volume available for air sacs to expand. We therefore  
133 measured their weight as they aged. We found females had a significant increase in weight with aging  
134 animals (2.15-2.8g,  $F_{4,39} = 5.44$ ,  $p = .002$ ) (Fig S4) while males displayed no significant differences, despite  
135 a slight decline with older ages ( $\sim 1.6$ g,  $F_{4,46} = 0.79$ ,  $p = .54$ ) (Fig S4). Previous work has shown that heavier  
136 animals have a higher threshold response to sound (Miller, 1977). Our work supports this, as females  
137 showed an increase in their body weight with age. Under natural conditions at approximately four weeks  
138 of age females begin laying their eggs. This could explain why we see such a large difference between the  
139 four week female age group as their bodies are probably the most dense. In captivity, locusts do not  
140 always have proper reproductive fitness (Uvarov, 1966) which is perhaps why we did not see a decline of  
141 weight with the oldest age groups.

142 The results of this study call attention to a number of important factors. First, there is a decline in  
143 hearing response with locust age. This means that the older locusts are more susceptible to being caught  
144 by predators. In addition, younger animals should be better at hearing each other (e.g., rustling of swarm  
145 mates). Secondly, animal reproductive condition should be a factor in studies. Our results clearly show  
146 that the female condition affects hearing more so than for the males. We suggest in this instance it is due  
147 to physical factors affecting the sound pathway. However, hormones and other factors may also create  
148 differences between sexes as they age. Furthermore, our results suggest, similar to others (Ridgel and  
149 Ritzman, 2005), that insects could be considered as a model of animal aging as they show aging effects,  
150 develop on a much faster time scale than vertebrates, and can be studied under controlled conditions.  
151 Our study emphasizes the need to pay attention to the age of invertebrate research animals, as age  
152 differences could confound results.

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## 156 **Methods**

### 157 **Animals**

158 Adult *Schistocerca gregaria* (Forskål) were obtained from Blades Biological Ltd, UK. The supplier  
159 provided animals with known final moult dates (either 1.5 or 3.5 weeks) keeping feeding conditions  
160 consistent between groups. Locusts were then fed organic lettuce and dried oats at the University of  
161 Strathclyde laboratory and were maintained on a 12 hr light-dark cycle at 24°C.

### 162 **Membrane Deflection**

163 Animals were weighed before trials. Membrane deflection trials followed a similar protocol to  
164 previous work (Gordon et al, 2014). Briefly, their right tympanal membrane was exposed to a micro-  
165 scanning Laser Doppler Vibrometer (PSV 300, Polytec, Waldbronn, Germany) with a close up unit (OFV  
166 056). A loudspeaker (ESS Air Motion Transformer, South El Monte, USA) was placed at least 10cm away.  
167 A microphone (Bruel & Kjaer 4138, Naerum, Denmark) was positioned to measure the sound pressure at  
168 the tympanal membrane. A broadband linear chirp was played at 60 and 70 dB SPL, generated by the  
169 laser vibrometer's control computer and then passed through an amplifier (TA-FE370, Sony, Tokyo,  
170 Japan). The FFT resolution was 12.5Hz and measurements averaged at least 15 times per point measured  
171 and later binned to 500Hz categories. Gain (displacement/sound pressure level) values were used for  
172 analysis to account for any differences in sound signal amplitude. Final sample size included (week-male,  
173 female): 2w-8m, 8f; 3w-6m, 6f; 4w-9m, 15f; 6w-11m, 8f; 8w-10m, 3f. Weeks four and above contained  
174 animals from different shipments. Data was analysed with an ANOVA in SPSS (IBM, Armonk, USA)  
175 grouping by sex or age.

### 176 **Neurophysiology**

177 Neurophysiology experiments followed the same protocol as previous work (Gordon et al, 2014).  
178 Briefly, the locust was mounted ventral-side up in dental beading wax (Kedment, DWS307, Purton, UK). A  
179 pair of hook electrodes, made from 50µm silver wire, were placed under the auditory nerve in the  
180 metathorax and insulated using petroleum jelly. The final sample size was: 2w--5m, 5f; 4w--5m, 5f; 8w--  
181 7m, 5f.

182 Sound was controlled and calibrated in a similar manner to the membrane deflection trials. The  
183 sound stimulus was created with a custom LabVIEW (National Instruments, version 8.5.1; Austin, USA)  
184 program, fed through a data acquisition system (National Instruments USB-6251 and BNC-2110). White  
185 noise (1-20kHz) was played for 30s before playing any of the experimental stimuli, to account for sensory  
186 adaptation. A sequence of tapered cosine-windowed (Tukey-windowed) pure-tone bursts were then  
187 played, ranging from 1-20kHz at 1kHz intervals over a 60dB (SPL) range from 40 to 100dB with 1dB step  
188 sizes.

189 Data were processed in LabVIEW, for details please see Gordon et al (2014). Briefly, the summed  
190 neural response of the auditory nerve was measured by calculating the root-mean-square (RMS) of the  
191 signal for the duration of the sound stimulus. Latency was calculated to the time the signal first exceeded  
192 20% of its maximum amplitude. Each individual dataset (several within an animal), comprising the  
193 responses to a full frequency range of 1-20kHz sound stimuli across all sound intensities, was normalized  
194 to the maximum amplitude of the RMS electrophysiological response in the dataset, to control for any  
195 change in signal intensity (excluding latency data, which remained as absolute values).

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201 Electrical Engineering at the University of Strathclyde.

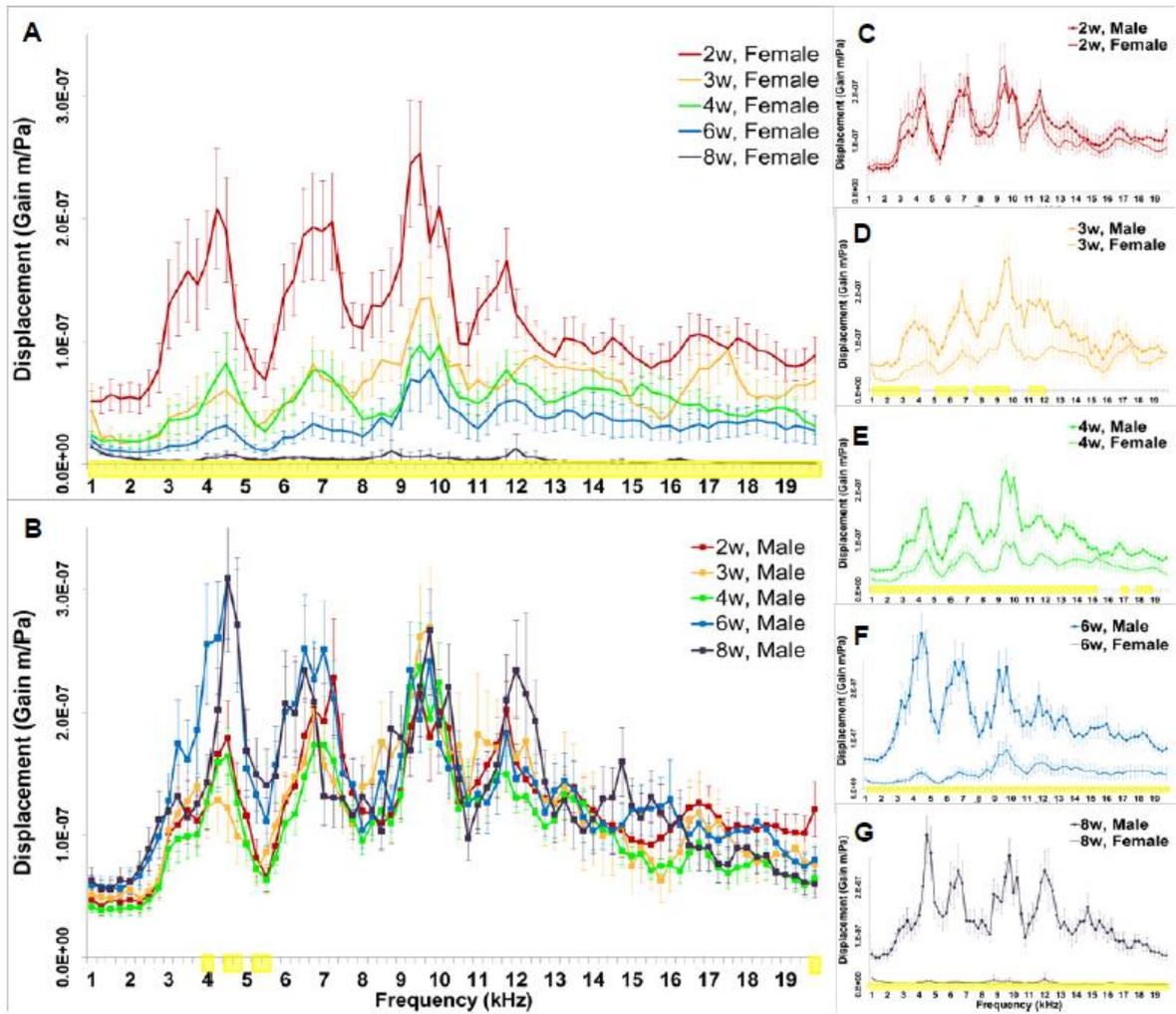
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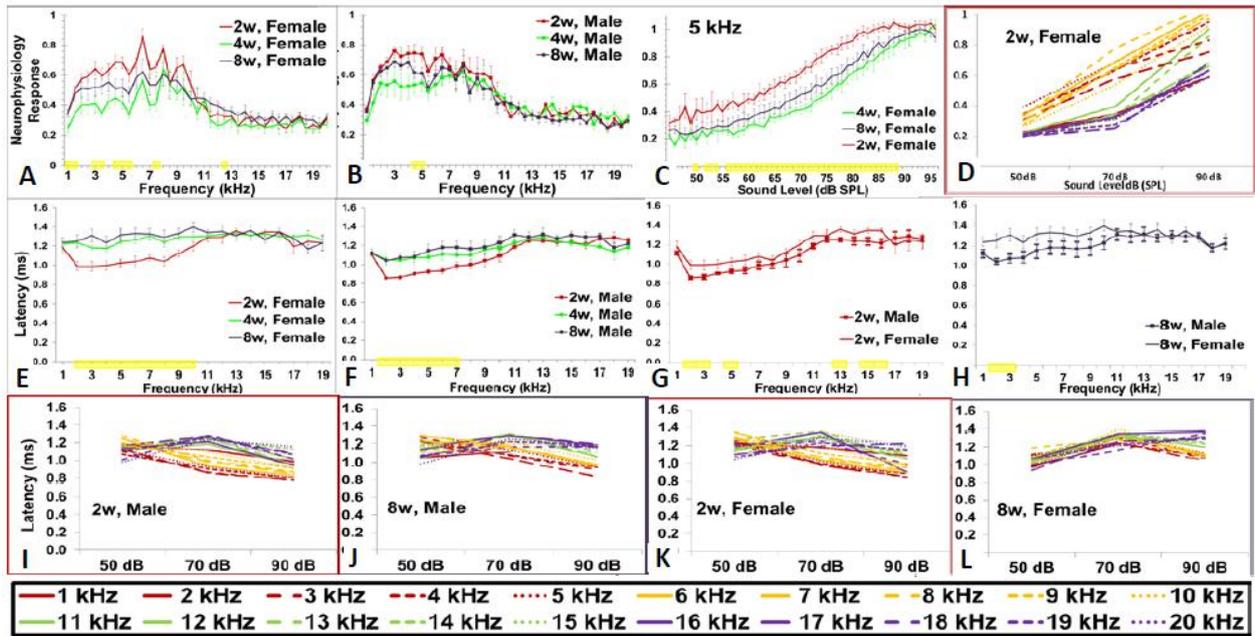
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229

230 **Figure 1.** Displacement (gain m/Pa) of the tympanal membrane with sound (1-20 kHz) of among ages for  
 231 (A) females, (B) males, and between sexes (C-G) at each age group: 2, 3, 4, 6, and 8 week post maturity  
 232 locusts. Age in weeks is indicated by colour, red is two weeks. Males are represented with lines with  
 233 squares. Yellow bar on the x-axis represents significance of 0.05 or less.

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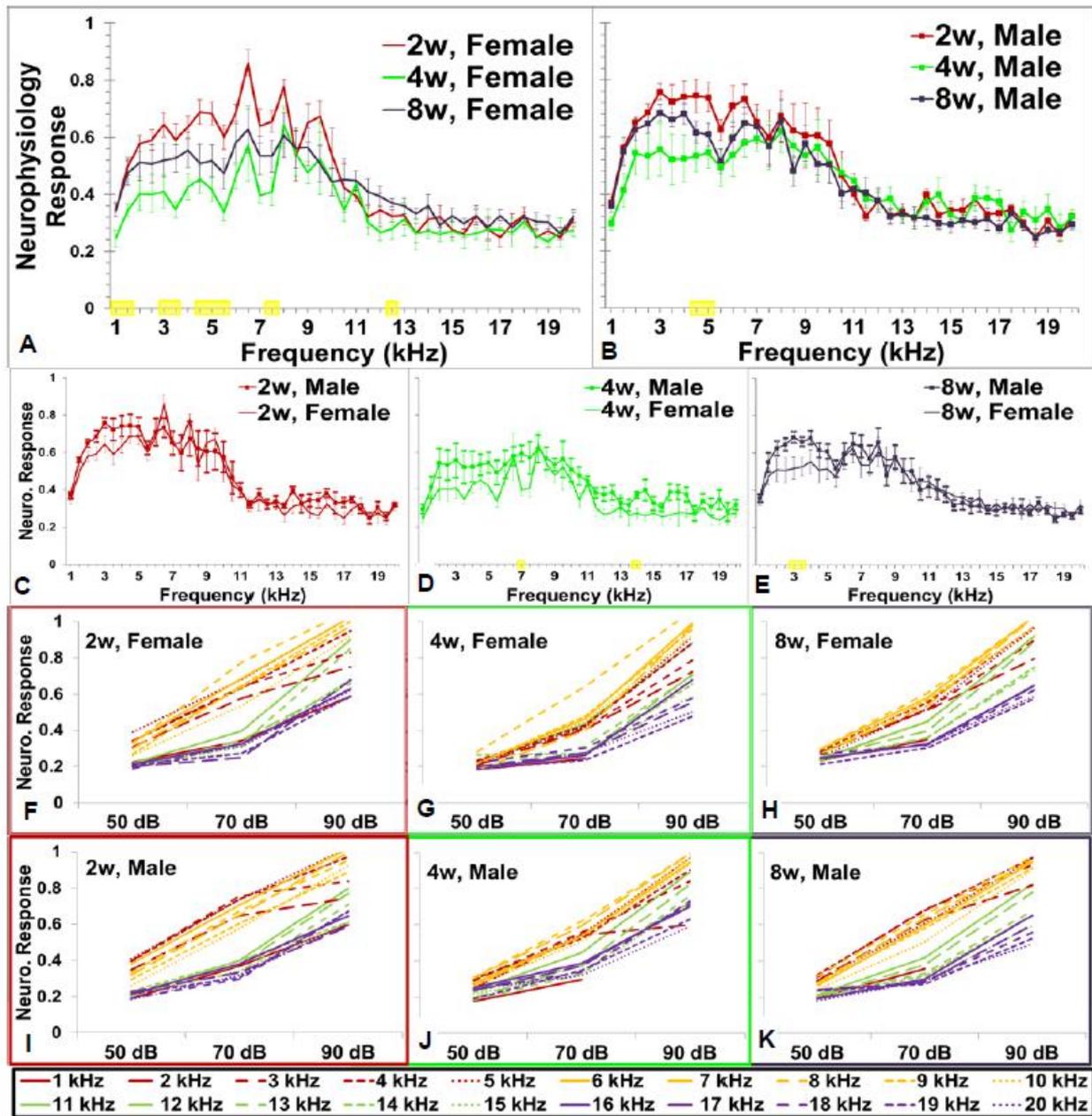


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236 **Figure 2.** Neurophysiological response of locusts. (A) Female and (B) male relative neural responses  
 237 across frequencies at 70 dB SPL. (C) Female response at 5 kHz across sound levels. (D) Female, 2 weeks  
 238 post maturity, response across frequencies at 50, 70, 90 dB SPL. Latency responses for (E) females and (F)  
 239 males at 70 dB SPL across frequencies. Latency response between males and females at 2 (G) and 8 (H)  
 240 weeks post maturity. Latency responses at three sound levels for (I) 2 week males, (J) 8 week males, (K) 2  
 241 week females, and (L) 8 week females. Yellow bars indicate significance < .05 for A-C, E-H. Key at the  
 242 bottom indicates the colour and pattern for each frequency for D, I-L.

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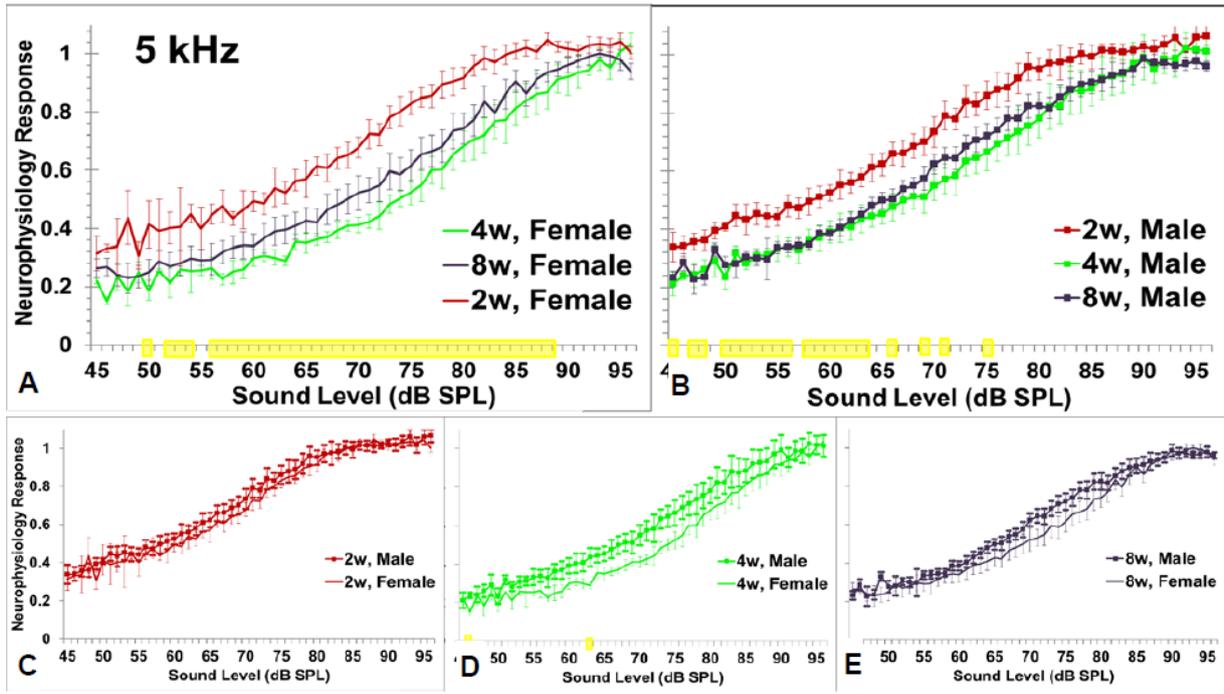
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246 **Figure S1.** Relative neurophysiological response at 70 dB SPL for (A) females, (B) males, (C-E) 2, 4, 8 week  
 247 post maturity locusts. Yellow bar on the x-axis represents significance of 0.05 or less. (F-K) Comparative  
 248 neurophysiology response at 50, 70, and 90 dB SPL within each age and sex group. Frequencies are  
 249 represented by colours: 1-5 kHz are red, 6-10 kHz are orange, 11-15 kHz are green, 16-20 kHz are purple.

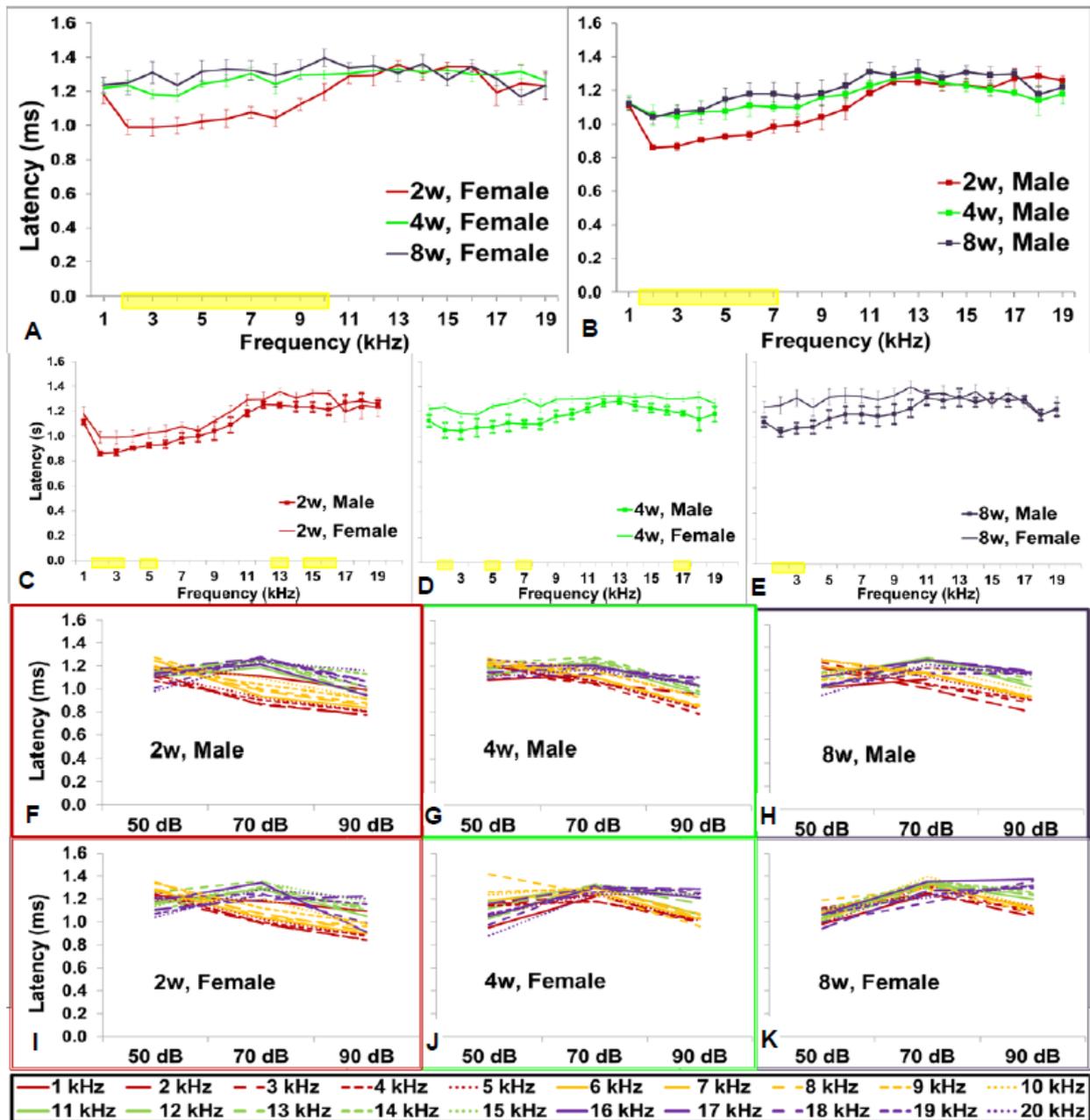
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252 **Figure S2.** Sample relative neurophysiological response for 5 kHz for (A) females, (B) males, (C-E) 2, 4, 8  
 253 week old locusts. Yellow bar on the x-axis represents significance of 0.05 or less.

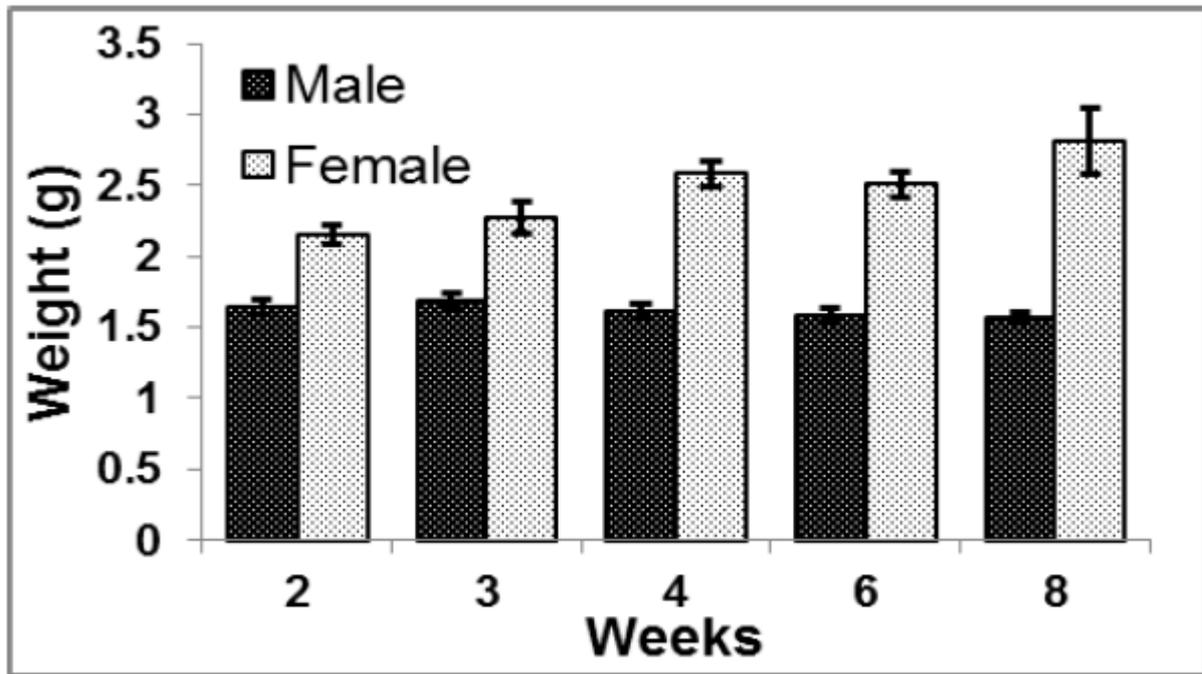
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255

256 **Figure S3.** Latency to the neurophysiological response at 70 dB SPL for (A) females, (B) males, (C-E) 2, 4,  
 257 8 week post maturity locusts. Yellow bar on the x-axis represents significance of 0.05 or less. (F-K)  
 258 Comparative latency response at 50, 70, and 90 dB SPL within each age and sex group. Frequencies are  
 259 represented by colours: 1-5 kHz are red, 6-10 kHz re orange, 11-15 kHz are green, 16-20 kHz are purple.

260



261

262 **Figure S4.** Weight (g) of males (dark bars) and females (light bars) for each age group. Weight  
263 significantly increased with female age ( $F_{4,39} = 5.44, p = .002$ ) but not males ( $F_{4,46} = 0.79, p = .54$ ).

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265

266