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RESEARCH ARTICLE

Ontogenetic investigation of underwater hearing capabilities in loggerhead sea turtles (*Caretta caretta*) using a dual testing approach

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ABSTRACT

Sea turtles reside in different acoustic environments with each life history stage and may have different hearing capacity throughout ontogeny. For this study, two independent yet complementary techniques for hearing assessment, i.e. behavioral and electrophysiological audiometry, were employed to (1) measure hearing in post-hatchling and juvenile loggerhead sea turtles *Caretta caretta* (19–62 cm straight carapace length) to determine whether these migratory turtles exhibit an ontogenetic shift in underwater auditory detection and (2) evaluate whether hearing frequency range and threshold sensitivity are consistent in behavioral and electrophysiological tests. Behavioral trials first required training turtles to respond to known frequencies, a multi-stage, time-intensive process, and then recording their behavior when they were presented with sound stimuli from an underwater speaker using a two-response forced-choice paradigm. Electrophysiological experiments involved submerging restrained, fully conscious turtles just below the air–water interface and recording auditory evoked potentials (AEPs) when sound stimuli were presented using an underwater speaker. No significant differences in behavior-derived auditory thresholds or AEP-derived auditory thresholds were detected between post-hatchling and juvenile sea turtles. While hearing frequency range (50–1000/1100 Hz) and highest sensitivity (100–400 Hz) were consistent in audiograms pooled by size class for both behavior and AEP experiments, both post-hatchlings and juveniles had significantly higher AEP-derived than behavior-derived auditory thresholds, indicating that behavioral assessment is a more sensitive testing approach. The results from this study suggest that post-hatchling and juvenile loggerhead sea turtles are low-frequency specialists, exhibiting little differences in threshold sensitivity and frequency bandwidth despite residence in acoustically distinct environments throughout ontogeny.

KEY WORDS: Sea turtle hearing, Auditory evoked potentials, Operant conditioning, Behavioral audiograms

INTRODUCTION

There is growing concern over anthropogenic sound in the world's oceans and its potentially harmful effects on marine animals. Anthropogenic noises can originate from a multitude of sources, including (but not limited to) shipping traffic, seismic surveys for petroleum exploration, military sonar operations and pile driving. These sounds can impact an animal in several ways: (1) altering

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behavior, (2) masking biologically significant sounds, (3) causing trauma to auditory apparatus (temporary or permanent) and (4) producing trauma to non-hearing tissue (barotraumas) (McCarthy, 2004). Increased awareness of anthropogenic sound input, in turn, has launched scientific inquiry into the underwater hearing capabilities of aquatic inhabitants, such as mammals, bony and cartilaginous fishes, sea turtles and invertebrates (Richardson et al., 1995; Casper et al., 2003; Bartol and Ketten, 2006; Casper and Mann, 2006; Hu et al., 2009; Wysocki et al., 2009; Mooney et al., 2010; Anderson and Mann, 2011; Popper and Fay, 2011; Christensen-Dalsgaard et al., 2012; Martin et al., 2012). While considerable hearing research has been conducted on fishes and marine mammals (Richardson et al., 1995; Wartzok and Ketten, 1999; Southall et al., 2007; Webb et al., 2008), much less is known about sea turtle hearing.

Much of the research on the hearing capacity of sea turtles derives from gross morphological dissections (see review by Bartol and Musick, 2003). Sea turtles receive sound through the standard vertebrate tympanic middle ear path, having a tympanum that is a continuation of the facial tissue, an air-filled middle ear cavity posterior to the tympanum with a connection via the Eustachian tube to the throat, and a connection via the middle ear bone (columella) with the oval window (Wever and Vernon, 1956; Wever, 1978; Lenhardt et al., 1985). The convergence ratio of the tympanic membrane to oval window in sea turtles is lower than in other semi-aquatic turtles (Lenhardt et al., 1985), and sea turtles lack an ossicular mechanism that acts as a lever, having only a single straight columella. Moreover, beneath the tympanum is a thick layer of subtympanal fat, a feature that distinguishes sea turtles from both terrestrial and semi-aquatic turtles (Wever, 1978). It has been suggested that this layer may enhance low-frequency sensitivity by increasing mass loading to the ear (Tonndorf, 1972). More recently, Ketten (Ketten, 2008) suggested that this additional fat layer may act as a low-impedance channel for underwater sound similar to the pathway found in odontocetes, where fats actually channel low frequency sounds to the inner ear. The auditory sense organ within the inner ear of the sea turtle cochlea is the basilar papilla (basilar membrane), which lies within the pathway of fluid displacement due to columella motion. In most reptiles, and presumably in sea turtles as well, the tectorial membrane extends over the hair cells of the basilar papilla. The amplified pressure waves are thought to bend the overlying tectorial membrane to stimulate the limbic hair cells of the papillae (Hetherington, 2008).

The few published electrophysiological studies on sea turtles indicate that they hear low-frequency sounds. Ridgway et al. (Ridgway et al., 1969) used both aerial and vibrational stimuli to obtain auditory cochlear potentials from juvenile green sea turtles (*Chelonia mydas*) for frequencies ranging from 50 to 2000 Hz. Bartol et al. (Bartol et al., 1999) collected auditory brainstem

responses (ABRs) from juvenile loggerhead sea turtles [*Caretta caretta* (Linnaeus 1758)] presented with vibratory stimuli, finding best sensitivity in the low-frequency region of 250–1000 Hz. The most sensitive threshold within this range was 250 Hz, and there was a rapid sensitivity decline above 1000 Hz. Bartol and Ketten (Bartol and Ketten, 2006) were the first to collect ABRs from submerged juvenile Kemp's ridley sea turtles (*Lepidochelys kempii*) and juvenile and sub-adult green sea turtles using aquatic stimuli. They found that *L. kempii* detect stimuli of 100–500 Hz with maximal sensitivity at 100–200 Hz, and *C. mydas* detect stimuli of 100–800 Hz. Martin et al. (Martin et al., 2012) acquired auditory evoked potentials (AEPs) from a single, submerged adult loggerhead and reported thresholds between 100 and 1131 Hz with highest sensitivity occurring at 100–400 Hz.

Whereas the studies above focus on electrophysiological evaluation of hearing in sea turtles, there is evidence that behavioral approaches to hearing assessment may in fact be more sensitive. Operant and classical conditioning techniques have been successfully applied to fishes, marine mammals and sea turtles to determine hearing capability ranges and maximal sensitivity ranges (e.g. lowest detectable level of a given stimulus or absolute threshold) (Patterson and Gulick, 1966; Popper, 1971; Coombs and Popper, 1982; McCormick and Popper, 1984; Yan and Popper, 1991; Kastak and Schusterman, 1998; Gerstein et al., 1999; Nachtigall et al., 2000; Houser and Finneran, 2006; Pacini et al., 2011; Gaspard et al., 2012; Martin et al., 2012). Many studies have involved the collection of both electrophysiological and operant conditioning data for hearing assessment, especially those that focus on marine fishes and mammal groups (Kastak and Schusterman, 1998; Sauerland and Dehnhardt, 1998; Szymanski et al., 1999; Casper et al., 2003; Wolski et al., 2003; Nachtigall et al., 2005; Yuen et al., 2005; Mulsow and Reichmuth, 2010; Reichmuth and Southall, 2011). In general, these studies indicate that operant conditioning thresholds are lower than those derived from electrophysiology for most tested frequencies. Given that behavioral approaches have not been employed extensively in sea turtles [but see Martin et al. (Martin et al., 2012), which includes behavioral results from one sea turtle], and there is evidence that behavioral approaches yield more sensitive thresholds, an important aim of the present study was to compare behavioral and electrophysiological hearing approaches for assessing hearing capabilities in sea turtles.

Although sea turtles reside in different acoustic environments throughout ontogeny and may have evolved different hearing capacities, life history comparisons in auditory detection have not been extensively explored in any sea turtle species. In fishes, studies have shown that threshold sensitivity increases (Corwin, 1983; Kenyon, 1996; Wysocki and Ladich, 2001), decreases (Egner and Mann, 2005) or does not change with ontogeny (Popper, 1971; Higgs, 2002; Higgs et al., 2003). While auditory threshold sensitivity in zebrafish does not change with ontogeny, Higgs and colleagues (Higgs, 2002; Higgs et al., 2003) found that the bandwidth of detectable frequencies increases with size. Bartol and Ketten (Bartol and Ketten, 2006), who collected the only data to date on the hearing capabilities of sea turtles of different life stages, detected a different pattern, finding that smaller juvenile green turtles have a wider range of hearing (100–800 Hz) than larger sub-adults (100–500 Hz). Sea turtles spend the majority of their lives in the ocean; their only land-linked behaviors are oviposition, hatching and occasional beach basking by green sea turtles. Like many marine fishes and mammals, sea turtles use a range of habitats during each developmental stage (reviewed by Bolten, 2003). Post-hatchlings become epipelagic, exploiting currents of different scales,

swimming along multidirectional paths (Mansfield et al., 2014). After ~7–10 years, which varies both among species and populations, a crucial ontogenetic habitat shift occurs whereby most sea turtles actively recruit to demersal, neritic habitats and are considered juveniles (McClellan and Read, 2007). Upon reaching sexual maturity, sea turtles migrate among foraging, courtship and nesting habitats, spatially and temporally overlapping with juveniles. The ambient acoustic environment changes with each ontogenetic habitat shift. Inshore acoustic signatures arise from waves breaking on land and flows around shoreline structures, sounds from benthic fauna, and low-frequency sound from shipping, recreational boating and seismic surveys, which are becoming increasingly commonplace (Hawkins and Myrberg, 1983; Hildebrand, 2009). In contrast, offshore open waters, where post-hatchlings reside, are generally quieter and dominated by sounds from pelagic fauna and air–water interactions. As most sea turtle species are migratory, occupying both inshore and offshore areas with unique and difficult-to-characterize soundscapes throughout ontogeny, a second aim of this study was to investigate whether loggerhead sea turtles exhibit an ontogenetic shift in hearing capability.

Given that little is known about hearing capabilities of sea turtles, especially throughout ontogeny, and behavioral approaches may yield different, perhaps more sensitive results, we pursued two primary objectives in this study: (1) to assess hearing in post-hatchling and juvenile loggerhead sea turtles (19–62 cm straight carapace length) to determine whether these migratory turtles exhibit an ontogenetic shift in underwater auditory detection and (2) to determine whether hearing frequency range and threshold sensitivity are consistent in behavioral and electrophysiological tests. This research will provide valuable data on a sea turtle species that is currently included on the IUCN Red List of Threatened Species (IUCN, 2013) and is potentially impacted by anthropogenic sounds.

RESULTS
Auditory evoked responses
AEPs were collected from six post-hatchling and seven juvenile loggerhead sea turtles (*C. caretta*). The Tucker-Davis Technologies (TDT) hardware and software produced consistent, clear sinusoidal signal waveforms at the location of the turtle's head (Fig. 1, Fig. 2A) and recorded well-defined AEPs at twice the stimulus frequency (Fig. 3). The magnitude of the AEP peak in the fast Fourier transform (FFT) decreased consistently with increased attenuation, with threshold occurring at the last decibel level where the AEP signal-to-noise ratio was 3 or more above neighboring signal-to-noise ratios (Fig. 3).

When AEP threshold responses were compared, no significant difference was detected between post-hatchlings and juveniles ($F_{1,17}=0.828$, $P=0.376$), and there was no significant age × frequency interaction ($F_{11,40}=1.075$, $P=0.405$). Post-hatchlings responded with the greatest sensitivity at 200 Hz (116 dB re. 1 µPa) and the lowest sensitivity at 1100 Hz (134 dB re. 1 µPa). Juveniles responded with the greatest sensitivity at 50, 100 and 400 Hz (117–118 dB re. 1 µPa) and the lowest sensitivity at 1100 Hz (140 dB re. 1 µPa) (Fig. 4).

Behavioral training
Behavioral audiograms were recorded from three post-hatchling and five juvenile *C. caretta* using a two-response forced-choice approach. One unexpected outcome of this study was that training sessions took significantly longer than expected and only a limited number of turtles completed all four training stages. For example, 23 juvenile turtles were considered for training. After more than
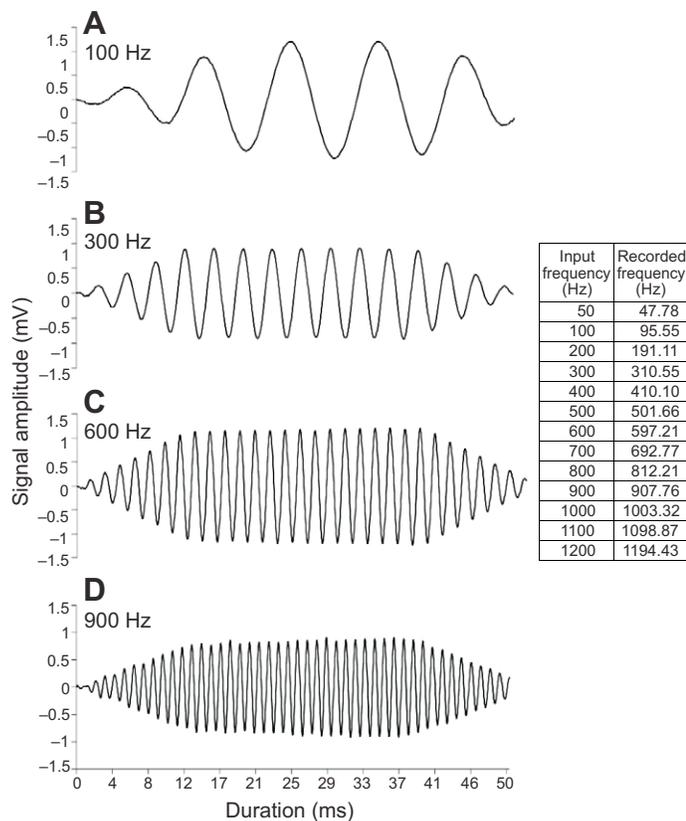
Input frequency (Hz)	Recorded frequency (Hz)
50	47.78
100	95.55
200	191.11
300	310.55
400	410.10
500	501.66
600	597.21
700	692.77
800	812.21
900	907.76
1000	1003.32
1100	1098.87
1200	1194.43

Fig. 1. Examples of acoustic stimuli (100, 300, 600 and 900 Hz) used throughout training and testing of hearing abilities of loggerhead sea turtles. The signals are actual hydrophone recordings collected at the turtle's head at a 116 dB re. 1 µPa level of sound. Note that signal distortion is low. Input frequencies used in the TDT system were slightly different than the frequencies recorded with the hydrophone at the turtle head location for audiometric experiments. Thus, actual input and recorded frequencies are listed in the table on the right.

4 months of consistent training, only five turtles (22%) completed stage 4 training with a ≥70% success rate for both sound and no-sound stimuli. Post-hatchling training required considerably less time (2 months), in part because training was less logistically cumbersome, i.e. the tank was partitioned and the animals were easier to manipulate, and those that did pick up the 'game' did so quickly relative to older turtles. A similar percentage of post-hatchlings (25%; 5/20) successfully completed the training exercises, but only three turtles consistently performed in behavioral trials and thus two were eliminated.

The extended training times, especially for juveniles, were not a product of equipment problems or limited contact time with the turtles but rather the pace at which the turtles mastered the training exercises. The training exercises do require the turtles to perform a fairly elaborate sequence of behaviors, which, to our knowledge, has not been attempted on any sea turtle. Although this multi-stage training is challenging for the sea turtle (and trainers) and only a small proportion of turtles progressed to experimental trials, this training is crucial for acquiring accurate behavioral audiograms.

Behavioral audiograms

Post-hatchling sea turtles responded to sounds in the range of 50–800 Hz while juveniles responded to a slightly extended range from 50 to 1000 Hz. Overall, post-hatchling turtles responded with the greatest sensitivity at 200 Hz (85 dB re. 1 µPa), with sensitivity
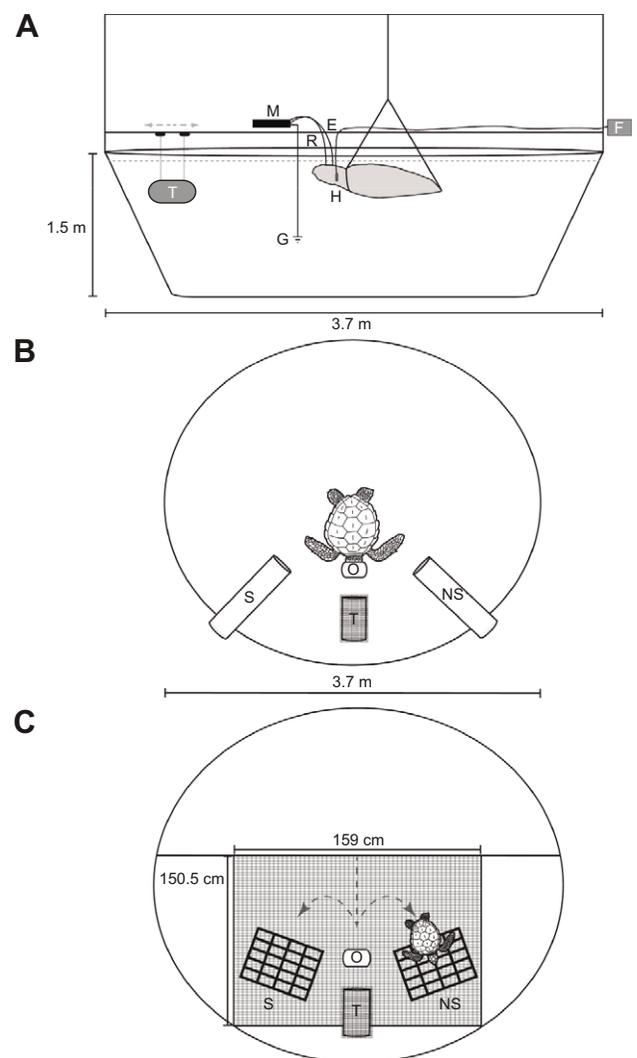

Fig. 2. Experimental setups for electrophysiological and behavioral trials. (A) During electrophysiological trials, the conscious turtle was submerged below the air–water interface facing the J9 speaker (T) with the hydrophone (H) positioned near its left ear, which connected to the hydrophone conditioning charge amplifier (F). The recording (E) and reference (R) electrodes were inserted in the dorsal side of the immobilized turtle's head and connected to the electrode headstage (M) secured in a watertight box mounted to the platform above the AEP tank. The ground electrode (G) was submerged in the tank water. (B) In the juvenile turtle behavioral setup, the turtle first inserted its head in the observer key (O), positioning it directly in front of the J9 speaker (T). An LED light suspended above the observer key was turned on to signal trial onset. When the stimulus was detected, the turtle swam to the 'signal' response key (S), and when the stimulus was not detected, the turtle swam to the 'no-signal' response key (NS). For both behavior trial setups, the response apparatuses (S, NS) were positioned equidistant from the observer key (O). (C) The post-hatchling behavior setup featured a submerged tank partition (74 cm deep) and involved the use of crates as a response key. Rather than biting the response key, the post-hatchling was trained to enter the response crate.

decreasing above and below 200 Hz. The lowest sensitivity within the post-hatchlings' auditory range occurred at 800 Hz (116 dB re. 1 µPa). Juveniles responded with the greatest sensitivity at 800 Hz (76 dB re. 1 µPa). The lowest sensitivity within their auditory range occurred at 500 Hz (108 dB re. 1 µPa) and 900 Hz (107 dB re. 1 µPa; Fig. 5). When behavioral threshold responses were compared, no
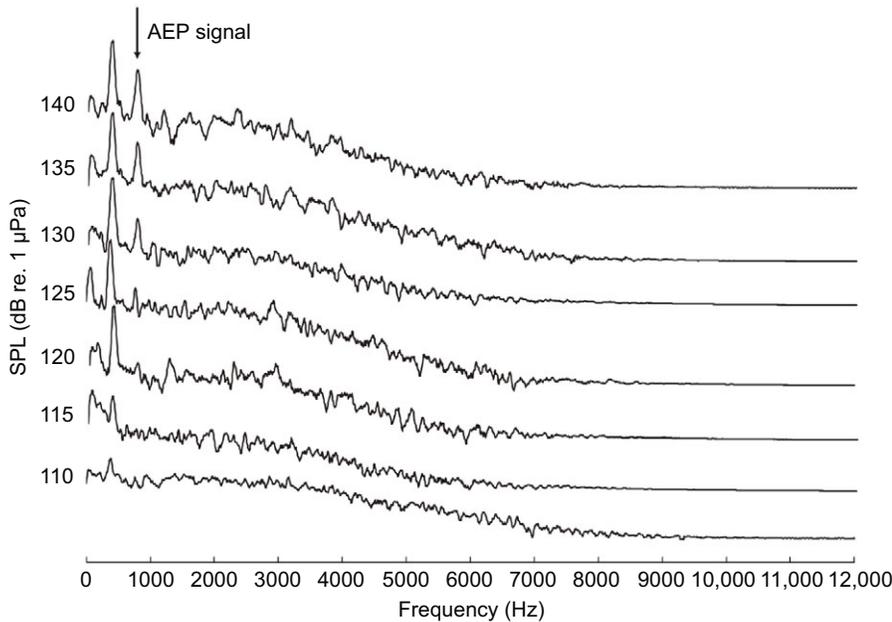
Fig. 3. Fast Fourier transformed (FFT) auditory evoked potential (AEP) traces recorded for a post-hatchling in response to a 410 Hz acoustic signal presented at descending SPLs of 140–110 dB re. 1 μPa. The AEP response is highlighted with an arrow and is approximately double the stimulus frequency (820 Hz). The acoustic signal was presented at opposing polarities (90 and 270 deg) for each SPL. The responses collected during opposing stimulus presentations were averaged prior to MATLAB FFT processing. The electrophysiological response decreased with decreasing acoustic stimulus intensity. For this post-hatchling, threshold was reached at 120 dB re. 1 μPa.

significant difference was detected between post-hatchlings and juveniles ($F_{1,9}$=0.010, P=0.923), and no age × frequency interaction was present ($F_{8,22}$=1.687, P=0.160). Post-hatchlings swam from the observer key to the response key in <16 s, with a mean (+s.d.) swimming speed of 0.140+0.090 body lengths (BL) s^{-1}, while juveniles swam from the observer key to the response key in <9 s, averaging 0.145+0.135 BL s^{-1}. Even when they approached threshold levels, turtles continued to respond quickly with no obvious change in swimming speed.

Comparison of electrophysiological and behavioral methods
No difference in threshold levels was detected for the two size classes; therefore, data were pooled to generate one AEP audiogram and one behavioral audiogram (Fig. 6). Over the majority of the frequency range, behavior-derived auditory thresholds were significantly lower than AEP-derived auditory thresholds, evidenced by the lack of overlap between 95% confidence intervals (Fig. 6). Only at the extremes of the frequency range, i.e. 50 and 1000 Hz, was overlap of

the 95% confidence intervals observed, though there was still a clear trend in higher AEP thresholds at these frequencies. The hearing frequency range measured in AEP trials extended from 50 to 1100 Hz, while the hearing frequency range for behavior trials spanned from 50 to 1000 Hz. Highest sensitivity occurred in the range of 100–400 Hz in both pooled AEP and behavioral audiograms.

DISCUSSION
This study provides the first comprehensive assessment of hearing in loggerhead sea turtles at different ontogenetic stages. It also includes measurements from two independent yet complementary approaches for hearing assessment, i.e. electrophysiological recordings and behavioral audiometry. Electrophysiological experiments involved the collection of AEPs from 13 fully conscious, restrained, submerged sea turtles presented with underwater sound from a J9 speaker. The behavioral hearing experiments entailed conditioning turtles to perform in a two-response forced-choice paradigm and recording behavioral
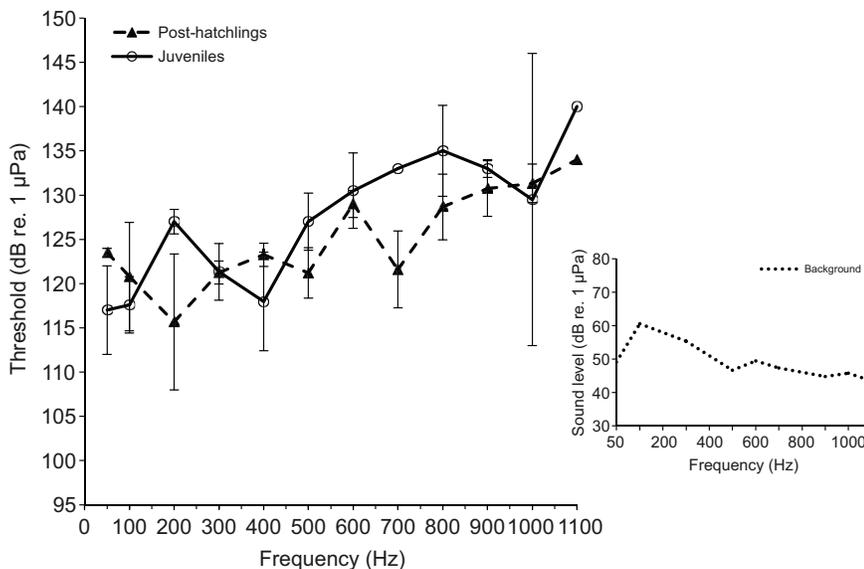

Fig. 4. Mean AEP audiograms for post-hatchling and juvenile loggerhead sea turtles. The inset indicates the ambient noise levels during trials. Error bars denote ±1 s.e.m.
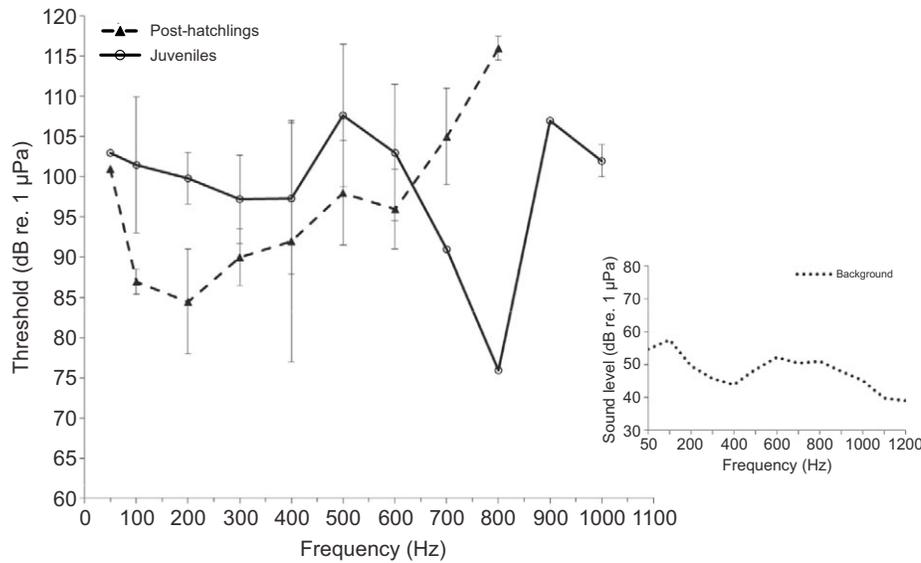
Fig. 5. Mean behavioral audiograms for post-hatchling and juvenile loggerhead sea turtles. The inset indicates the ambient noise levels during trials. Error bars denote ±1 s.e.m.

responses of eight turtles when presented with underwater sound generated by the same J9 speaker. Results from both approaches indicate that post-hatchling and early juvenile loggerhead sea turtles respond only to low frequencies (<1200 Hz), with little difference in bandwidth frequency and threshold levels. However, our results indicate that behavioral measurements are significantly more sensitive than AEP measurements for threshold determinations.

Electrophysiological assessment of sea turtle hearing sensitivity

We observed consistent, clearly definable AEP FFT peaks at twice the stimulus frequency; these peaks decreased with decreasing acoustic stimulus intensity and were consistent with previous studies on sea turtles and other aquatic species (Egner and Mann, 2005; Mooney et al., 2010; Martin et al., 2012). This doubling frequency occurred because of responses from two groups of hair cells oriented in opposite directions; one group responded to the pressure peak of the sinusoidal sound wave (forward movement of inner ear fluid) and the other group responded to the pressure trough (rearward fluid movement). In contrast to Bartol and Ketten (Bartol and Ketten, 2006), who found hearing range differences between smaller and larger green sea turtles, no difference in hearing range between post-

hatchling and juvenile loggerhead sea turtles was detected in the present study; both size classes responded to frequencies between 50 and 1100 Hz. While our results revealed no significant difference in threshold between life history stages over this frequency range, dissimilarities in peak sensitivity were observed. Post-hatchlings responded with the greatest sensitivity at 200 Hz (116 dB re. 1 μPa), whereas juveniles responded with the greatest sensitivity at 50, 100 and 400 Hz (117/118 dB re. 1 μPa). The high variance at 1000 Hz for juveniles (see Fig. 4) was a product of one turtle with an exceptionally low threshold reading (113 dB re. 1 μPa).

The AEP-derived hearing range and threshold levels reported here are consistent with previous studies conducted on sea turtles using a variety of approaches. Ridgway et al. (Ridgway et al., 1969) surgically implanted electrodes to measure cochlear potentials in the green sea turtle in response to aerial and vibratory stimuli and found that these turtles detect a limited frequency range (200–700 Hz) with best sensitivity in the low tone region of approximately 400 Hz. Bartol et al. (Bartol et al., 1999) collected ABRs from juvenile loggerhead sea turtles using vibratory stimuli delivered directly to the dermal plates over the tympanum and found best sensitivity in the low-frequency region of 250–1000 Hz, especially 250 Hz, which was the lowest frequency tested. Using a speaker suspended in air
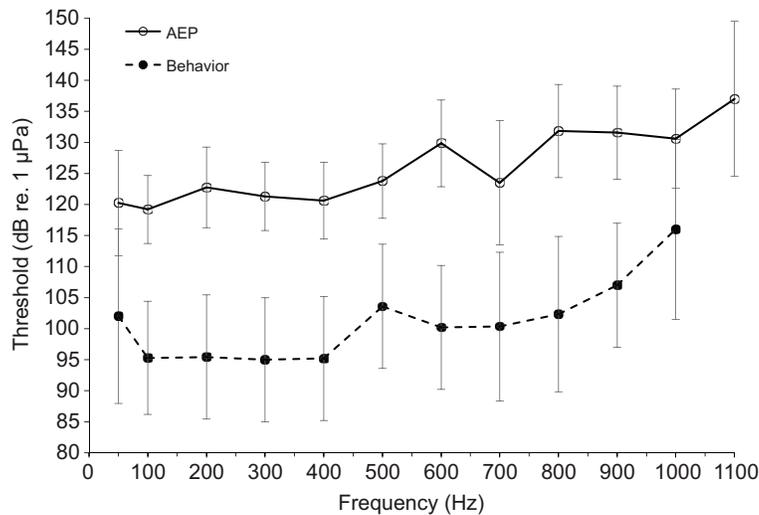

Fig. 6. Behavior and AEP audiograms based on data pooled by size class. Error bars denote 95% confidence intervals of mean threshold levels. No overlap in confidence intervals is present for 100–900 Hz, indicating that AEP thresholds are significantly higher than behavior thresholds for this range.

and submerged turtles, Bartol and Ketten (Bartol and Ketten, 2006) recorded ABRs and found that *C. mydas* detected stimuli of 100–800 Hz with best sensitivity occurring at 200–700 Hz, and *L. kempii* detected stimuli of 100–500 Hz with maximal sensitivity at 100–200 Hz. Martin et al. (Martin et al., 2012) collected AEPs from a submerged adult loggerhead using an underwater sound source and reported thresholds between 100 and 1131 Hz with the highest sensitivity occurring at 200–400 Hz (110 dB re. 1 µPa).

Operant conditioning assessment of sea turtle hearing sensitivity

Here we demonstrate that this two-response forced-choice framework is an effective experimental tool for measuring hearing sensitivity in sea turtles, but it requires a significant training period to implement. In this study, training lasted 2–5 months, varying by year class, and ~81% of the turtles did not complete the four-stage training procedure. If turtles did not show signs of interest in squid after three sessions, they were removed from further training exercises. Martin et al. (Martin et al., 2012) experienced similar difficulties training one adult loggerhead to perform in a go/no-go paradigm, with training taking 1 year. They also reported erratic and unpredictable behavior even after prolonged training. While our turtles occasionally required 'refresher' training to maintain engagement in the exercises, we generally found consistent responses during trials, suggesting that training may be more difficult with older turtles or with turtles kept in captivity for extended periods. [The 31-year-old turtle in the Martin et al. (Martin et al., 2012) study was part of an aquarium exhibit.] Interestingly, once the turtles were trained, they played the operant conditioning 'game' very effectively, making choices promptly (<9–16 s) and exhibiting no hesitation in response key selection even at threshold levels, based on behavioral video analyses.

In the present study, post-hatchlings and juveniles responded to sounds between 50 and 800–1000 Hz with no difference in threshold being detected between the two ontogenetic stages over this range. Overall, post-hatchling turtles responded with the greatest sensitivity at 200 Hz (85 dB re. 1 µPa), with sensitivity decreasing above and below 200 Hz. Juveniles responded with the greatest sensitivity at 800 Hz (76 dB re. 1 µPa). Based on a behavioral audiogram from an adult loggerhead turtle, Martin et al. (Martin et al., 2012) found a threshold range of 50 to 800 Hz with the best sensitivity at 100 Hz (98 dB re. 1 µPa), which is consistent with post-hatchlings in our study. Juveniles in our study had a slightly broader frequency range (50–1000 Hz) and a higher peak sensitivity frequency. However, when the size classes were pooled, which is reasonable given that no difference in size class was detected, maximum sensitivity occurred at 100–400 Hz (≈95 dB re. 1 µPa) (see Fig. 6), which again is similar to data from Martin et al. (Martin et al., 2012).

The reason for the unexpectedly high sensitivity at 800 Hz for juveniles in the present study is unclear. A power spectrum analysis at this frequency did not reveal any significant resonant frequencies that would drive the sensitivity thresholds to the observed levels, and the animals were not exhibiting any abnormal behaviors at this frequency. Moreover, we were not able to attribute this increase in sensitivity to recording artifact or equipment issues. Clearly, more behavioral data are needed at this frequency to determine whether this intriguing pattern is indeed present in a larger sample size of juvenile turtles.

Comparison of hearing assessment approaches

One important finding of this study is that thresholds are significantly higher (less sensitive) for AEP trials than behavioral

trials. Though the audiograms displayed similar trends for both size classes and, in the case of the post-hatchlings, were almost identical in shape, there was an average threshold difference of 28 dB re. 1 µPa between the two methods. This is not surprising given that behavioral audiograms have been shown to provide a more sensitive estimation of threshold than electrophysiological thresholds in other marine animals, such as fish and marine mammals (Fay, 1988; Richardson et al., 1995; Yuen et al., 2005). AEPs are global measures of minute electrical signals from physiologically distant origins, and consequently auditory responses can be subsumed in the baseline noise of the system (body), resulting in underestimates of auditory threshold (Kenyon et al., 1998). Behavioral audiograms are not as constrained by signal-to-noise issues, as they involve an easily observable active behavioral response to sound stimuli.

Martin et al. (Martin et al., 2012) tested one adult loggerhead and did not find a consistent difference between behavioral and AEP audiogram thresholds. Rather, behavioral thresholds were lower than AEP thresholds between 100 and 400 Hz (4–14 dB difference) but higher than AEP thresholds at 800 Hz (≈4 dB difference). They also found slightly different ranges for the two methods, with a behavioral range of 50 to 800 Hz and an AEP range of 100 to 1131 Hz. Martin et al. (Martin et al., 2012) suggested that these differences may be a product of multiple factors, including: (1) duration of stimulus presentation (a 2 s duration signal was employed for behavioral tests and a 50 ms tone repeated 11 times per second was used for AEP trials); (2) variability in behavior of the sea turtle during trials; (3) variability in the perceived signal due to the proximity of the reflective water surface; (4) high background noise, making detection of the AEP signal difficult at low frequencies; and (5) general differences in the two techniques. In the present study, some of these issues were avoided by using a similar stimulus presentation for AEP and behavioral tests (50 ms tone bursts delivered at 11–14 presentations s^{-1}), using younger turtles that retained their training more consistently, having a quieter testing facility (<65 dB of background noise as opposed to 100 dB), and employing a more quantitative approach for determining AEP thresholds (FFT to background noise ratios as opposed to visual inspection), albeit some studies have demonstrated that there is no difference between visually determined and FFT-ratio approaches (e.g. Mann et al., 2001).

While behavioral approaches produce the most sensitive thresholds, collecting behavioral data is not always practical when compared with AEP data collection. In the case of sea turtles, behavioral trials require considerable training (2–5 months), maintenance of animals in captivity for extended periods, and large tank facilities for larger life history stages. Efforts to minimize human influences on the sea turtle during experimental trials are also necessary. For this study, a researcher delivered auditory stimuli, recorded data, and monitored the subject using the live video feed in a separate enclosure. The trainer remained in the tank room during trials, delivering food rewards via clawed mechanical tools behind curtains to further minimize human–animal interaction. Another obstacle is that the capture of sea turtles is unpredictable and long-term husbandry permits are required for behavioral trials, which are extremely difficult to obtain. In contrast, AEP data collection is rapid (1–3 h), repeatable and field portable, eliminating many of the husbandry challenges of behavioral studies. Furthermore, AEPs can be collected from injured or sick animals that could not otherwise perform training exercises.

Given the difficulties associated with behavioral work with sea turtles, it is understandable why AEPs are generally the preferred

method for assessing hearing in sea turtles. However, based on the results from the present study, AEP thresholds should be interpreted with caution. We found a mean difference of 28 dB re. 1 µPa between the two approaches for both post-hatchlings and juveniles, with a maximum difference of 59 dB re. 1 µPa at 800 Hz (juveniles). Thus, based on results reported here, behavioral experiments provide a more sensitive approach for defining threshold and are a better indicator of absolute threshold levels. Behavioral hearing tests also ascribe a crucial behavioral component to hearing trials, which is lacking in AEP studies. It is interesting to note that hearing range and the general shape of the audiograms were fairly consistent between the two approaches, suggesting that AEPs alone can provide an accurate picture of hearing range and a broad-stroke indication of threshold sensitivities when behavioral tests are not possible.

Do hearing capabilities change throughout ontogeny?

The results of the present study indicate that threshold sensitivity does not differ significantly for post-hatchling and juvenile loggerhead sea turtles, and this was consistent for both behavioral and electrophysiological hearing assessment testing. Moreover, post-hatchlings and juveniles had detectable bandwidth frequencies extending from 50 Hz to 800–1100 Hz. Examining hearing in one 31-year-old loggerhead, Martin et al. (Martin et al., 2012) employed different approaches than those used in the present study, and it is difficult to directly compare AEP results between the two studies because of threshold determination differences. However, it is possible to compare behavioral audiogram data between the two studies and collectively consider animals from 1 to 31 years old. When such comparisons are made, the behavioral audiogram reported in Martin et al. (Martin et al., 2012) is similar in shape and threshold to the behavioral audiograms presented here between 50 and 400 Hz. The only major discrepancy occurs at 800 Hz, where Martin et al. (Martin et al., 2012) report a threshold of 148 dB re. 1 µPa, and we report values of 116 dB re. 1 µPa for post-hatchlings and 76 dB re. 1 µPa for juveniles. Therefore, when both the present study and Martin et al. (Martin et al., 2012) are considered collectively, there does not appear to be a clear ontogenetic shift in hearing capabilities in loggerhead sea turtles from post-hatchlings to adults.

Little is currently known about how sea turtles use hearing in their natural environment; thus, it is difficult to fully interpret the observed lack of difference in hearing capabilities throughout ontogeny. Based on the available data, sea turtles are clearly low-frequency specialists, and it is certainly feasible that they tune to similar low-frequency acoustic stimuli throughout ontogeny. For example, detecting low-frequency acoustic signatures of waves and other naturally occurring sources may be beneficial at all sea turtle life history stages, including when post-hatchlings navigate away from nesting beaches to occupy the epipelagic zone. Even if there were intermediate stages that did not require a low-frequency detection system, it would nonetheless be beneficial to retain the system if it were needed for later life stages, such as adult stages where it might be beneficial for finding nesting beaches. Moreover, it is also possible that hearing plays only a limited sensory role throughout ontogeny relative to other modalities, such as vision, olfaction and magnetic orientation (Bartol and Musick, 2003; Lohmann et al., 2013); therefore, a precisely tuned auditory system may not be required for different life stages, only a basic hearing sense. To understand the biological significance of hearing in sea turtles, future studies that characterize the sound stimuli present in sea turtle habitats are needed, as they promise to provide valuable information on the types of acoustic signatures sea turtles may be targeting.

MATERIALS AND METHODS

Animals

Electrophysiological and behavioral hearing assessments were conducted on post-hatchling [19.0–32.2 cm straight carapace length (SCL)] and juvenile (44.1–62.0 cm SCL) loggerhead sea turtles (*Caretta caretta*) from June 2009 to March 2011 at the NOAA Fisheries Galveston Laboratory Sea Turtle Facility (TX, USA). Animals originated from beaches in southeastern Florida including Juno, Melbourne (Archie Carr National Wildlife Refuge) and Pompano. Upon hatching, animals were subjected to a health evaluation and immediately transported to the NOAA Fisheries Service Galveston Laboratory. These sea turtles were physically separated from conspecifics in partitioned, elongate fiberglass raceways (Higgins, 2003) and were fed a crocodilian pellet and squid diet (restricted quantities) throughout the duration of the study. Experiments were performed in 3.7 m diameter, 1.5 m deep fiberglass tanks containing filtered natural seawater (≈15,000 l per tank). All experimental protocols were approved by the Old Dominion University Institutional Animal Care and Use Committee (protocol no. 08-006), and animals were held under federal and state permits (US Fish and Wildlife Service permit no. TE676379-4; Florida Fish and Wildlife Conservation Commission permit no. MTP-015; Texas Parks and Wildlife Department permit no. SPR-0390-038).

Acoustic stimulus delivery and SPL monitoring equipment

A TDT System 3 RP2.1 processor and RV8 Barracuda were used with TDT SigGen and BioSig programs (Alachua, FL, USA) to deliver acoustic stimuli via a J9 underwater speaker (NUWC-USRD, Newport, RI, USA). The J9 speaker, which was submerged 29.5 cm below the air–water interface, was powered by a Crunch PowerZone 500 W amplifier (Maxxsonics Inc., Lake Zurich, IL, USA) and was suspended using marine-grade cables, blocks and micro-captive ball traveler cars, which moved on a titanium track for speaker positioning (Harken, Pewaukee, WI, USA). Acoustic stimuli used for electrophysiological (AEPs) and behavioral testing consisted of 50 ms tone bursts (10 ms rise–fall time) ranging from 50 to 1200 Hz; frequencies above 1200 Hz did not elicit responses. A submerged Reson TC4013 hydrophone (−211 dB re. 1 µPa ± 1 V µPa^{-1}) and Reson CCA1000 (EC6067) conditioning charge amplifier (Reson A/S, Slangerup, Denmark) monitored SPLs at the turtle's ear at a 25,000 Hz sampling rate (100 averages). Examples of hydrophone-recorded waveforms at the sea turtle's head, along with a table of input and actual hydrophone-recorded frequencies, are included in Fig. 1. All subsequent reported data reflect input frequencies for simplicity. The tone bursts were presented in descending order of intensity (5 dB steps). AEP and behavioral trials started at ≈140 and ≈120 dB re. 1 µPa, respectively. Tone bursts were presented 11–14 times s^{-1} for AEP and behavior trials. Turtles responded to stimuli presented in opposite polarities (90 and 270 deg) during electrophysiological trials to help isolate responses from extraneous electrical body noise. Polarity was not modified during behavioral trials. Ambient noise levels in the experimental tanks were recorded with the hydrophone before and after each trial.

Electrophysiological approach (AEPs)

The same TDT system was used to acquire time-locked bioelectrical and hydrophone data. Bioelectrical signals were amplified 20× and averaged (250 recordings) to remove extraneous noise. To minimize motion artifacts, turtles were restrained with a custom acrylic canvas harness (Canvas and More, Little Bay, Norfolk, VA, USA). Two subdermal needle electrodes (post-hatchlings: size 6 mm, Rochester Electro-Medical, Lutz, FL, USA; juveniles: size 1.2 cm, Astro-Med, West Warwick, RI, USA) were inserted below the frontoparietal and frontal scutes while the animal was conscious and restrained (Bartol et al., 1999). Each insertion site was secured with liquid bandage material to prevent water intrusion. The ground electrode was submerged in the experimental tank, which is consistent with ground electrode placement used in other studies of aquatic animal hearing (Kenyon et al., 1998; Casper and Mann, 2006; Mann et al., 2001; Hu et al., 2009; Anderson and Mann, 2011; Martin et al., 2012). After electrode placement,

individuals were lowered in their canvas restraint via a pulley system, sufficiently submerging the animal to cover the tympanic scutes but shallow enough to facilitate voluntary breathing (Fig. 2A). To minimize stress on the animal, the overhead lights were turned off during AEP trials. The distance between the J9 transducer and the animal varied with year class (post-hatchlings: 90 cm; juveniles: 71.5–80.5 cm). A TDT RA16 Medusa base station and RA4LI electrode pre-amplifier were used with the TDT system described above to collect AEPs. The sampling rate for AEPs was 25 kHz and a high-pass (10–50 Hz), low-pass (3 kHz) and 60 Hz notch filter were used during recording to remove extraneous frequencies.

Average AEP waveforms were converted to ASCII formats in the BioSig module of the TDT software and imported into MATLAB (MathWorks, Inc., Natick, MA, USA) for processing. MATLAB routines were developed in-house to analyze the AEP data and required several successive operations: (1) an FFT (primary frequency component identification) was used to locate the source frequency and AEP signal [located at twice the source frequency as a result of simultaneous responses from two groups of hair cells oriented in opposite directions (Mooney et al., 2010)]; (2) both signals were isolated using a Butterworth bandpass filter (order 4); (3) the two signals were then subtracted from the original signal to produce a waveform that was nearly exclusively noise; (4) a mean of the magnitude of the FFT of the noise signal near the frequency of the AEP frequency was used to determine the noise level; and (5) the ratio of the magnitude of the FFT of the AEP signal (with noise) to the noise amplitude derived from step 4 was plotted along with ratios of other FFT frequencies ±300 Hz from the AEP signal. Threshold was defined as the lowest SPL level tested where the ratio derived from step 5 was at least three more than the other neighboring (±300 Hz) ratios. This criterion provided a conservative analysis of threshold, ensuring that we evaluated responses above the noise floor.

Operant conditioning approach

Individual turtles were exposed to an extensive multi-step conditioning procedure to establish associations between signal presence/absence and response keys prior to experimental trials. The goal of our operant conditioning approach was to require turtles to vary behavior according to small acoustic stimuli differences, permitting a behavioral measure of acoustic sensitivity. Behavioral audiograms were recorded using a two-response forced-choice approach (Gerstein et al., 1999), whereby each trial consisted of a single 'signal' or 'no-signal' presentation requiring the turtle to select the corresponding response key indicating the presence or absence of the acoustic stimulus.

A stimulus delivery and data acquisition system was developed in-house using National Instruments (Austin, TX, USA) hardware, including a PXI 1033 chassis, PXI-1428 image acquisition board, PXI-6250 M series multifunction data acquisition board and SCC-68 connector block, and LabVIEW software (National Instruments, Austin, TX, USA). The behavioral setup included an observer key (plastic ring) positioned 30 cm in front of the J9 speaker with two response keys (PVC pipes for juveniles or plastic crates for post-hatchlings) located equidistant from the observer key near the walls of the 3.7 m diameter, 1.5 m deep tank described earlier (Fig. 2B,C). The observer key positioned the turtle directly in front of the J9 speaker, ensuring the turtle received the calibrated SPL for each sound presentation, and equidistant from the response keys.

Training involved four progressive stages. (1) Stage 1 involved luring the turtle to insert its head in the observer key to trigger the overhead LED light, signaling trial onset. (2) Once the turtle consistently placed its head in the observer key, the turtle was trained to swim to the correct side of the tank when sound/no sound was presented in non-random blocks. (3) After the turtle completed several successful swimming sequences in stage 2, it was taught to bite or enter the correct response key depending on whether sound/no sound was presented in non-random blocks. (4) Finally, the turtle was trained to swim to and bite or enter the appropriate response key during randomized signal presentation. Irrespective of presentation order, turtles were rewarded with food (squid) for each correctly bitten or entered response key. Once a reward was consumed, the LED light was extinguished until the turtle entered the observer key for the next training sequence. Before moving to randomized presentations (stage 4), turtles had to achieve a 70% success rate both for sound and no-sound stimuli during non-random

presentations (stage 3). The turtles were not considered fully trained and ready for behavioral threshold trials until they completed the final training stage and achieved a 70% success rate for randomized sound and no-sound presentations. For training purposes, a 300 Hz signal was generated at an SPL of 120–130 dB re. 1 μPa.

A set of three to 10 warm-up trials were conducted prior to threshold data collection to assess the motivation and performance of the turtles tested on a given day. If the test animal exhibited low motivation, the turtle was removed and returned to its holding tank. For those animals that exhibited high motivation and performance during warm-up trials, sound/no-sound trial blocks were used, whereby five 'signals' and five 'non-signals' (10 total) were presented at random. Correct responses were rewarded with squid. Acoustic stimuli were presented in descending intensity levels, and threshold was defined as the SPL where the animal failed to respond correctly ≥70% of the time for a given intensity–frequency combination. A trial was terminated if the turtle did not respond within a 30 s window, and was then re-started. Occasionally, a block had to be terminated before completion because of prolonged low test-subject motivation, aggressive behavior toward experimental equipment, or incorrect animal positioning (e.g. individual swimming away from observer key just before stimulus delivery).

While the basic behavioral experimental protocol was employed for all turtles, some components of the setup were altered slightly over the 3-year study to accommodate experimental improvements and size differences among the turtles. In the case of one juvenile turtle, which was tested early in the study, the response chutes were located on opposite sides of the tank 3.5 m apart, with the chute openings at a water depth of 18.5 cm. To allow for a more rapid association between stimulus and reward and to reduce training times, the response chutes were positioned closer together for subsequent juvenile turtles, again with the openings at a water depth of 18.5 cm. All response keys for juvenile turtles consisted of PVC pipes containing a squid reward so as not to bias the turtle to select one chute over another based on olfactory cues. Once the turtle bit the submerged end of the correct response key, squid was delivered to the turtle using flexible shaft mechanical fingers (General Specialty Tools and Instruments, NY, USA) inside the PVC pipe with no humans in the line of sight of the turtles. For the post-hatchlings, which were significantly smaller than the juveniles, the tank was partitioned in half using plastic grating to limit the free-swimming range of the turtle and expedite data acquisition. The post-hatchling turtles were too small to bite PVC response key chutes. Therefore, plastic crates into which the turtles could swim were used. Once the turtles swam into the correct crate, they were rewarded with squid by an observer who was only visible after the turtle entered the crate (Fig. 2C).

The LabVIEW-based stimulus delivery and data acquisition system described above was used for automated and manual triggering of a 24 V DC LED light, acoustic stimuli and real-time video acquisition. When a turtle inserted its head into the observer key, a signal was sent automatically or manually to (1) turn on the light, (2) initiate video recording and (3) trigger the TDT RP2.1 processor to initiate the appropriate RPvds routine for sound delivery. After the turtle made a response key selection, the light, video recording and sound stimulus were switched off. The system control center was located in a room separate from the experimental tanks, ensuring the turtle's behavior was not influenced by the presence of researchers.

Video recording

During all behavioral trials, digital video was recorded using a UC-685-CL color digital CCD video camera (UNIQ Vision, Santa Clara, CA, USA) outfitted with a 3.5 mm wide angle lens (Navitar, Rochester, NY, USA) positioned above the tank. The camera permitted real-time monitoring of trials by the researcher in a room out of view of the turtle. As mentioned above, video recording was triggered automatically or manually using the stimulus delivery and data acquisition system when the turtle positioned its head in the observer key and was terminated when the turtle completed a response choice. To improve the resolution of the video footage, two 500 W halogen lights with red spectral filters were positioned above the tank, illuminating the turtle and tank below. A red filter was used because sea turtles have reduced sensitivity to red wavelengths (Levenson et al., 2004), and thus the additional lighting did not interfere with the LED trial onset

cue. Video files were acquired at 10 frames s^{-1} and were analyzed using IrfanView version 4 (Wiener-Neustadt, Austria) and Streams 5 software (IO Industries, London, ON, Canada). The amount of time that lapsed between signal onset and engagement of the response key, i.e. response time, was documented and swimming trajectories were tracked.

Tank mapping

Sound pressure and particle motion tank mapping was performed using two spatially separated hydrophones positioned along each of three mutually perpendicular axes following a protocol similar to that described elsewhere (Gade, 1982; Mooney et al., 2010; Christensen-Dalsgaard et al., 2012). Particle velocities and accelerations at threshold near the turtle's head during signal presentations were on the order of 10^{-8}–10^{-5} m s^{-1} and 10^{-5}–10^{-1} m s^{-2}, respectively, for behavioral and AEP experiments.

Statistical analysis

All statistical analyses were performed using SPSS (PASW Statistics 18, SPSS Inc., Chicago, IL, USA). Two-way (year class and frequency) repeated-measures ANOVAs were used to compare threshold responses between post-hatchlings and juveniles in both behavioral and electrophysiological trials. Assumptions of normality, homogeneity of variance and sphericity were met, and thus no data transformations were required. Ninety-five percent confidence intervals were calculated for behavioral and electrophysiological trials. When no overlap was observed between confidence intervals, data were assumed to be significantly different.

Competing interests

The authors declare no competing financial interests.

Author contributions

A.L.L., S.M.B. and I.K.B. collaborated on the experimental approach, data analysis and preparation of the manuscript. A.L.L. designed and built the experimental apparatus, performed the experiments and collected the data.

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