

Ontogenetic development of hearing sensitivity to airborne sound in the female red-eared slider, *Trachemys scripta elegans*

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ABSTRACT:

Ontogenetic development of hearing sensitivity has been verified in many groups of vertebrates, but not turtles. Turtles exhibit sexual dimorphism in hearing. To examine the development of hearing in female turtles, auditory brainstem responses (ABR) were compared by assessing the hearing-sensitivity bandwidth, ABR threshold, and latency of female *Trachemys scripta elegans* aged 1 week, 1 month, 1 yr, and 5 yr. The hearing-sensitivity bandwidths were 0.2–1.1, 0.2–1.1, 0.2–1.3, and 0.2–1.4 kHz in each age group, respectively. Below 0.6 kHz, the ABR threshold decreased from the 1-week to 1-yr age group, with a significant difference between age groups. No significant difference was detected between the 1- and 5-yr age groups (within a stimulus frequency of 0.2–0.6 kHz). Above 0.6 kHz, ABR thresholds decreased significantly from the 1-yr to 5-yr age group (within a stimulus frequency of 0.7–1.0 kHz). There was no significant difference between the 1-month and 1-yr age groups (within a stimulus frequency of 0.7–1.0 kHz), or between the 1-week and 1-month age groups (within a stimulus frequency of 0.7–1.0 kHz, except 0.9 kHz). Thus, female turtle hearing shows frequency-segmented development.

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I. INTRODUCTION

From insects to mammals, acoustic communication is crucial for survival, successful reproduction, and many other life history strategies (Gerhardt and Huber, 2002; Köppl *et al.*, 2014; Suthers *et al.*, 2016). Usually, changes in the auditory system cause changes in auditory sensitivity during ontogeny; these can have a profound impact on an organism's hearing (Werner *et al.*, 2011). Studies of the ontogeny of hearing sensitivity have revealed changes in the auditory system with aging and have improved our understanding of auditory adaptation in different acoustic environments. The development of hearing sensitivity allows accurate and efficient acoustic communication among individuals (Werner *et al.*, 2011).

Ontogenetic development of hearing sensitivity has been verified in fishes (Wysocki and Ladich, 2001; Higgs, 2002), frogs (Boatright-Horowitz and Simmons, 1995), lizards (Werner *et al.*, 1998), birds (Aleksandrov and Dmitrieva, 1992; Brittan-Powell and Dooling, 2004), mammals (Rübsamen *et al.*, 1989; Linnenschmidt and Wiegrebe, 2019), and humans (Johnson *et al.*, 2008). There are different patterns in hearing sensitivity throughout ontogeny. These include (1) increases (Corwin, 1983; Rübsamen, 1992) or the absence of significant changes (Wang *et al.*, 2015) in hearing-sensitivity bandwidth; (2) increases (Kenyon, 1996; Wysocki and Ladich, 2001), decreases

(Egner and Mann, 2005), or negligible change (Higgs *et al.*, 2002; Higgs *et al.*, 2003) in threshold sensitivity; and (3) increases (Reimer, 1995) or decreases (Boatright-Horowitz and Simmons, 1995) in the most sensitive frequencies.

Turtles, like amphibious animals, face changes between terrestrial and aquatic hearing, and their acoustic environment changes during ontogeny; therefore, they may have evolved plasticity in hearing to adapt to complex acoustic environments (Christensen-Dalsgaard *et al.*, 2012; Martin *et al.*, 2012). Despite considerable research into auditory system development in many groups of vertebrates, little is known about it in chelonians. At present, few studies comparing the hearing sensitivity of different age groups in chelonians, and these reached different conclusions (Bartol and Ketten, 2006; Yudhana *et al.*, 2010; Lavender *et al.*, 2014). However, these studies had small sample sizes ($n < 7$), narrow age ranges, and unclear sexual categorization; all these factors may affect the results. Our previous study provided the first evidence that turtle hearing shows sexual dimorphism, with the hearing of females showing greater sensitivity (Wang *et al.*, 2019b). Thus, further research into the ontogenetic development of hearing sensitivity in turtles is required.

The red-eared slider (*Trachemys scripta elegans*) is a semi-aquatic, freshwater turtle, well adapted to a variety of habitats, including rivers, streams, and even brackish water (salinity 5.3–14.6‰; Ma and Shi, 2017). It is an important and potentially powerful model for studying hearing in turtles. Hearing has been widely studied in this species with

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regard to the general ultrastructure of the auditory receptors (auditory hair cells; Sneyry, 1988), functional morphology of cochlear hair cell stereociliary bundles (Hackney *et al.*, 1993), structure of the sound receiver organ (the tympanic disk; Christensen-Dalsgaard *et al.*, 2012), habitat-related auditory plasticity (demonstrating higher sensitivity in water than in air; Christensen-Dalsgaard *et al.*, 2012), responses of auditory hair cell afferent fibers (Schnee *et al.*, 2013), morphology of the middle-ear cavity (Willis *et al.*, 2013), and sexually dimorphic hearing sensitivity (higher sensitivity in females than in males; Wang *et al.*, 2019). The findings of these studies provide an appropriate foundation and a reliable model organism to assess the ontogenetic development of hearing sensitivity in chelonians. Moreover, *T. scripta elegans* is farmed in many provinces of China; hence, sufficient experimental specimens were available.

The auditory brainstem response (ABR) measurement is a noninvasive and rapid method used to measure hearing sensitivity; its use has been validated for frogs (Wang *et al.*, 2016; Cui *et al.*, 2017), toads (Wang *et al.*, 2019a), and reptiles (Brittan-Powell *et al.*, 2010; Yudhana *et al.*, 2010). Our aim was to determine if development is accompanied by changes in turtle hearing sensitivity by measuring ABR, to assess ontogenetic changes groups ranging from post-hatchling to reproductive *T. scripta elegans* adults. To do this, we focused on the hearing-sensitivity bandwidth, threshold sensitivity, and latency.

II. MATERIALS AND METHODS

A. Experimental animals

Considering that sexually dimorphic hearing sensitivity has already been confirmed in turtles, only female *T. scripta elegans* individuals were used. We used age groups of 1 week ($n = 10$), 1 month ($n = 11$), 1 yr ($n = 10$), and 5 yr ($n = 10$). All animals were purchased from farms in Hainan Province, China, and maintained in aquaria until the experiments were conducted. Body mass and carapace length are shown in Fig. 1. Because we could not determine the sex of

1-week-old and 1-month-old individuals using external morphology, their sex was determined using a paraffin section of a gonad. Age of individuals <1 yr old were determined by time since hatching. Ages of 5-yr-old individuals were determined after the experiments, by observing paraffin sections of toe phalanges. Prior to electrode placement, each turtle was deeply anesthetized using a solution of 0.5% Pelltobarbitalum Natricum (CAS No.: 57-33-0, Xiya Reagents, Shandong, China) dissolved in 0.9% sodium chloride. The anesthetic was administered *via* hind limb intramuscular injection at an initial dose of 0.003 ml g^{-1} . Additional doses (each at 20% of the initial dose) were administered in cases when the subject was not deeply anesthetized (Wang *et al.*, 2019b). The electrophysiological experiments began after the subject showed no pain response to stimulating the hind leg muscles with forceps. The animal treatment procedures were approved by the Animal Research Ethics Committee of Hainan Provincial Education Centre for Ecology and Environment, Hainan Normal University (HNECEE-2018-001) and were carried out in strict accordance with institutional guidelines.

B. ABR measurements

1. ABR procedures

ABR recordings were acquired after the turtles were deeply anesthetized. Acoustic signals were played through a portable amplified field speaker with a frequency response from 55 Hz–20 kHz (JBL GT7-6, Harman International Industries, Inc., USA) with a centered 0° azimuth with respect to the animal, at a distance of 5 cm from the ear pinna.

ABR recordings were acquired using a TDT RA4LI low-impedance digital headstage and RA4PA Medusa pre-amp with three needle electrodes (27 ga, 13 mm in length, Rochester Electro-Medical, Inc., Lutz, Florida, USA), inserted subdermally above the tympanum (recording electrode); on the top of the head under the frontal scale (reference electrode); and in the ipsilateral front leg (ground electrode). Electrode impedances were less than 3 k Ω . The

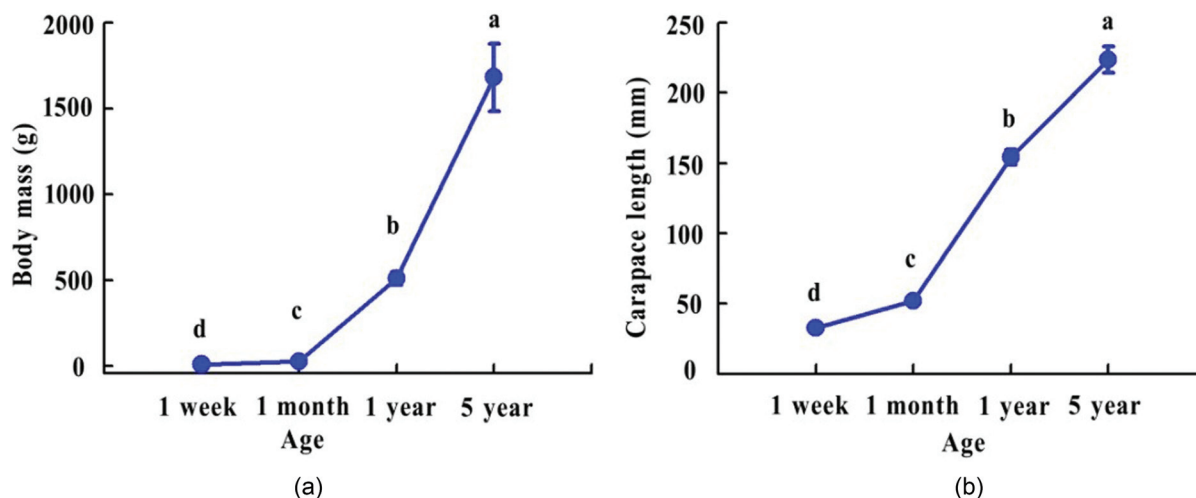


FIG. 1. *Trachemys scripta elegans* body mass (a) and carapace length (b) varied with age. Points and error bars reflect the mean \pm SD.

responses were amplified (20×), filtered (0.03–3 kHz), and averaged using BioSig software and the System III hardware (TDT) data-acquisition system (Scott *et al.*, 2017). All recordings were undertaken in a soundproof booth lined with echo-attenuating, acoustic foam.

2. Stimulus generation and presentation

Stimuli and ABR recordings were generated digitally and controlled using a TDT RZ6 Multi-I/O Processor, linked *via* fiber optic cables to a TDT RA4LI low-impedance digital headstage and RA4PA Medusa preamp, and linked to a laptop computer running BioSig/SigGen software (Tucker-Davis Technologies, Inc., Florida, USA). Two types of stimuli, tone pips and clicks, were generated, and delivered through the portable amplified field speaker, which was driven by the TDT RZ6 Multi-I/O Processor. Stimuli were synthesized digitally from 0.2 to 1.5 kHz with 100 Hz increments (9 ms duration, 2 ms rise/fall time, a sample rate of 24 414 Hz, with alternating polarity) at a rate of 4 per second, attenuated in 5 dB steps from 85 dB to 30 dB sound pressure level (SPL). All stimulus frequencies were played from low to high frequency. Clicks were 0.1 ms in duration with a 249 ms interstimulus interval, attenuated in 5 dB steps from 85 dB to 30 dB SPL, and presented at a rate of 4/s. We assumed that 80 dB was a higher level than the ABR thresholds of all turtles for the stimuli used. The ABR thresholds and latencies were determined using visual inspection, as described by Brittan-Powell *et al.* (2002). ABR latencies were recorded as the time from stimulus onset to the first negative waveform valley.

3. Calibration

ABR stimulus levels were calibrated using a sensor signal conditioner (model 480C02, PCB Piezotronics, Inc., New York, USA) with a 1/4" microphone (model 426B03 PCB Piezotronics, Inc., New York, USA) positioned at the location of the turtle's head. The distance between the speaker and the turtle's head was fixed at 5 cm. Calibrations were made using a TDT RZ6 Multi-I/O Processor and BioSigRP (Tucker-Davis Technologies, Inc., Florida, USA), which repeatedly played the signal at the same rate used while recording ABR, and simultaneously recorded the hydrophone signal at a sampling rate of 24 414 Hz.

C. Morphological data measured

Before ABR recording, the body mass of all specimens was recorded using an electronic balance [SE 3001FZH, Ohaus instrument (Shanghai) Co., Ltd., Shanghai, China, or SI-234, Denver instrument (Beijing) Co., Ltd., Beijing, China]. The carapace length was measured using a Mitutoyo digital caliper (500–196–30, 0.01 mm, Mitutoyo Corp., Japan, or 500–151–30, 0.01 mm, Mitutoyo Corp., Japan).

D. Data analysis and statistics

Prior to statistical analysis, the assumptions of normality and homogeneity of variance in the data were examined using the Shapiro-Wilk and Levene tests, respectively. ABR morphologies, ABR thresholds, and latencies obtained from female *T. scripta elegans* in response to tone or click stimuli were sorted and analyzed using SPSS22.0 (IBM Corp., Chicago, IL, USA). Data on body mass and carapace length among the different age groups were analyzed using a one-way analysis of variance (ANOVA). A repeated-measures factorial ANOVA was used to determine the significance of the ABR threshold and latency at each stimulus frequency among different age groups. Differences of the ABR threshold or latency at the same stimulus frequency among different age groups was analyzed using multivariate analysis of a general linear model followed by a Tukey *post hoc* test. Results were expressed as mean \pm SD. P values <0.05 were considered statistically significant.

III. RESULTS

A. Morphological characteristics

Body mass was influenced by age ($F = 648.78$, $df = 3$, $P < 0.001$), and differed among age groups ($P < 0.001$) [Fig. 1(a)]. Carapace length increased with age ($F = 2734.05$, $df = 3$, $P < 0.001$), and differed among age groups ($P < 0.001$) [Fig. 1(b)].

B. Hearing-sensitivity bandwidth and ABR threshold

T. scripta elegans had U-shaped ABR-derived audiograms and a threshold that was above 35 dB at all stimulus frequencies [Fig. 2(a)]. The hearing-sensitivity bandwidths were 0.2–1.1 kHz, 0.2–1.1 kHz, 0.2–1.3 kHz, and 0.2–1.4 kHz in the 1-week, 1-month, 1-yr, and 5-yr age groups, respectively [Fig. 2(a)]. The greatest sensitivity frequency was 0.2–0.9 kHz, and the lowest sensitivity was at 0.6 kHz [Fig. 2(a)].

There was significant difference in ABR threshold among the different age groups ($F = 43.88$, $df = 3$, $P < 0.001$). ABR threshold was compared at the same stimulation frequency among the different age groups. Below 0.6 kHz, the ABR thresholds decreased rapidly with increasing age (from 1 week to 1 yr) in the 0.2–0.6 kHz range, with differences between age groups ($P < 0.05$), but there was no significant difference between the 1- and 5-yr age groups ($P > 0.05$; Table I). Above 0.6 kHz, the ABR threshold decreased from the 1-yr to 5-yr age group (using a stimulus frequency of 0.7–1.0 kHz; $P < 0.05$; Table I). There was no difference between the 1-month and 1-yr age groups (within a stimulus frequency of 0.7–1.0 kHz; $P > 0.05$), or the 1-week and 1-month age groups (within a stimulus frequency of 0.7–1.0 kHz, except at 0.9 kHz; $P > 0.05$; Table I).

C. Latency

The latency for tone burst was below 5 s in all age groups [Fig. 2(b)]. There was significant difference in

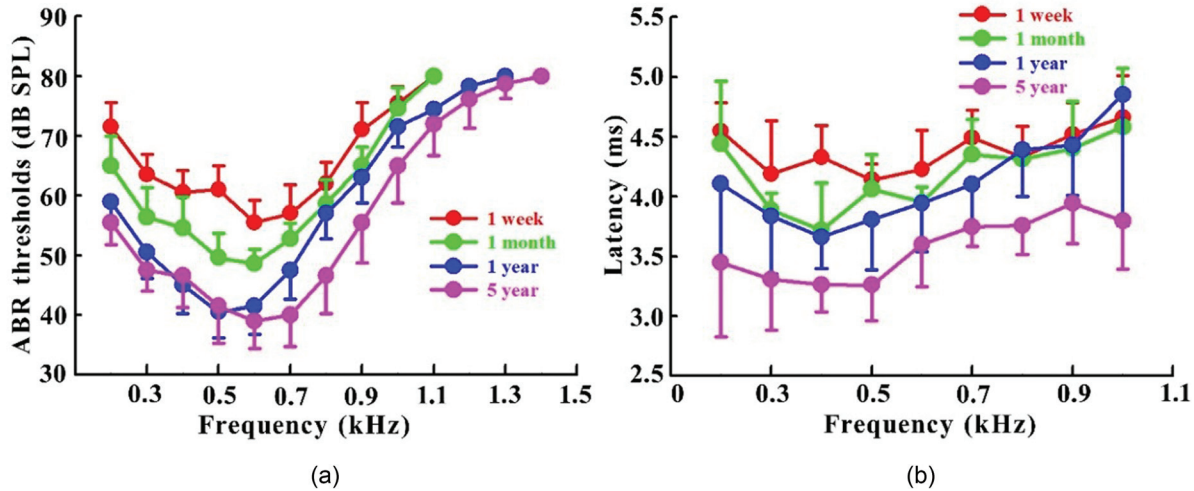


FIG. 2. Auditory brainstem response (ABR) threshold (a) and latency (b) change with tone burst stimulus frequency; responses varied between *Trachemys scripta elegans* age groups. Points represent the threshold (mean \pm SD).

latency among the different age groups ($F = 19.18$, $df = 3$, $P < 0.001$). The latencies of the 1-week age group were significantly higher than in the 1-month age group at a stimulus of 0.4 kHz ($P < 0.05$) and were significantly higher than in the 1-yr age group (using a stimulus frequency of 0.4 kHz, 0.5 kHz, 0.7 kHz; $P < 0.05$; Table II). The latencies of the 1-month age group were significantly higher than in the 1-year age group (using a stimulus frequency 0.7 kHz; $P < 0.05$; Table II). The latency was significantly lower in the 5-year age group when compared to the other three age groups (using a stimulus frequency of 0.3–0.5 kHz, 0.8 kHz, 1.0 kHz; $P < 0.05$; Table II).

IV. DISCUSSION

At present, few studies have addressed the development of hearing in chelonians. The most important finding is that,

TABLE I. Differences in the auditory brainstem response (ABR) threshold for tone stimulus at each stimulus frequency for *Trachemys scripta elegans* at different age groups. Note: Significant differences in ABR threshold among age groups at the same stimulus frequency are indicated by different lowercase letters (a, b, c). Different superscript lowercase letters within columns indicate significant differences ($P < 0.05$) between groups at the same stimulus frequency. The same superscript lowercase letters indicate no significant differences between groups at the same stimulus frequency ($P > 0.05$).

Frequency (kHz)	ABR threshold			
	1-week age	1-month age	1-year age	5-year age
0.2	71.5 \pm 4.1 ^a	65.0 \pm 5.0 ^b	59.0 \pm 3.9 ^c	55.5 \pm 3.7 ^c
0.3	63.5 \pm 3.4 ^a	56.4 \pm 5.0 ^b	50.5 \pm 4.4 ^c	47.5 \pm 3.5 ^c
0.4	60.5 \pm 3.7 ^a	54.5 \pm 5.2 ^b	45.5 \pm 4.7 ^c	46.5 \pm 5.3 ^c
0.5	61.0 \pm 3.9 ^a	49.5 \pm 4.2 ^b	40.5 \pm 4.4 ^c	41.5 \pm 6.3 ^c
0.6	55.5 \pm 3.7 ^a	48.6 \pm 2.3 ^b	41.5 \pm 4.7 ^c	39.0 \pm 4.6 ^c
0.7	57.0 \pm 4.8 ^a	52.7 \pm 2.6 ^{ab}	47.5 \pm 4.9 ^b	40.0 \pm 5.3 ^c
0.8	62.0 \pm 3.5 ^a	58.6 \pm 3.9 ^a	57.0 \pm 4.2 ^a	46.5 \pm 6.3 ^b
0.9	71.0 \pm 4.6 ^a	65.0 \pm 3.2 ^b	63.0 \pm 4.2 ^b	55.5 \pm 6.9 ^c
1.0	75.5 \pm 2.8 ^a	74.5 \pm 3.5 ^a	71.5 \pm 3.4 ^a	65.0 \pm 6.2 ^b
1.1	80.0 \pm 0.0 ^a	80.0 \pm 0.0 ^a	74.5 \pm 1.6 ^b	72.0 \pm 5.4 ^b

in hawksbill turtles (*Eretmochelys imbricata*), 3-yr-old individuals had a wider frequency range and exhibited a larger amplitude response than 2-yr-old individuals (Yudhana *et al.*, 2010). Loggerhead sea turtles (*Caretta caretta*) exhibited little difference in threshold sensitivity and frequency bandwidth throughout ontogeny (Lavender *et al.*, 2014). Bartol and Ketten (2006) reported the 2-yr-old loggerhead sea turtles responded to sounds ranging 100–700 Hz while the 3-yr-old loggerhead sea turtles responded to sounds between 100 and 400 Hz. In subadult green turtles (*Chelonia mydas*), smaller individuals had a wider hearing-sensitivity bandwidth (0.1–0.8 kHz) than larger individuals (0.1–0.5 kHz; Bartol and Ketten, 2006). Unlike these studies, our aim was to investigate the ontogenetic development of hearing from the post-hatchling to reproductive adult stage in chelonians and the effect of sex on the hearing sensitivity in turtles. We found that the upper limit of the hearing-sensitivity bandwidth shifted to a higher level with aging. The ABR threshold decreased during ontogeny. In general, ABR latency also reduced with aging. Below 0.6 kHz, the ABR threshold did not differ between the

TABLE II. Differences in the latency for tone stimulus at each stimulus frequency for *Trachemys scripta elegans* at different age groups. Note: Significant differences in latency among age groups at the same stimulus intensity are indicated by different lowercase letters ($P < 0.05$).

Frequency (kHz)	Latency			
	1-week age	1-month age	1-year age	5-year age
0.2	4.55 \pm 0.24 ^a	4.44 \pm 0.52 ^a	4.03 \pm 0.62 ^{ab}	3.45 \pm 0.62 ^b
0.3	4.19 \pm 0.45 ^a	3.89 \pm 0.14 ^a	3.79 \pm 0.49 ^a	3.31 \pm 0.42 ^b
0.4	4.33 \pm 0.27 ^a	3.72 \pm 0.39 ^b	3.63 \pm 0.26 ^b	3.26 \pm 0.23 ^c
0.5	4.14 \pm 0.13 ^a	4.06 \pm 0.29 ^{ab}	3.74 \pm 0.37 ^b	3.26 \pm 0.30 ^c
0.6	4.23 \pm 0.33 ^a	3.95 \pm 0.12 ^{ab}	3.90 \pm 0.41 ^{ab}	3.60 \pm 0.35 ^b
0.7	4.49 \pm 0.23 ^a	4.35 \pm 0.29 ^a	4.03 \pm 0.25 ^b	3.75 \pm 0.16 ^b
0.8	4.33 \pm 0.26 ^a	4.31 \pm 0.12 ^a	4.36 \pm 0.40 ^a	3.76 \pm 0.24 ^b
0.9	4.52 \pm 0.26 ^a	4.40 \pm 0.40 ^a	4.35 \pm 0.35 ^{ab}	3.94 \pm 0.34 ^b
1.0	4.66 \pm 0.35 ^{ab}	4.58 \pm 0.49 ^{ab}	4.85 \pm 1.10 ^a	3.80 \pm 0.41 ^b

1- and 5-yr age groups; above 0.6 kHz, it was significantly lower in the 5-yr age group than that in the 1-yr age group. Thus, female turtle hearing shows frequency-segmented development.

There are differences in hearing development across vertebrate classes. In the bicolor damselfish (*Stegastes partitus*), the auditory thresholds decrease exponentially with increasing age, rapidly approaching the adult levels (Kenyon, 1996). Hearing sensitivity changes only slightly during the growth of the lusitanian toadfish (*Halobatrachus didactylus*; Vasconcelos and Ladich, 2008). In the budgerigar (*Melopsittacus undulatus*), hearing is poor at hatching, and thresholds improve markedly in the first week; by one week before fledging, ABR audiograms of young budgerigars are very similar to those of adult birds (Brittan-Powell and Dooling, 2004). In some mammals, hearing sensitivity is weak at birth and gradually develops during the first week of life (Brown *et al.*, 1978; Brown and Grinell, 1980). Conversely, other species, such as the bat (*Phyllostomus discolor*; Linnenschmidt and Wiegrebe, 2019) and humans (Birnholz and Benacerraf, 1983), have a well-developed auditory system at birth and even before birth. Hysteresis of the ontogenetic auditory development in *T. scripta elegans* at higher frequencies (>0.6 kHz) differs from those reported above, and the time required to achieve a final level of auditory maturation varies among species. Given that hearing sensitivity can exert a strong influence on the adaptation to different acoustic environments, understanding the ontogenetic development of hearing will provide new insights into the function of auditory systems among vertebrate classes.

Three possible mechanisms of developmental plasticity of peripheral hearing sensitivity in vertebrates have been reported. First, in frogs, the size of the tympanic membrane may be linked to differences in hearing sensitivity (Feng *et al.*, 2006), and in some species of lizards, increased body size (or age) is accompanied by functional changes in the auditory periphery (Werner *et al.*, 1998). However, the size of the tympanic membrane is not related to sexual dimorphism in hearing sensitivity in turtles (Wang *et al.*, 2019). Therefore, growth of the tympanic membrane during ontogeny may not explain the developmental plasticity of hearing sensitivity in turtles. Second, in geckos, age-related changes in middle-ear sound conduction occur (Werner *et al.*, 1998). In geckos, precocial bird species, and some altricial rodents, once the structural development of the middle ear is complete, adult-like sound conduction is exhibited (Cohen *et al.*, 1992; Doan *et al.*, 1994; Doan *et al.*, 1996; Werner *et al.*, 1998). Research into the hearing of *T. scripta elegans* has shown that habitat-related plasticity of hearing sensitivity is related to the size of the middle ear (Christensen-Dalsgaard *et al.*, 2012). Consequently, age-related changes in middle-ear sound conduction may also contribute to age-related alterations in the threshold sensitivity in *T. scripta elegans*. Third, the sensory epithelium of the cochlear receptor organ may increase in size throughout life. In frogs and fishes, the area of auditory receptor epithelium increases with age, and cochlear growth is accompanied by an increase in the

number of hair cells on the sensory surface (Li and Lewis, 1974; Popper and Hoxter, 1984; Werner *et al.*, 1998; Wang *et al.*, 2015). This suggests that the number of hair cells is related to developmental plasticity of hearing sensitivity. Future morphological and anatomical research should address these questions.

V. CONCLUSIONS

Female turtle hearing shows frequency-segmented development. The findings of this study provide a developmental timetable for use in future studies on the anatomical development of the auditory system in turtles, and provide greater clarity on the patterns of development of the vertebrate auditory system. However, it remains unknown whether age-related changes in middle-ear structures and in the auditory receptor epithelium lead to differences in peripheral hearing sensitivity in this species. Further, as sexually dimorphic hearing sensitivity has been reported in turtles, it is worth investigating whether they also exhibit sexual dimorphism in the ontogenetic development of hearing sensitivity. Future morphological and anatomical studies should address these questions.

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