

1 **Developmental plasticity of hearing sensitivity in red-eared slider *Trachemys***
2 ***scripta elegans***

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9 **Abstract**

10 Developmental plasticity of hearing sensitivity (DPHS) has been verified in some
11 groups of vertebrates. Turtles face a trade-off between terrestrial and aquatic hearing
12 in different acoustic environments throughout ontogeny. However, how chelonian
13 hearing sensitivity changes throughout ontogeny is still unclear. To verify DPHS in
14 turtles, auditory brainstem responses (ABR) were compared using hearing thresholds
15 and latencies in female red-eared slider (*Trachemys scripta elegans*) aged 1 week, 1
16 month, 1 year, and 5 years, and the results showed hearing sensitivity bandwidths of
17 approximately 200–1100, 200–1100, 200–1300, and 200–1400 Hz, respectively. The
18 lowest threshold sensitivity was approximately 600 Hz. Below 600 Hz, ABR
19 threshold decreased rapidly with increasing age (1 week to 1 year), with significant
20 differences between age groups, but no significant difference between the 1- and
21 5-year age groups (stimulus frequency, 200–600 Hz). Above 600 Hz, ABR threshold
22 was the lowest in the 5-year age group. These findings show that aging was
23 accompanied by hearing sensitivity changes, suggesting rapid, frequency-segmented
24 development during ontogeny. This variability in hearing sensitivity differs from that
25 reported in other vertebrates, and allows adaptation to acoustically distinct
26 environments throughout ontogeny. Our findings further elucidate the developmental
27 patterns of the vertebrate auditory system.

28 **Keywords:** Auditory brainstem response; Developmental plasticity; Hearing
29 sensitivity; *Trachemys scripta elegans*

30 **Running title:** Acoustic communication of turtle

31 **1. Introduction**

32 From insects to mammals, acoustic communication is crucial for survival,
33 successful reproduction, and many other life-history strategies [1-3]. Usually, changes
34 in the auditory system cause changes in sensitivity during ontogeny; these can have a

35 profound impact on an organism's hearing [4, 5]. Moreover, the development of
36 hearing sensitivity allows accurate and efficient acoustic communication in
37 individuals [4].

38 Developmental plasticity of hearing sensitivity has been verified in fishes [6, 7],
39 frogs [8], lizards [9], birds [10, 11], mammals [12, 13], and humans [14], suggesting
40 that auditory processing matures with age, and that this process differs between
41 species. Turtles, like other amphibious animals, face a trade-off between terrestrial
42 and aquatic hearing, and their acoustic environment changes during ontogeny; they
43 may have evolved variability in hearing capacity to adapt to complex environments
44 [15, 16]. Although there has been considerable research into auditory system
45 development in some groups of vertebrates, much less is known about it in chelonians.
46 At present, we know of only three studies on the developmental plasticity of hearing
47 sensitivity in chelonians, and these reached different conclusions [17-19]. However,
48 these studies had small samples ($n < 7$), narrow age ranges, and unclear sexual
49 categorization, factors that may affect their findings. Our previous study provided the
50 first evidence that sexually dimorphic hearing sensitivity has evolved in turtles, with
51 the hearing of females shows greater sensitivity [20]. It is necessary to further study
52 ontogenetic changes in hearing sensitivity in chelonians.

53 Red-eared slider (*Trachemys scripta elegans*) is a semi-aquatic freshwater turtle,
54 well adapted to living in various habitats, including rivers, streams, and even brackish
55 water (Salinity 5.3–14.6‰) [21]. *Trachemys scripta elegans* is an important and
56 potentially powerful model for researching hearing. Aspects of hearing, including the
57 general ultrastructure of the auditory receptors (auditory hair cells) [22], the
58 functional morphology of cochlear hair cell stereociliary bundles [23], the structure of
59 the sound receiver organ (the tympanic disc) [15], habitat-related auditory plasticity
60 [15], the response properties of auditory hair cell afferent fibers [24], the morphology
61 of the middle-ear cavity [25], and sexually dimorphic hearing sensitivity [20], have
62 been widely studied in this species. Those results provide an appropriate foundation
63 and a reliable model organism to assess the developmental plasticity of hearing
64 sensitivity in chelonians. Moreover, *T. scripta elegans* is farmed in many provinces of
65 China; hence, sufficient numbers of experimental specimens were available.

66 Auditory brainstem response (ABR) measurement is a noninvasive and rapid
67 method to measure hearing sensitivity; its use has been validated for frogs [26, 27],
68 toads [28], and reptiles [18, 29]. Our aim was to demonstrate that aging is

69 accompanied by changes in turtle hearing sensitivity by measuring ABR, to assess
70 ontogenetic changes in post-hatchling-to-reproductive *T. scripta elegans* adults. To do
71 this, we focused on the hearing sensitivity bandwidth, threshold sensitivity, and
72 latency.

73

74 **2. Materials and methods**

75 **2.1 Experimental animals**

76 Considering that sexually dimorphic hearing sensitivity has evolved in turtles, only
77 female *T. scripta elegans* individuals were used. We used age groups of 1 week (n =
78 10), 1 month (n = 11), 1 year (n = 10), and 5 years (n = 10). All animals were
79 purchased from farms in Hainan Province, China, and maintained in standard aquaria
80 at 20–25 °C until experiments were conducted. Body mass and carapace length are
81 shown in Figure 1. Because we could not determine sex using external morphology,
82 sex was determined by paraffin section of toe phalanges of 1-week and 1-month old
83 individuals. Ages of individuals < 1 year old were determined by time since hatching.
84 Ages of 5-year-old individuals were determined after the experiments by observing
85 paraffin sections. Prior to electrode placement, each turtle was deeply anesthetized
86 using a solution of 0.5% pelltobarbitalum natricum (CAS No.: 57-33-0, Xiya
87 Reagents, Shandong, China) dissolved in 0.9% sodium chloride. The anesthetic was
88 administered via hind limb intramuscular injection at an initial dose of 0.003 mL g⁻¹.
89 Additional doses (each at 20% of the initial dose) were administered in cases when
90 the subject was not deeply anesthetized [20]. The electrophysiological experiments
91 began after the subject showed no pain response to stimulating the hind leg muscles
92 with forceps.

93 **2.2 ABR measurements**

94 **2.2.1 ABR procedures**

95 ABR recordings of approximately 100 min were made using a TDT RZ6 Multi-I/O
96 Processor, linked via fiber optic cables to a Tucker-Davis Technologies (TDT) RA4LI
97 low-impedance digital headstage and RA4PA Medusa preamp; and analyzed using
98 BioSig and SigGen software (Tucker-Davis Technologies, Inc., FL, USA). A portable
99 amplified field speaker (frequency response 55–20 000 Hz, JBL GT7-6, Harman
100 International Industries, Inc., USA) was located in a sound-proof booth lined with
101 echo-attenuating acoustic foam, linked via fiber optic cables to a TDT RZ6 Multi-I/O
102 Processor. Standard platinum alloy subdermal needle electrodes (27 ga, 13 mm length,

103 Rochester Electro-Medical, Inc., Lutz, FL, USA) were inserted sub-dermally above
104 the tympanum (recording electrode), on the top of the head under the frontal scale
105 (reference electrode), and in the ipsilateral front leg (ground electrode), and the other
106 end of each needle was connected to the TDT RA4LI low-impedance (<3 k Ω) digital
107 headstage.

108 **2.2.2 Stimulus generation and presentation**

109 Sound stimuli were generated using a TDT RZ6 Multi-I/O Processor, which
110 directly drove the speaker, running TDT SigGen software. Each individual tone burst
111 (9 ms duration, 2 ms rise/fall time, with a sample rate of 24 414 Hz and alternating
112 polarity) was synthesized digitally from 0.2 kHz to 1.5 kHz in 100 Hz increments, and
113 attenuated in 5 dB steps from 90 dB to 35 dB, and presented at a rate of 4/s. Clicks
114 were 0.1 ms in duration with a 249 ms interstimulus interval, attenuated in 5 dB steps
115 from 90 dB to 35 dB, and presented at a rate of 4/s. Each ABR wave represented the
116 average response to 200 stimulus presentations. Signals from the electrodes were
117 amplified (20 \times) and filtered (high pass: 30 Hz; low pass: 3 kHz; notch filtered: 50 Hz).
118 Sound stimuli were replicated once.

119 **2.2.3 Calibration**

120 ABR stimulus levels were calibrated in the free field using a TDT RZ6 Multi-I/O
121 Processor and BioSigRP (Tucker-Davis Technologies, Inc., Florida, USA), linked to a
122 sensor signal conditioner (model 480C02, PCB Piezotronics, Inc., Depew, NY, USA)
123 with a 1/4 inch microphone (model 426B03 PCB Piezotronics, Inc., Depew, NY, USA)
124 positioned approximately at the tip of jaw of the turtle, but when the turtle was absent.
125 The distance between the speaker and the microphone was approximately 5 cm. The
126 speaker repeatedly played the signal at the same rate used while recording ABRs, and
127 simultaneously recorded the microphone signal at a sampling rate of 24 414 Hz.

128 **2.2.4 ABR thresholds and latencies define**

129 The ABR thresholds and latencies were determined using visual inspection similar
130 to that previously described [29]. The threshold measurement was defined as the
131 stimulus level below which no repeatable responses could be recognized [30, 31]. In
132 order to reduce artificial error, all turtle ABR thresholds were determined by the same
133 experienced person. We assumed that the 80 dB level was above the ABR threshold of
134 all turtles studied, for the stimuli used.

135 **2.3 Morphological data measured**

136 Before ABR recordings, the body mass of all specimens was recorded by an

137 electronic balance (SI-234, Denver Instrument (Beijing) Co., Ltd.), while carapace
138 length was measured using a Mitutoyo digital caliper (500-196-30, Mitutoyo Corp.,
139 Japan).

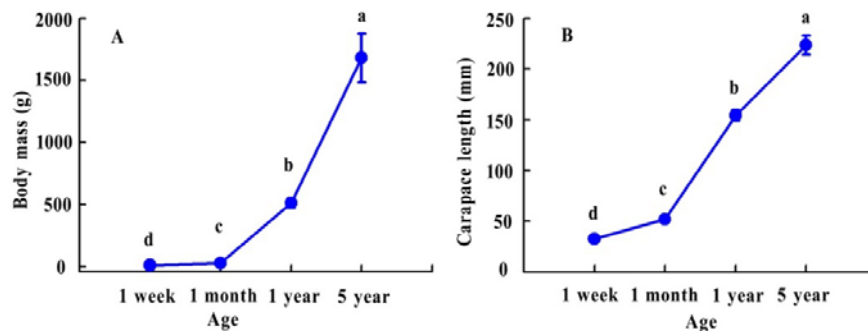
140 **2.4 Data analysis and statistics**

141 ABR morphologies, sensitivity thresholds, and latencies obtained from female *T.*
142 *scripta elegans* in response to tone and click stimuli were sorted and analyzed using
143 IBM SPSS 22.0 (IBM Corp., Chicago, IL, USA). Data on body mass and carapace
144 length of different age groups were analyzed using one-way ANOVA followed by
145 Tukey multiple comparison testing. General linear model multivariate analysis
146 following by Tukey multiple comparison testing was used to determine the
147 significance of differences in ABR thresholds and latencies among age groups at each
148 stimulus frequency. Results are expressed as mean \pm SD, and $P < 0.05$ was considered
149 to reflect statistically significant difference.

150 **3. Results**

151 **3.1 Morphological characteristics**

152



153

154 Figure 1. *Trachemys scripta elegans* body mass (A) and carapace length (B) varied
155 with age. Points and error bars reflect the mean \pm SD.

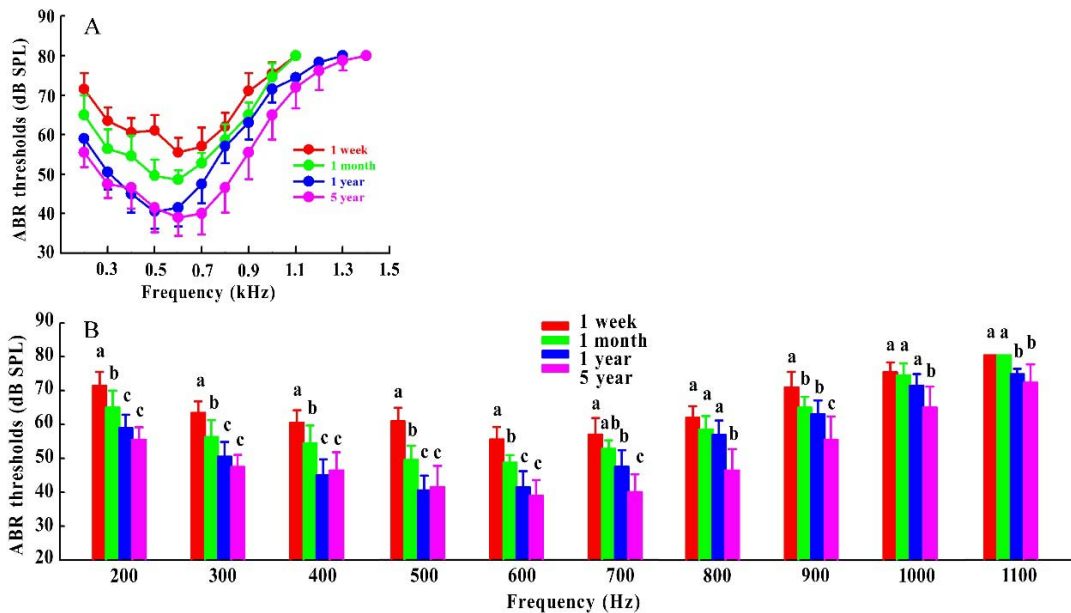
156 Body mass was significantly influenced by age ($F = 8119.45$, $df = 3$, $P < 0.001$),
157 and differed significantly among age groups ($P < 0.001$). Carapace length became
158 significantly longer with age ($F = 5816.28$, $df = 3$, $P < 0.001$), and differed
159 significantly among age groups ($P < 0.001$).

160

161 **3.2 Hearing sensitivity bandwidth and ABR thresholds**

162 The hearing sensitivity bandwidths were 200–1100 Hz, 200–1100 Hz, 200–1300
163 Hz, and 200–1400 Hz in the 1-week, 1-month, 1-year, and 5-year age groups,
164 respectively (Fig. 2A). The greatest sensitivity frequency was about 200–900 Hz,

165 and the lowest sensitivity measured was at about 600 Hz (Fig. 2A).



166

167 Figure 2. ABR threshold varied with stimulus frequency and age group in *Trachemys*
168 *scripta elegans* (A); ABR threshold by stimulus frequency and age group in *T. scripta*
169 *elegans* (B).

170 Below 600 Hz, the ABR threshold decreased rapidly with increasing age (1 week to
171 1 year), with significant differences among age groups, but no significant difference
172 between the 1- and 5-year age groups, for which the stimulus frequency ranged from
173 200 Hz to 600 Hz (Fig. 2B). Above 600 Hz, the ABR threshold was significantly
174 lower in the 5-year age group than in other groups (Fig. 2B).

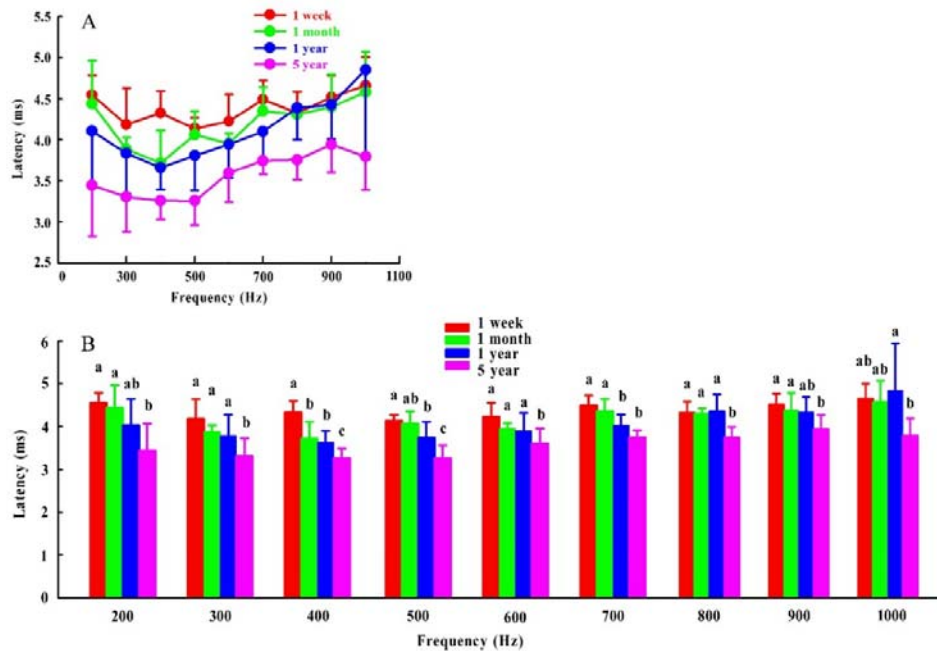
175

176 3.3 ABR latency

177 The ABR latency for tone bursts at 75 dB was <5 ms in all age groups (Fig. 3A).

178 The ABR latency for tone bursts was lowest in the 5-year age group (Fig. 3B).

179



180

181 Figure 3. Latency against tone-burst frequency at 75 dB, by age group, in *Trachemys*
182 *scripta elegans* (A); Latency by stimulus frequency and age group in *T. scripta*
183 *elegans* (B).

184

185 4. Discussion

186 Herpetologists have long considered turtles and tortoises to be “the silent group”,
187 neither vocalizing nor hearing particularly well [32, 33]. However, the hearing
188 sensitivity of chelonians has recently begun to attract attention, and behavioral and
189 electrophysiological studies have revealed that turtles and tortoises are low-frequency
190 specialists (usually <1000 Hz) [16, 34, 35]. We found that the turtles could not hear
191 frequencies >1400 Hz, and that the upper limit of the hearing sensitivity bandwidth
192 shifted higher during ontogeny.

193 Although habitat-related auditory plasticity has been reported for *T. scripta elegans*
194 [15], little is known about the development of the auditory system in this group. To
195 our knowledge, ours is the first study to demonstrate developmental plasticity of
196 peripheral hearing sensitivity from the post-hatchling to reproductive adult stage in
197 chelonians. We found that the ABR threshold was significantly lower in the 1-week to
198 1-year age groups than in the 5-year age group. Remarkably, frequency sensitivity
199 was developmentally segmented by 600 Hz: below this, the ABR threshold did not
200 differ among the 1- to 5-year age groups; above this, it was significantly lower in the
201 1-year than 5-year age group. We thus conclude that ontogenetic hearing sensitivity

202 development is rapid and frequency-segmented in *T. scripta elegans*. Our findings
203 differ somewhat from those reported elsewhere. For instance, 3-year-old Hawksbill
204 turtles (*Eretmochelys imbricata*) were sensitive to a wider frequency range and
205 exhibited a larger amplitude response than the 2-year-olds [18]; loggerhead sea turtles
206 (*Caretta caretta*) exhibited little difference in threshold sensitivity and frequency
207 bandwidth throughout ontogeny [19]; and in subadult Green turtles (*Chelonia mydas*),
208 smaller individuals had a wider hearing sensitivity bandwidth (100–800 Hz) than
209 larger individuals (100–500 Hz) [17]. Our study improves on earlier studies,
210 potentially providing more reliable results, because we used a larger age range, and,
211 more importantly, we first determined the sex of individuals.

212 In the Bicolor damselfish (*Pomacentrus partitus*) the auditory thresholds decrease
213 exponentially with increasing age, rapidly approaching adult levels [36]. Hearing
214 sensitivity changes only slightly during growth of the Lusitanian toadfish
215 (*Halobatrachus didactylus*) [37]. In budgerigars (*Melopsittacus undulatus*), hearing is
216 poor at hatching, and thresholds improve markedly in the first week; by 1 week before
217 fledging, ABR audiograms of young budgerigars are very similar to those of adult
218 birds [11]. In some mammals, hearing sensitivity is weak at birth and gradually
219 develops during the first weeks of life [38, 39], although other species, such as
220 *Phyllostomus discolor* bats [13] and humans [40], have a well-developed auditory
221 system at birth and even before birth. Our findings for *T. scripta elegans*, which
222 include hysteresis in ontogenetic auditory development, therefore differ from those
223 reported in other vertebrates. Our results further suggest that the time required to
224 achieve a final level of auditory maturation varies among species.

225 Three possible mechanisms of developmental plasticity of peripheral hearing
226 sensitivity in vertebrates have been reported. First, in frogs, the size of the tympanic
227 membrane may be linked to differences in hearing sensitivity [41], and in some
228 species of lizards, increased body size (or age) is accompanied by functional changes
229 in the auditory periphery [9]. For *T. scripta elegans*, the tympanic disc is the key
230 sound receiver [15]. However, we have found that the size of the tympanic membrane
231 is not related to sexual dimorphism in hearing sensitivity in turtles [20]; thus, growth
232 of the tympanic membrane during ontogeny may not explain the developmental
233 plasticity of hearing sensitivity that we report herein. Second, age-related changes in
234 middle-ear sound conduction occur [9]; once the structural development of the middle
235 ear is complete, adult-like sound conduction is exhibited [9, 42-44]. Research into

236 habitat-related plasticity of hearing sensitivity has shown that *T. scripta elegans* is
237 more sensitive to sound underwater than in the air, and that this is related to the large
238 size of the middle ear [15]. Consequently, age-related changes in middle-ear sound
239 conduction may also contribute to age-related alterations in threshold sensitivity in *T.*
240 *scripta elegans*. Third, the sensory epithelium of the cochlear receptor organ may
241 increase in size throughout life. In frogs and fishes, the area of auditory receptor
242 epithelium increases with age, and cochlear growth is accompanied by an increase in
243 the number of hair cells on the sensory surface [9, 45-47]. This suggests that the
244 number of hair cells is related to developmental plasticity of hearing sensitivity in *T.*
245 *scripta elegans*. Future morphological and anatomical research should address these
246 questions.

247 Adult female turtles spend more time on land during the reproductive period, when
248 laying and incubating; their improved hearing may enable them to adapt to the
249 complex terrestrial environment. Hence, the rapid and frequency-segmented
250 development of the chelonian auditory system during ontogeny reflects adaptation to
251 acoustically distinct environments, and prepares the adults for reproduction.

252 In conclusion, we found that there was rapid and frequency-segmented
253 development of the auditory system during ontogeny in *T. scripta elegans*. This differs
254 from what has been reported for other vertebrates. Our findings provide greater clarity
255 on the patterns of development of the vertebrate auditory system. It is still unknown
256 whether age-related changes in middle-ear structures and in the auditory receptor
257 epithelium lead to developmental plasticity of peripheral hearing sensitivity in this
258 species. Further, as sexually dimorphic hearing sensitivity has been found in turtles, it
259 is worth investigating whether they also exhibit sexual dimorphism in developmental
260 plasticity of hearing sensitivity. Future morphological and anatomical studies should
261 address these questions.

262

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266 **Competing interests**

267 The authors declare that there are no competing interests.

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272

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