Hearing Capabilities of Loggerhead Sea Turtles (*Caretta caretta)* throughout Ontogeny: An Integrative Approach involving Behavioral and Electrophysiological Techniques

Final Project Report

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Executive Summary

While some electrophysiological auditory studies have been conducted on sea turtles, little is currently known about sea turtle hearing capabilities throughout ontogeny or how electrophysiological data correlate with behavioral responses, a necessary step for comprehensive hearing assessment. For this study we employed two independent but complementary approaches, i.e., behavioral and electrophysiological audiography, to assess hearing in two different size classes (i.e., post-hatchling and juvenile) of loggerhead sea turtles *Caretta caretta*. Behavioral trials involved first 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 LabVIEW-based stimulus delivery and data acquisition system. A two-response, forced-choice approach was used, whereby the turtles selected one chute when sound was detected and another when it was not. Electrophysiological experiments involved submerging restrained, fully conscious turtles just below the air-water interface so that their ears were underwater but breathing was not restricted, and recording auditory evoked potentials (AEPs) using a Tucker-Davis Technologies system when sound stimuli were presented using an underwater speaker. Sound pressure levels (SPLs) and particle motions (i.e., particle velocity and particle acceleration) were also recorded. No ontogenetic differences in behavior-derived thresholds and sensitivity ranges were detected, and there was no difference in response speed (body lengths s^{-1}) between hatchlings and juveniles or between suprathreshold and threshold trials. The only signifcant response speed difference was between correct and incorrect trials, with turtles swimming slower when making an incorrect choice relative to a correct choice. As was the case for behavior data, AEP-derived thresholds and sensitivity ranges were similar for post hatchling and juvenile sea turtles. At behavioral thresholds, particle accelerations and particle velocities were ~10^{-4} – 10^{-3} m s^{-2} and ~ 10^{-8} – 10^{-7} m s^{-1}, respectively, which are at or below the detection limits of the most sensitive fishes. Based on these low particle motions, negative buoyancy of the turtle, and the anatomy of the sea turtle ear, which lacks an otolith-based accelerometer system, the pressure component and not particle motion component of sound mostly likely drove the observerd thresholds, though this was not tested directly in this project. While the hearing frequency range detected in both behavior and AEP experiments were consistent (50 – 1200 Hz), both post-hatchlings and juveniles had significantly higher AEP-derived (mean = 126.6 re 1 µPa over hearing range) than behavior-derived (mean = 97.1 re 1 µPa over hearing range) auditory thresholds. This is an important finding for it indicates that AEP tests are less sensitive than behavioral tests and should not be used to set the standard for sound exposure levels in the field. Collectively, data from this project help define the hearing frequency range and threshold of two ontogenetic stages of turtles and provide a means to evaluate future electrophysiological audiograms. However, more research in the areas of hearing loss/damage, hair cell regeneration, masking, and *in situ* behavioral responses to sound are needed to better define the impact of human-made sound sources on sea turtles.

Introduction

There is growing concern over anthropogenic sound in the world's oceans and the potentially harmful effect it has on protected marine organisms. Anthropogenic noises can originate from a multitude of sources, including (but not limited to) shipping traffic, seismic surveys for petroleum exploration, military sonar operations, pile driving, etc. These sounds have the potential to impact an animal in several ways: alteration of behavior, masking of biologically significant

sounds, trauma to hearing (temporary or permanent), and trauma to non-hearing tissue (barotraumas) (McCarthy, 2004).

Sea turtles are one group of endangered marine organisms that are likely to be impacted by anthropogenic sound production. Sea turtles spend the majority of their lives in the ocean; their only land-linked behaviors are egg deposition and hatching. Like many marine fishes and mammals, sea turtles use a range of habitats for each developmental stage (see review by Bolton, 2003). Once hatchlings reach the sea, they are pelagic, moving primarily with ocean currents. After a period of years, which varies both among species and populations, a critical ontogenetic habitat shift occurs whereby most sea turtles actively recruit to a demersal, neritic habitat and are considered juveniles. Finally, upon reaching maturity, all sea turtles maintain a discrete foraging area (this region frequently overlaps with the juveniles), migrating only to return to their natal nesting beach. The exceptions to this life history model appear to be leatherback and olive ridley sea turtles (East Pacific populations). These sea turtles remain pelagic as both juveniles and adults, returning to the neritic zone only for reproduction (Bolton, 2003). The acoustic environment changes with each ontogenetic habitat shift. In the inshore environment, where juvenile and adult sea turtles generally reside, the ambient environment is noisier than the open ocean environment of the hatchlings; this inshore environment is dominated by low frequency sound from shipping and recreational boating (Hawkins and Myrberg, 1983) and seismic surveys, which are becoming more commonplace (Hildebrand, 2005).

Much of the research on the hearing capacity of sea turtles is limited to gross morphological dissections and electrophysiological studies (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 between 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 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. These characteristics are not conducive for aerial sound detection. The dense layer of fat under the tympanum may act as a low-impedence channel for underwater sound (similar to that pathway found in odontocetes where fats actually channel the low frequency sounds to the inner ear; Ketten et. al., 1999). The retention of air in the middle ear of these sea turtles, which is compressible and can act as a pressure-to-particle motion amplifier, suggests that they are able to detect sound pressures, similar to the role that swimbladders play in fishes (Fay and Popper, 1999; Sand and Karlsen, 2000). The auditory sense organ within the inner ear of the sea turtle cochlea is the basilar papilla (basilar membrane). This membrane is large and composed of dense connective tissue in sea turtles (rather than a thin basilar membrane found in terrestrial turtles) (Wever, 1978; Hetherington, 2008). This basilar papilla is positioned opposite the round window and lies within the pathway of fluid displacement due to columella motion. In most reptiles, and presumably in sea turtles as well, the tectorial membrane lays over the hair cells of the basilar papilla. The amplified pressure waves are thought to bend the overlying tectoral membrane to innervate the hair cells on the papillae (Hetherington, 2008).

Previous electrophysiological studies have been performed on two species of juvenile sea turtles: greens (*Chelonia mydas*) (Ridgway et al., 1969) and loggerheads (*Caretta caretta*) (Bartol, 1999). Ridgway et al. (1969) used both aerial and vibrational stimuli to obtain auditory

cochlear potentials from juvenile green sea turtles. Thresholds were not measured; instead cochlear response curves of 0.1 μV potential were plotted for frequencies ranging from 50 to 2000 Hz. They found that green sea turtles detect a limited frequency range (200-700 Hz) with best sensitivity at the low tone region of about 400 Hz. Though this investigation examined two separate modes of sound reception, i.e., air conduction and bone conduction, sensitivity curves were relatively similar, suggesting that the inner ear is the main structure for determining frequency sensitivity. To measure electrophysiological responses to sound stimuli, Bartol et al. (1999) collected auditory brainstem responses (ABRs) from juvenile loggerhead sea turtles. Vibratory stimuli were delivered directly to the dermal plates over the loggerhead sea turtle's tympanum. Thresholds were recorded for both tonal and click stimuli. Best sensitivity was found in the low frequency region of 250-1000 Hz. The decline in sensitivity was rapid after 1000 Hz, and the most sensitive threshold tested was at 250 Hz. More recently, Bartol and Ketten (2006) collected underwater ABRs from hatchling and juvenile loggerhead and juvenile green sea turtles. For these experiments, the speaker was suspended in air while the turtle's tympanum remained submerged underwater. All turtles tested responded to sounds in the low frequency range, from at least 100 Hz (lowest frequency tested) to no greater than 900 Hz. Interestingly, the smallest turtles tested, i.e., hatchling loggerheads, had the greatest range of hearing (100-900 Hz) while the larger juveniles responded to a much narrower range (100 - 400 Hz). Hearing sensitivity of green sea turtles also varied with size; smaller greens had a broader range of hearing (100-800 Hz) than that detected in larger subjects (100-500 Hz).

Behavioral sea turtle hearing data are limited to behavioral responses of juvenile loggerheads to sound in their natural environment (Moein et al., 1995; O'Hara and Wilcox, 1990). The studies by Moein et al. (1995) and O'Hara and Wilcox (1990) both were performed to facilitate development of an acoustic repelling device for sea turtles. O'Hara and Wilcox (1990) attempted to create a sound barrier for loggerhead turtles at the end of a canal using seismic airguns. The test results indicated that airguns are effective as a deterrent for a distance of about 30 m when the sound output of the system is approximately 220 dB re 1 μPa at 1 m in the 25-1000 Hz range. However, this study did not account for the reflection of sound off the canal walls, and the stimulus frequency and intensity levels were ambiguous. Moein et al. (1995) investigated the use of pneumatic energy sources (airguns) to repel juvenile loggerhead sea turtles from hopper dredges. A net enclosure was erected in the York River, VA to contain the turtles and an airgun was stationed at each end of the net. Sound frequencies of the airguns ranged from 100-1000 Hz at three decibel levels (175, 177, and 179 dB re 1 μPa at 1 m). Avoidance of the airguns was observed upon first exposure. However, after three separate exposures to the airguns, the turtles habituated to the stimuli.

To fully understand the hearing capabilities of sea turtles and ultimately determine how sea turtles will respond to various anthropogenic noise sources, both behavioral audiograms and AEPs need to be collected over an ontogenetic range of turtles. As indicated earlier, sea turtles reside in different acoustic environments with each life history stage and may well have different hearing capacity throughout ontogeny. These potential differences have not been explored extensively in any species of sea turtle. An integrated study involving two independent but complementary acoustic assessment tools, i.e., behavioral audiograms and AEPs, is important to fully evaluate hearing capabilities of sea turtles. The AEP technique has been the preferred auditory assessment method for sea turtles in the past because it can be employed with greater ease than behavioral trials, which require considerable hours of training, maintaining animals in captivity for extended periods, and large tank facilities for larger life history stages. Although AEPs provide a valuable measure of hearing ability and have advantages ranging from rapid, non-invasive data collection to high repeatability, they have limitations. AEPs are global measures of minute electrical signals from physiologically distant origins, and consequently they

can underestimate auditory threshold (Kenyon et al., 1998). More fundamentally, AEP measurements are not validated, i.e., there is a disconnect between the measured electrophysiological response and behavior. Although behavioral techniques also have problems (most of which stem from the difficulties of behavioral training), they complement AEPs nicely, providing a separate, potentially more sensitive measure of hearing threshold and ascribing a critical behavioral component to hearing trials, which is lacking in AEP studies.

For this study, we employed a two method approach for hearing assessment, with four goals: (1) collect behavioral audiograms from loggerhead sea turtles *Caretta caretta* of different ontogenetic life stages; (2) collect auditory evoked potential responses (AEPs) from the same turtles considered in behavioral experiments; (3) determine if hearing frequency range and threshold recorded in behavioral and AEP experiments are consistent; and (4) determine if hearing capabilities change during ontogeny. A secondary objective included in the initial proposal was to attempt to collect sufficient data to make interspecific hearing capability comparisons. However, this objective was dropped, as we discovered that behavioral training required such an incredible time investment for loggerheads that it was simply not feasible to include other species of turtles in the study. Moreover, a large number of green and Kemp's Ridley sea turtles were simply not available at the NOAA Fisheries Service Galveston Laboratory for the extended periods required for training.

Methods

Animal Maintenance

Experiments were conducted at the NOAA Fisheries Service Galveston Laboratory (Texas, USA), which maintains approximately 400 captive-reared loggerheads [4-50+ cm straight carapace length (SCL)] from Florida nests for scientific studies. For this study, several year classes of sea turtles *Caretta caretta* were considered (Cc2005 – Cc2009; number indicates the year eggs were hatched), ranging in size from 15.5 to 62.0 cm, SCL (Fig. 1,Table 1). The youngest year class (Cc2009) was held in baskets positioned in raceway tanks, the middle year class (Cc2007) was held in raceway tanks with dividers, and the oldest year class (Cc2005) was held in larger 1,000-gallon tanks (Fig. 2). All experiments were conducted in 3.7 m diameter, 1.5 m deep tanks located in a separate lab facility (Fig. 2). All turtles were held under several Federal and State Permits (USFWS Permit #TE676379-3, FWC Permit TP #015, TPWD Permit #SPR-0390-038).

Figure 1. *Loggerhead sea turtles (Caretta caretta) used for this study ranged from ~15.5 to 62.0 cm straight carapace length (SCL).*

Table 1. *Morphological data for sea turtles (Caretta caretta) used in AEP and behavior studies. Turtles in the Cc2005 and Cc2007 classes were pooled and considered 'juveniles'. Turtles in the Cc2009 class were considered 'post-hatchlings'.*

Year Class	Turtle	SCL (cm)	Weight (kg)	AEPs	Behavior
Cc2005	TTN 219	50.6-60.0	16.4-27.75	X	
	TTN 233	51.0-59.0	18.52-28.50	X	X
	YYN 528	48.1-62.0	11.75-25.20	X	
	TTN 298	52.9-61.0	17.33-26.45	X	
Cc2007	YYN 822	46.1-49.5	10.5-14.4	X	X
	YYN 869	47.2-52.8	11.4-15.95		X
	YYN 874	44.1-48.0	10.4-14.75		X
	YYN 886	45.0-50.5	11.8-16.75	X	X
Cc2009	RW 16, POS 6	19.0-31.6	0.95-3.75	X	X
	RW 16, POS 9	19.3-32.2	0.95-4.25	X	X
	RW 16, POS 13	15.5-32.1	0.95-4.10	X	X

Behavioral Experiments

Training - Individual turtles were subjected to a multi-step conditioning procedure to establish associations between experimental apparatus and signal presence/absence. This approach required significant training of turtles. A LabVIEW-based data acquisition and stimulus delivery (DASD) system, developed and assembled by our team, was used for behavioral conditioning exercises (Fig. 3). The DASD system displayed live video of the sea turtle so that behavior could be observed in a room out of view of the turtle. A reinforced tube integrated with an Omega pressure transducer served initially as the observer key (Fig. 4). When the turtle bit down on the observer key, the DASD system sent triggers to turn on a light above the turtle tank and initiate sound delivery from a J9 underwater transducer (Naval Undersea Warfare Center, Underwater Sound Reference Division, Newport, RI), which had a range of 40 Hz – 20 kHz (more details on the DASD system are included below in the 'behavioral trials' section). The light served as a valuable cue for the turtle that it had successfully bit down on the observer key to commence the trial. Because of dietary restrictions of the turtles, training periods were performed for a maximum of 2 hours a day for each turtle.

Figure 2. *Facilities at the NOAA Sea Turtle Laboratory, Galveston, TX. Raceway tanks (left) and 1000-gallon tanks (middle) were used to hold younger and older sea turtles, respectively. A separate building with two experimental tanks (right) was used for behavioral and electrophysiology experiments.*

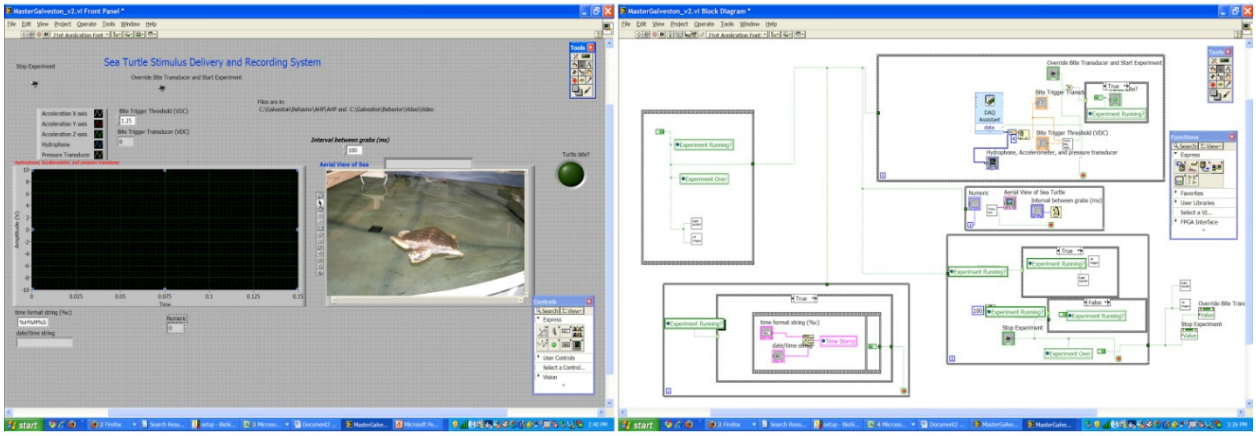


Figure 3. *Front panel (left) and block diagram (right) for one of several LabVIEW VI's developed for behavioral training and subsequent audiogram recording.*

While this triggering approach worked well for many of the turtles, we noticed that some turtles entangled themselves in the reinforced tubing of the observer key, requiring us to revise our triggering approach in year two of the study. The revised approach involved using a smaller plastic ring trigger as opposed to a looped tube, which eliminated the entanglement issue while still keeping the turtle positioned reliably in front of the speaker for the initiation of sound delivery (Fig. 4). The turtle triggered the light and speaker when it placed its head through the ring rather than biting the reinforced tubing.

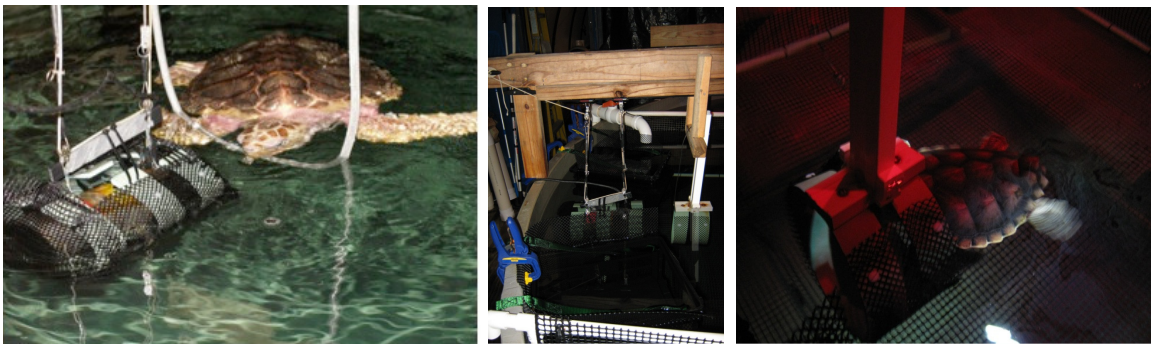


Figure 4. *Initial trigger used for behavioral training involving a reinforced tube with an integrated pressure transducer (left) and the revised trigger consisting of a plastic ring that triggered light and sound delivery when the turtle inserted its head into the ring structure (middle and right).*

Training involved several progressive stages: 1) teaching turtles to position their head within the observer key/bite key; 2) teaching the turtles to position their head within the observer key/bite key and swim to the correct area of the tank when sound/no sound is presented *non randomly*; 3) teaching the turtles to position their head within the observer key/bite key, swim to the appropriate area of the tank depending on whether sound/no sound is presented *non randomly*, and bite the response key, i.e., reward shoot; and 4) teaching the turtles to position their head within the observer key/bite key, swim to the appropriate area of the tank depending on whether sound/no sound is presented *randomly*, and bite the response key. A food reward (squid) was used to reinforce behaviors in stages 1 and 2. To train the turtles to respond correctly in stage 3,

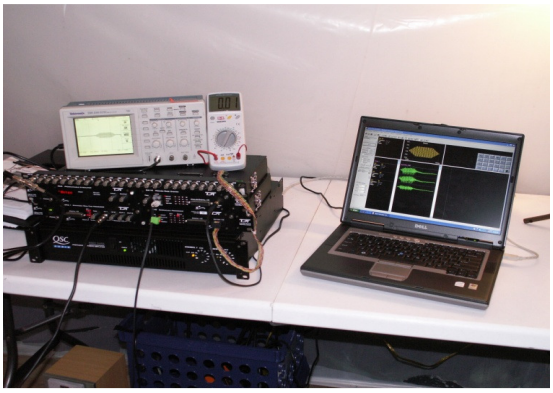
each turtle was presented with two possible responses: (1) biting one pipe located in one area of the tank if a sound is heard and (2) biting another pipe in another area of the tank if no sound is heard. When the correct pipe was chosen, the turtle's behavior was reinforced with a food reward (squid) delivered through a reward chute. Before moving to stage 4, turtles had to achieve an 80% success rate both for sound and no sound stimuli during *non-random* presentations. The turtles were not considered fully trained and ready for behavioral audiogram trials until they completed stage 4 training and achieved a 70-80% success rate for both sound and no sound stimuli during *random* presentations. A frequency of 300 Hz with a sound pressure level (SPL) of 120-140 dB re 1 µPa was used in all training exercises. This frequency and SPL were selected because loggerheads are known to hear sounds within this range at this level of sensitivity based on previous underwater AEP work (Bartol and Ketten, 2006).

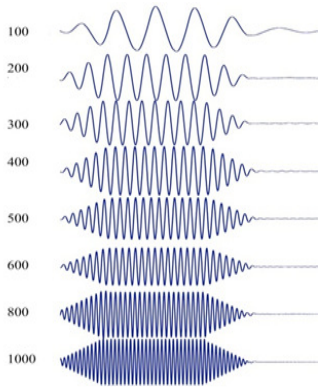
Behavioral trials – Once the turtles were trained, behavioral trials began using a two-response, forced-choice approach (Blough and Blough 1977), whereby the turtles were required to vary behavior according to small acoustic stimuli differences, permitting a behavioral measure of acoustic sensitivity. Three separate size classes of loggerheads were considered: (1) Cc2005 (N=1), (2) Cc2007 (N=3), and (3) Cc2009 (N=3) (Table 1). The relatively low sample size is a reflection of the extended training period required and the inability of many turtles to learn and retain their training. Because the Cc2005 class had a low sample size and the turtles in this class were similar in size to the Cc2007 class, we considered the Cc2005/Cc2007 turtles one size class (juveniles) and the Cc2009 another class (post-hatchlings) in our analyses.

The behavioral setup included an observer key (plastic ring) positioned 30 cm in front of the J9 speaker with two response chutes (PVC pipes or plastic crates) located equidistant from the observing key near the walls of a 3.7 m, 1.5 m deep tank. As was the case with training exercises, one chute was designated the 'sound' chute and triggered when a sound was detectable by the turtles and the other chute was designated the 'no sound' chute and triggered when a sound was not detectable. While the basic experimental protocol was employed for all year classes tested, some components of the setup were altered slightly to accommodate size differences among the turtles. In the case of the Cc2005 turtle, the observer key was located in the middle of the tank and 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 (Fig. 6). In the case of Cc2007 turtles, the observer key was located near the side of the tank 30 cm in front of the J9 with the response chutes on either side of the J9, again with their openings at a water depth of 18.5 cm. For both the Cc2005 and Cc2007 year classes, all response keys consisted of PVC pipes containing a squid reward so as not to bias the turtle in selecting one chute over another based on olfactory cues. The squid was delivered to the turtle using flexible shaft mechanical fingers (General Tools) with no humans in the line of sight of the turtles. For the Cc2009 year class, which was significantly smaller than the other size classes, the tank was partitioned in half using plastic grating. Like the Cc2007 arrangement, the observer key was located near the tank wall 30 cm in front of the J9 speaker. However, the Cc2009 turtles were too small to bite PVC response key chutes so the chutes were replaced with plastic crates that the turtles could swim into and receive a food reward via the mechanical finger system, again with no humans in sight.

Tucker Davis Technologies (TDT) System 3 hardware (RP2.1 processor, RV8 Barracuda processor, and RA16 Medusa base station) and software (BioGen, BioSig) together with the USRD J9 underwater speaker powered by a 500 W amplifier (Crunch Electronics) were used to deliver acoustic stimuli, collect hydrophone data, and calculate sound pressure levels. The acoustic stimuli used for behavioral testing consisted of tone bursts (50 ms duration with 10 ms rise-fall time) of known frequencies (50 Hz to 1200 Hz in 100 Hz steps) presented in descending

order of intensity (5 dB steps)(Fig. 5). Actual frequencies recorded using a hydrophone are listed in Figure 5. All subsequent reported data reflect input frequencies for simplicity.





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

Figure 5. *TDT system (left), example of tone burst stimuli (middle), and input and actual recorded frequencies (right) produced using TDT system for behavioral audiogram experiments.*

A data acquisition and stimulus delivery (DASD) system was custom-designed in-house for this study, which allowed automated and manual triggering of a 24V DC LED light, acoustic stimuli, and real-time video acquisition (Fig. 6). The system was controlled with a LabVIEW VI (National Instruments) written by I. Bartol and run on a Dell Latitude laptop. All equipment comprising the system was interfaced with the laptop using a NI SCC-68 breakout box and a NI PXI-1033 chassis configured with a NI PXI-1428 image acquisition board and NI PXI-6250 M series multifunction data acquisition board (National Instruments). When a turtle inserted its head within a plastic ring (observer key) positioned 30 cm in front of the J9 underwater speaker, a signal was sent automatically or manually to (1) turn on the LED light, (2) initiate video recording, and (3) trigger the TDT RP2.1 processor to initiate the appropriate RPvds routine for sound delivery. The control center for the DASD system was set up in a room separate from the experimental tanks, so that turtle behavior was not influenced by the presence of researchers.

The observer ring arrangement was an important component of the experimental setup (Fig. 7). First, it allowed us to present consistent and repeatable SPLs at the start of each trial. The observer ring was positioned 30 cm from the J9 to prevent the turtle from swimming between the J9 and ring and approaching the ring from the backside. Wider spacing between the ring and J9 and other key designs (e.g., tubing) allowed turtles to trigger the ring from other approach lines besides 'head-on', eliminating our ability to present a consistent, repeatable signal of known SPL at the observer key. Second, the ring allowed for control over the turtle's orientation relative to the speaker. In earlier designs, we noticed that the turtles would frequently contact the key at various angles relative to the longitudinal axis of the J9 while swimming, with their momentum carrying them to the reward chute in line with their swimming trajectory. This confounded a true sound-driven selection process. The ring eliminated this trajectory issue because it required the turtles to enter the ring at a controlled, low speed, parallel to the longitudinal axis of the J9 and equidistant to the reward chutes, making the reward chute selection a deliberate choice rather than a momentum-driven selection.

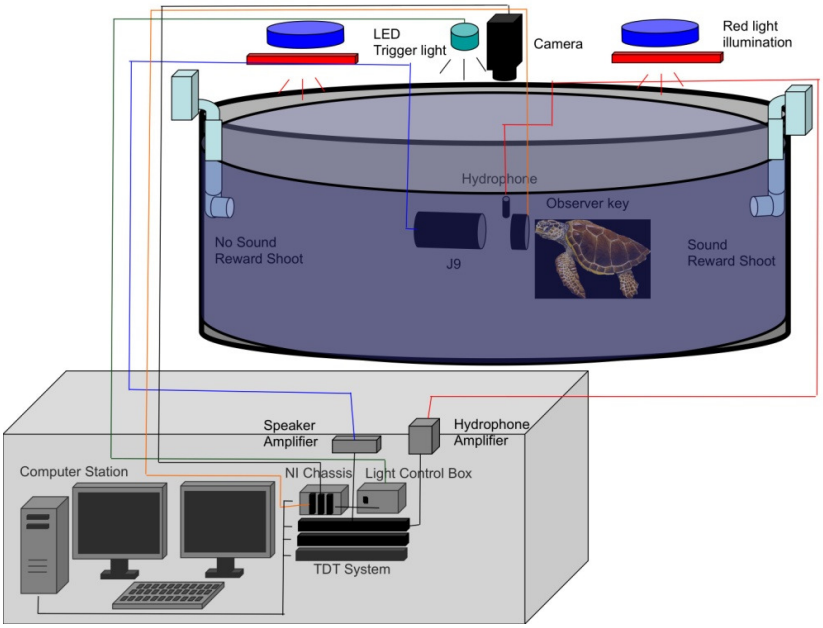


Figure 6. *Schematic of data acquisition and stimulus delivery (DASD) system used for behavioral trials for the Cc2005 year class of turtles. The items in the gray box were set up in a separate room from the experimental tank so that turtles were not influenced by researchers.*

To monitor SPL levels during experimental trials, a TC4013 hydrophone (Reson A/S, Slangerup, Denmark) was positioned near the observer key and output from the hydrophone's CCA100 conditioning charge amplifier (Reson A/S) was imported in real-time into the TDT system. Moreover, ambient noise levels in the experimental tanks were recorded with the hydrophone before and after each trial. To prevent damage to the J9 from sea turtle bites, a protective cage was constructed out of plastic screen material and PVC around the speaker. As was the case with behavioral training, the LED light, which was triggered when the turtle inserted its head within the observer ring, served as an important cue for the turtle that the trial had commenced (Fig. 7).

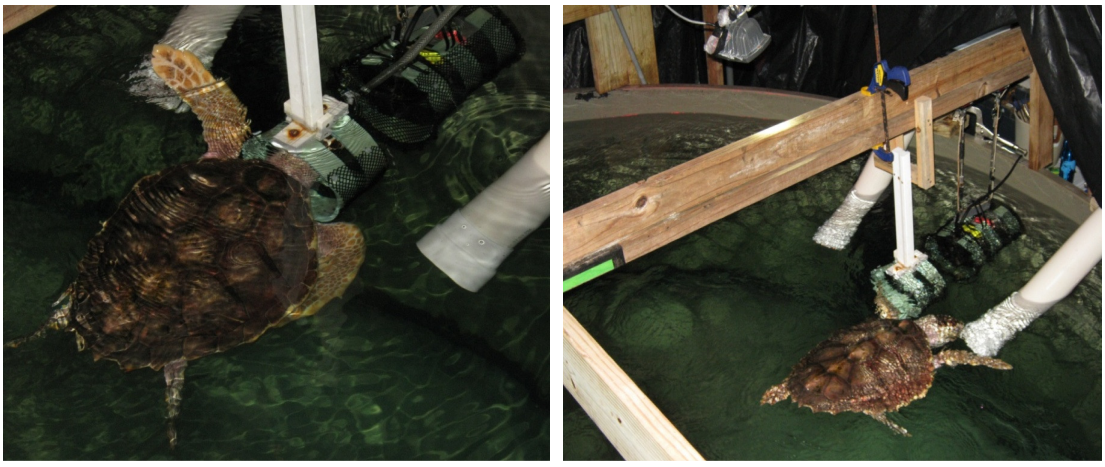


Figure 7. *Freely swimming turtles enter observer ring positioning themselves head on relative to the J9 and equidistant from the response chutes (left). A white light above the tank is triggered to signal trial onset, initiating acoustic presentation and video recording. The turtle indicates a response by biting either a 'no signal' or 'signal' chute (right).*

As mentioned above, the turtles underwent an intensive training period to condition them to respond correctly to sound and no sound, i.e., swim to the 'sound' chute when a sound was detected and swim to the 'no sound' chute when a sound was not detected (Fig. 7). Therefore, during experimental trials, the turtles were familiar with the 'game' and, in most cases, promptly made a response pipe selection shortly after triggering the light and sound stimuli via the observer key.

Threshold was defined as the SPL level where the animal failed to respond correctly $\geq$70% of time for either the 'sound' or 'no sound' presentations.

Video Recording – During all behavioral trials, digital video was recorded using a UC-685-CL camera (UNIQ Vision) outfitted with a 3.5 mm wide angle lens (Navitar, Rochester, NY) positioned above the tanks. Recording was initiated automatically or manually with the DASD system when the turtle positioned its head within the observer ring and was terminated when the turtle selected a response chute. To improve the resolution of the video footage, two 500W halogen lights with red spectral filters were positioned above the tank, illuminating the turtle in the tank below (Fig. 6). A red filter was used because sea turtles have reduced sensitivity to red wavelengths (Levenson et al., 2004) and thus the added lighting did not interfere with the LED trial onset cue. The camera was triggered at 10 fps. Video frames were analyzed using Streams 5 (IO Industries) or Irfanview software. Response times of the turtles were determined for each trial and swimming trajectories of the turtles were also tracked. To account for differences in turtle size and distance to response chutes in the trials, response times were converted to response speeds and expressed in body lengths s^{-1} for subsequent statistical analyses.

Electrophysiological Experiments

Nine sea turtles were considered for electrophysiological studies (6 of these were turtles from behavioral trials) (Table 1). An effective protocol for restraining and submerging the turtles was developed. This protocol involved wrapping each turtle in custom canvas slings (Little Bay Canvas and More, Norfolk, VA) and positioning the turtle via pulleys and blocks just below the air-water interface to facilitate voluntary breathing (Fig. 8). The canvas sling was a critical component of the protocol because it restricted movement and reduced motion artifacts. Prior to lowering the turtles in the tank, two subdermal electrodes (i.e., recording and reference) were inserted. A third ground electrode was placed in the water. The recording and reference electrodes were positioned dorsally along the frontoparietal scute and sealed with liquid bandage (Fig. 8). Because of significant differences in size among the sea turtles, different electrodes were used; F-E2 and F-E7 120mm needle electrodes (Grass Astro-Medical, West Warwick, RI) were used with the Cc 2005 and Cc2007 turtles and 6 mm subdermal electrodes (Rochester Electro-medical, Lutz, FL) were used with the Cc2009 turtles.

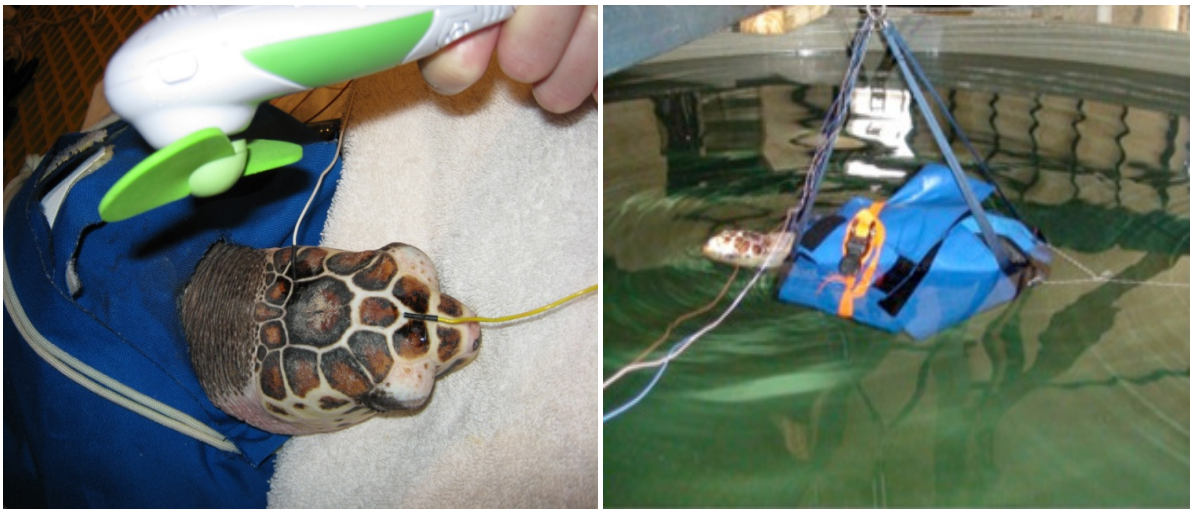


Figure 8. *Recording (white wire) and reference (yellow wire) electrodes were inserted along the frontoparietal scute and sealed with liquid bandage (left). A canvas restraint system was used to reduce motion artifacts during AEP recordings and position the turtle's ear just below the air-water interface (right).*

Tone bursts (50 ms duration with 10 ms rise-fall time) of known frequencies (50 Hz to 1200 Hz were presented in descending order of intensity (5 dB steps) and in opposite polarities to reduce extraneous noise using the TDT system, J9 speaker, and amplifier described in the 'behavioral trials' section. The J9 speaker was positioned 29.5 cm deep (center axis of speaker to water surface) in the center of the tank 70-90 cm from the sea turtle head. In contrast to behavioral trials, sound presentations could be performed quickly, as the turtle did not need to swim to response chutes after each presentation. Bioelectrical signals (auditory evoked potentials (AEPs)) were amplified by 20x using a RA4Li gain amplifier (TDT) and averaged over 250 presentations to remove myogenic and electrical noise. The sampling rate for AEPs was 25,000 Hz and a high pass (10-50 Hz), low pass (3 kHz), and 60 Hz notch filter were used during recording to remove unwanted frequencies. Sound pressure levels at the turtle's head were also recorded using a TC4013 hydrophone (Reson A/S, Slangerup, Denmark), CCA100 conditioning charge amplifier (Reson A/S), and the TDT system. Moreover, ambient noise levels in the experimental tanks were recorded using the hydrophone before and after each trial.

Average AEP waveforms were converted to ASCII formats in the Biosig module of the TDT software and imported into Matlab for processing. Several Matlab routines were developed in house to analyze the AEP data. The Matlab routines performed several successive operations: (1) an FFT 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 (Egner and Mann, 2005)); (2) both signals were isolated using a Butterworth bandpass filter (order 2-4); (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) and the noise amplitude derived from step 4 were plotted in dB. We defined AEP thresholds as the last SPL level tested where the ratio derived from step 5 was at least 3 dB above other FFT relative amplitudes. This 3 dB cutoff provided a conservative analysis of threshold, ensuring that we were examining responses outside the noise.

Tank Mapping

Sound pressure levels and particle motions were mapped within the experimental tanks. The J9 underwater speaker was positioned in the same locations that were used for behavioral and electrophysiological experiments and sound pressure levels (SPL) were recorded at positions along a transect from the J9 speaker to the opposite side of the tank, including observer ring (behavioral trials) and turtle ear (electrophysiological trials) locations for all three size classes. In addition to positions along a transect extending from the J9 speaker to the tank's edge, recordings were made at the locations of all response keys used in behavioral experiments. The most critical locations were the observer ring and turtle ear for behavioral and electrophysiological trials, respectively, as these were the locations where the turtle was located relative to the speaker during the onset of behavioral trials and throughout the electrophysiology trials.

The J9 underwater speaker, Crunch amplifier, and TDT system were used to produce sound stimuli at all frequencies and attenuation levels considered for electrophysiological and behavioral experiments. At each location described above, two TC4013 hydrophones (Reson A/S, Slangerup, Denmark) positioned 2 cm apart along each of three mutually perpendicular axes (*x, y, z*) were used to record sound pressure levels generated by the J9 speaker. A sampling probe made of PVC with a rotatable end was constructed so that hydrophones could be placed along orthogonally oriented axes while keeping the center point between hydrophones constant. The hydrophones were interfaced with two separate CCA100 conditioning charge amplifiers (Reson A/S) and data were recorded at 25 kHz using the TDT system. Water levels were maintained in the tanks at the exact levels used during experimentation.

The mean value recorded by the two TC4013 hydrophones was considered the SPL level at the sampling location. Particle accelerations along each axis were determined using the following equation (Kalmijn, 1988; Wahlberg et al., 2008):

$$a = \frac{-\Delta sig}{\rho \Delta r}$$

where *a* is particle acceleration (m s^{-2}), Δsig is the magnitude of the difference between the waveforms of the two hydrophones (Pa), ρ is density of the seawater (kg m^{-3}), and r is the distance between the hydrophones (m). For sinusoidal waveforms, particle acceleration is related to particle velocity according to the following equation (Kalmijn, 1988):

$$a = v \times 2\pi f$$

where *a* is particle acceleration (m s^{-2}), *v* is particle velocity (m s^{-1}), and *f* is frequency (Hz). Using this equation, particle velocities were also determined.

Results

Behavioral Experiments

Finding 1: Sea turtles required extended training time before they were ready for behavioral audiogram trials.

One unexpected outcome of this study was that training sessions took significantly longer than expected. The average training time for the Cc 2005/2007 and 2009 classes were 6 months and 3.7 months, respectively. A representative spreadsheet of training progress for one turtle is included below (Table 2). Post-hatchling turtles generally picked up the training quicker than

older turtles, requiring 16 training sessions on average compared to 31 training sessions for juveniles. The extended training time was 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 critical for acquiring accurate behavioral audiograms. To further complicate matters, some turtles demonstrated that they could respond to the *training* session stimulus correctly, but could not maintain this state consistently during *experimental* runs and thus were removed from our analyses, lowering our sample size. Therefore, when doing future auditory behavioral research with sea turtles, it is important to factor in the time investment required to train sea turtles, a period of time that is by no means trivial.

Finding 2: Post-hatchlings (Cc2009) and juveniles (Cc2007, Cc2005) detected a similar range of frequencies with some variation in sensitivities within the hearing range.

Irrespective of size class, all turtles responded to sounds in the range of 50-1200 Hz and failed to respond to sounds above 1200 Hz. A sample data sheet for a behavioral trial for one turtle is included in Table 3 along with the resulting behavioral audiogram in Fig. 9. Overall, post hatchling turtles (Cc2009) responded with the greatest sensitivity at 200 Hz (84.5 dB re 1 µPa), with sensitivity decreasing above and below 200 Hz. The lowest sensitivity within the post-hatchlings' auditory range occurred at 800 Hz (112 dB re 1 µPa). Juveniles (Cc2007 and Cc2005) responded with the greatest sensitivity at 800 Hz (76 dB re 1 µPa), with high sensitivity at 400 Hz (88.5 dB re 1 µPa), 700 Hz (91 dB re 1 µPa), and 1200 Hz (86 dB re 1 µPa). The lowest sensitivity within their auditory range occurred at 50 Hz (117 dB re 1 µPa) (Fig. 10).

Table 2. *Example of a training sequence for a Cc2007 sea turtle. The point at which training was achieved is highlighted in yellow.*

Rew. Not Shown & Correct (N)	Rew. Not Shown & Correct (Y)	% Cor (n)	Total Attempted (n)	# Cor (n)*	% Cor (y)	Total Attempted (y)	# Cor (y)*	SPL	Session Duration	Time	Date
			7			5			30 min	14:45	14-Jun-10
33	36	89	9	8	73	11	8	122	25 min	17:35	26-Jun-10
14	60	43	14	6	80	5	4	118	30 min	12:50	30-Jun-10
46	77	46	13	6	85	13	11	119	55 min	9:20	1-Jul-10
77	92	77	13	10	92	13	12	117	45 min	13:45	5-Jul-10
100	77	100	13	13	77	13	10	118	60 min	10:20	7-Jul-10
100	85	100	13	13	85	13	11	117	45 min	7:35	13-Jul-10
100	11	100	6	6	11	9	1	119	115 min	5:00	2-Sep-10
50	50	50	4	2	50	6	3	117	80 min	20:05	9-Sep-10
50	57	50	16	8	64	14	9	118	85 min*	6:20	12-Sep-10
70	100	70	10	7	100	10	10	117	90 min	18:35	13-Sep-10
7	83	41	29	12	83	6	5	117	120 min	19:40	14-Sep-10
17	100	50	6	3	100	4	4	116	35 min	20:20	15-Sep-10
60	100	60	5	3	100	5	5	118	50 min	20:55	20-Sep-10
80	100	80	5	4	100	5	5	118	70 min	19:50	21-Sep-10
50	70	50	10	5	70	10	7	117	60 min	6:20	23-Sep-10
40	60	40	5	2	60	5	3	120	45 min	20:15	27-Sep-10
75	62	75	12	9	62	13	8	118	70 min	4:50	29-Sep-10
90	N/A	90	10	9	N/A	N/A	N/A	N/A*	55 min	6:50	2-Oct-10
0	50	0	0	0	50	2	1	116	30 min	4:35	4-Oct-10
81	55	81	16	13	55	22	12	115	60 min	6:15	7-Oct-10
89	44	89	9	8	44	9	4	116	70 min	6:20	22-Oct-10
90	70	90	10	9	70	10	7	119	55 min	5:45	8-Nov-10
100	80	100	5	5	80	5	4	120	25 min	7:20	21-Nov-10
80	90	80	10	8	90	10	9	120	60 min	4:15	17-Dec-10

Table 3. *Behavior trials for one post hatchling loggerhead (Cc2009). Once the turtle was successfully trained, behavioral testing proceeded in blocks of trials, with 10 repetitions for each dB level. Threshold was determined when the animal fell below the 80% correct criteria for either the yes or no response.*

Frequency	dB	% Correct Stimulus	% Correct No Stimulus	Frequency	dB	% Correct Stimulus	% Correct No Stimulus	Frequency	dB	% Correct Stimulus	% Correct No Stimulus
50 Hz	114	80	100	**300 Hz**	119	80	100	**700 Hz**	120	80	80
50 Hz	110	80	80	**300 Hz**	118	100	70	**700 Hz**	111	100	80
50 Hz	105	100	100	**300 Hz**	110	80	80	**700 Hz**	105	100	80
50 Hz	101	60	80	**300 Hz**	104	80	80	**700 Hz**	104	100	100
50 Hz	99	40	100	**300 Hz**	99	80	80	**700 Hz**	99	60	100
100 Hz	119	100	80	**300 Hz**	95	80	80	**700 Hz**	95	40	80
100 Hz	114	80	100	**300 Hz**	90	80	80	**800 Hz**	119	100	100
100 Hz	107	80	100	**300 Hz**	86	60	100	**800 Hz**	114	20	100
100 Hz	100	100	100	**400 Hz**	128	100	100	**900 Hz**	114	80	100
100 Hz	96	80	100	**400 Hz**	124	100	100	**900 Hz**	105	100	N/A
100 Hz	91	80	100	**400 Hz**	117	80	100	**1000 Hz**	124	80	80
100 Hz	88	60	100	**400 Hz**	111	80	100	**1000 Hz**	118	100	100
100 Hz	84	20	80	**400 Hz**	107	60	100	**1000 Hz**	115	60	80
200 Hz	123	100	80	**600 Hz**	117	80	100	**1000 Hz**	109	80	100
200 Hz	117	80	100	**600 Hz**	112	80	100	**1000 Hz**	104	80	80
200 Hz	107	80	100	**600 Hz**	108	100	100	**1000 Hz**	99	60	100
200 Hz	98	80	100	**600 Hz**	103	60	100	**1000 Hz**	95	80	80
200 Hz	85	80	80								
200 Hz	78	80	100								

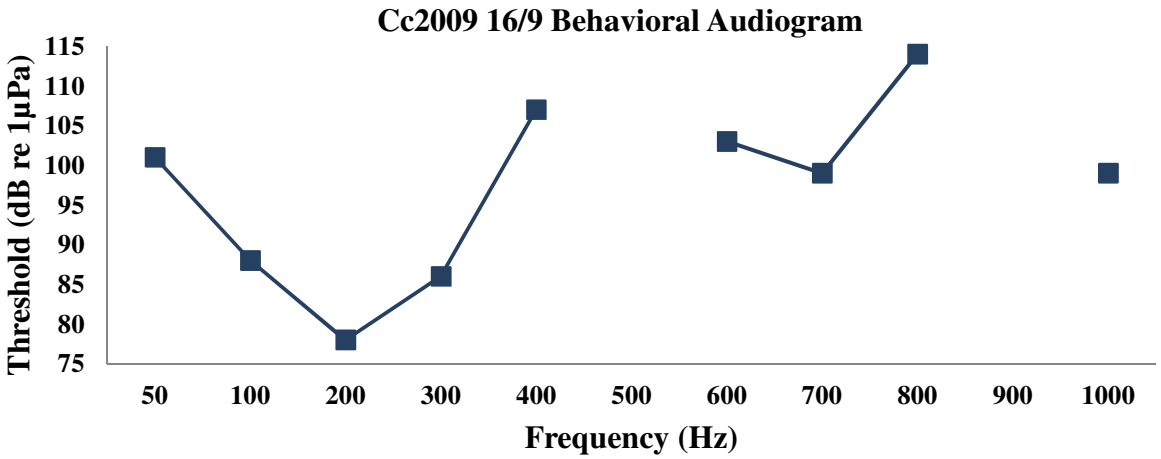


Figure 9. *Behavior audiogram generated from data collected in Table 3.*

Thresholds for the post-hatchlings and juveniles were consistently above the ambient background noise in the experimental tanks, indicating that the observed thresholds are absolute and not masked thresholds (Fig. 10).

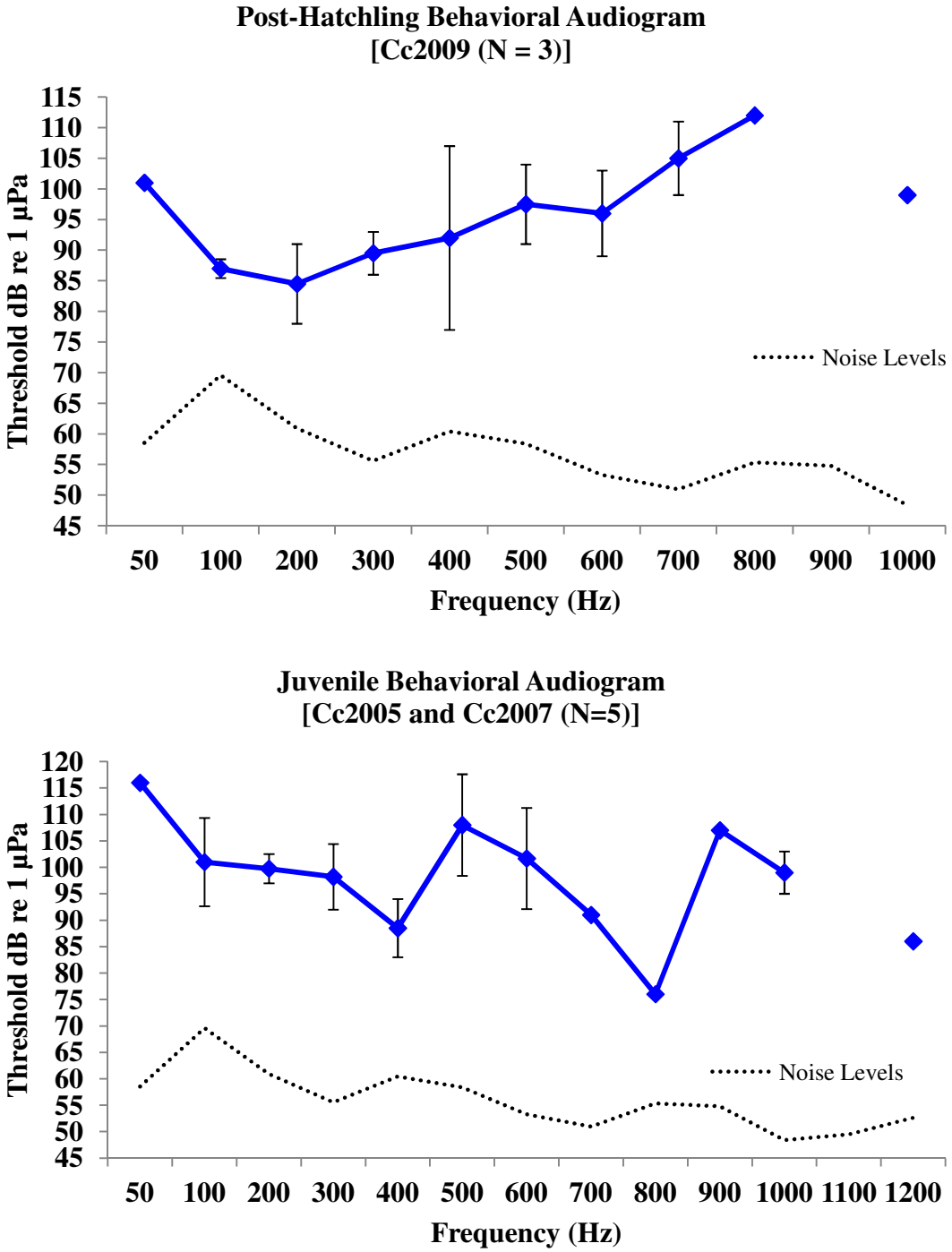


Figure 10. *Mean behavioral audiograms for the Cc2009 year class (top) and C2005 and Cc2007 year classes (bottom) of sea turtles. The Cc2005 and Cc2007 year classes were pooled because they were similar in size. Error bars denote ±1 S.E. of the mean. The black dotted line represents ambient noise conditions in the experimental tank.*

Finding 3: No statistical significance in behavioral thresholds was detected between post-hatchling (Cc2009) and juvenile (Cc2007, Cc2005) turtles.

While there was some variation in the audiograms between post-hatchling and juvenile sea turtles, a paired two-tailed t-test revealed no significant difference in threshold between the two year classes (df = 9, t_{crit} = 2.262, t_{stat} = 0.306, P = 0.767). The mean thresholds across the hearing frequency range for the post-hatchlings and juveniles were 97.91 dB re 1 µPa and 96.35 dB re 1 µPa, respectively.

Finding 4: Response speeds were greater for incorrect than correct trials. However, response speeds did not differ with size class or between suprathreshold and threshold trials.

A 3-factor ANOVA was performed on response speeds derived from video footage during behavioral trials (Table 4). To remove a size or distance bias from the analysis, all response speeds were expressed in body lengths s^{-1}. Correct choice response speeds were significantly faster than incorrect choice response speeds, suggesting that turtles took slightly more time to select a response key when they were incorrect than when they were correct. In general, turtles did not waste time in making selections, even when they approached threshold levels, i.e., when they triggered the observer key they swam quickly to the response key generally in < 10 s for juveniles and < 15 s for post hatchlings.

Table 4. *3-factor ANOVA for response speeds (body lengths s^{-1}) recorded in behavioral experiments. Factors: (1) size = post-hatchling and juvenile; (2) threshold = above threshold and at threshold; (3) response = correct response and incorrect response irrespective of whether stimuli was sound or no sound.*

Tests of Between-Subjects Effects

Dependent Variable:Rank of BLperSec

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1.872E7	7	2674309.248	2.771	.007
Intercept	2.919E9	1	2.919E9	3023.932	.000
Size	425311.782	1	425311.782	.441	.507
Threshold	427321.568	1	427321.568	.443	.506
ResponseYN	7140132.572	1	7140132.572	7.397	.007
Size * Threshold	964662.403	1	964662.403	.999	.318
Size * ResponseYN	19519.589	1	19519.589	.020	.887
Threshold * ResponseYN	381608.157	1	381608.157	.395	.530
Size * Threshold * ResponseYN	91887.676	1	91887.676	.095	.758
Error	3.283E9	3401	965209.890		
Total	1.321E10	3409			
Corrected Total	3.301E9	3408			

a. R Squared = .006 (Adjusted R Squared = .004)

This quick and deliberate response is an indication that the turtles were well trained and understood the 'game'. Interestingly, no significant difference in response speeds was detected between post-hatchling and juvenile turtles and between supratheshold and threshold trials.

Electrophysiological Experiments

Finding 1: AEP waveforms were clearly visible in the FFT spectrum and were consistently found at twice the stimulus frequency.

The TDT hardware and software produced consistent, clear sinusoidal signal sources and recorded well-defined AEPs at twice the stimulus frequency (Fig. 11). The magnitude of the AEP peak in the FFT decreased consistently with increased attenuation, with threshold occurring at the last dB level where the FFT relative amplitude was at least 3 dB greater than FFT relative amplitudes for other non-AEP frequencies (Fig. 12).

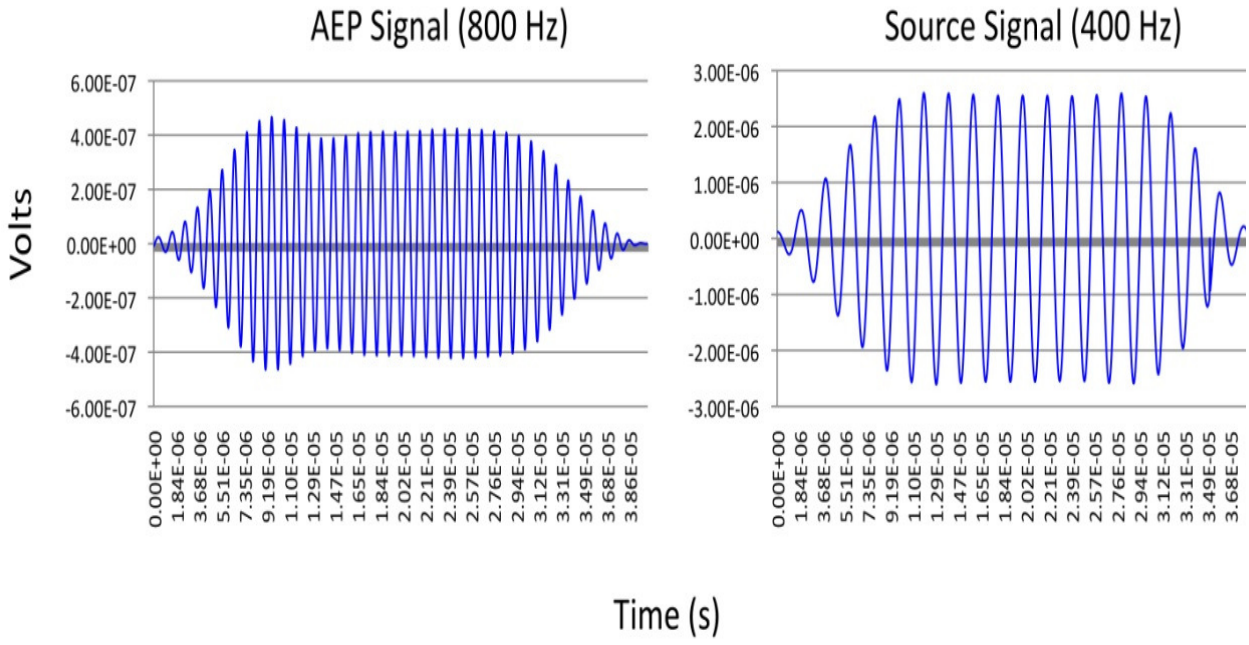


Figure 11. *Example of AEP signal (left) recorded from a source signal (right) of 400 Hz. Note that the AEP signal is twice the frequency of the source signal, which is a result of simultaneous responses from two groups of hair cells oriented in opposite directions.*

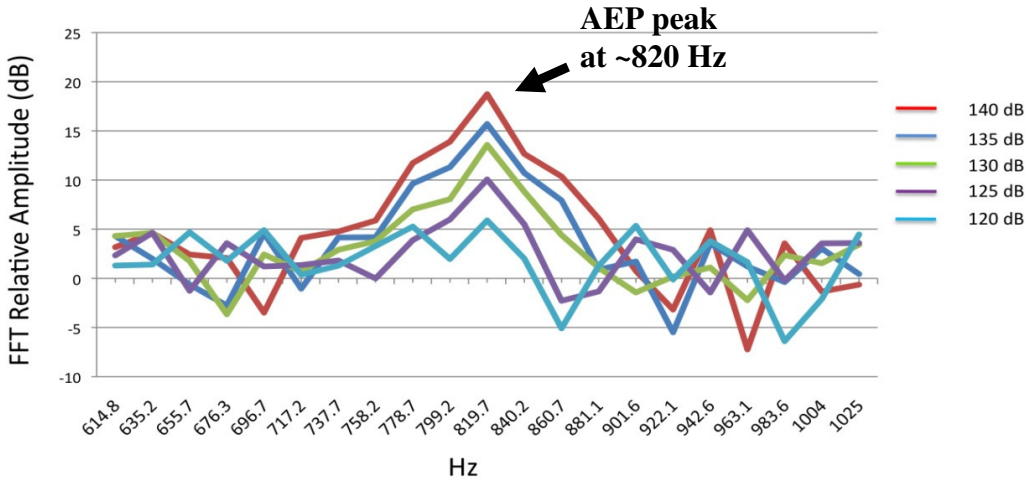


Figure 12. *Magnitude of AEP FFT relative to the local noise level for a Cc2009 turtle (RW16, POS6) presented with sound stimuli of 410 Hz at dB levels from 120 – 140 dB re 1 µPa. The AEP peak is approximately twice the source frequency as expected. Threshold is defined as the last dB level where the AEP FFT relative amplitude is at least 3 dB greater than FFT relative amplitude for other frequencies. In the figure above, 125 dB is the threshold level, as the peak for 120 dB is not greater than 3 dB above the surrounding FFT relative amplitudes.*

Finding 2: AEP waveforms did vary among individuals, but there was no significant difference between post-hatchling and juveniles AEP-based thresholds.

Not surprisingly there was variation in individual AEP-based audiograms, as can be seen in the three post-hatchlings tested (Fig. 13). When the mean AEP-based audiograms for post-hatchlings and juveniles were compared, no significant differences were detected (paired two-tailed t-test: $df = 11$, $t_{crit} = 2.228$, $t_{stat} = 0.302$, $P = 0.767$). The mean threshold for post-hatchlings and juveniles over the auditory range was 126.27 and 126.92 dB re 1 µPa, respectively. Post-hatchlings (Cc2009) responded with the greatest sensitivity at 200 Hz (116 dB re 1 µPa), with lowest sensitivity at 1000 Hz (135 dB re 1 µPa). Juveniles responded with the greatest sensitivity at 50 Hz (110 dB re 1 µPa), with lowest sensitivity at 1000 Hz (142 dB re 1 µPa) (Fig. 14).

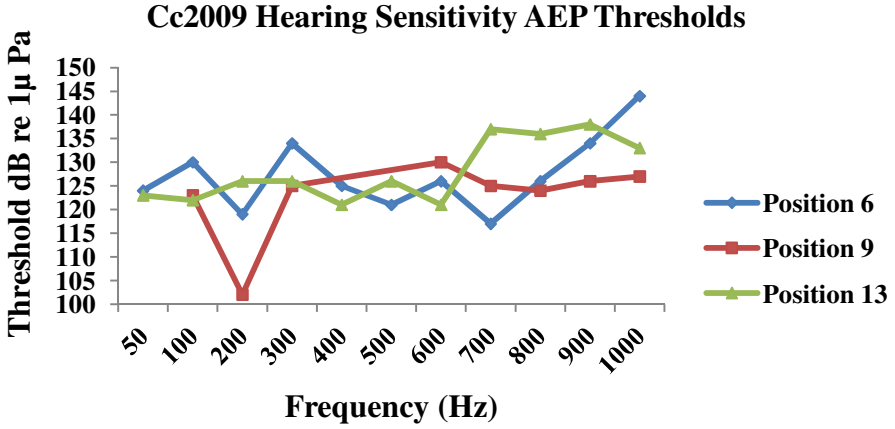


Figure 13. *Example of electrophysiology audiograms for the three Cc2009 sea turtles (RW 16, Pos. 6, 9, and 13) (see Table 1).*

Finding 3: The AEP-derived hearing frequency range was similar for post-hatchling and juvenile turtles.

The hearing range for post-hatchlings and juveniles was 50 – 1100 Hz (Fig. 14). Thresholds for the post-hatchlings and juveniles were consistently above the ambient background noise in the experimental tanks, indicating that the observed thresholds are absolute and not masked thresholds (Fig. 14).

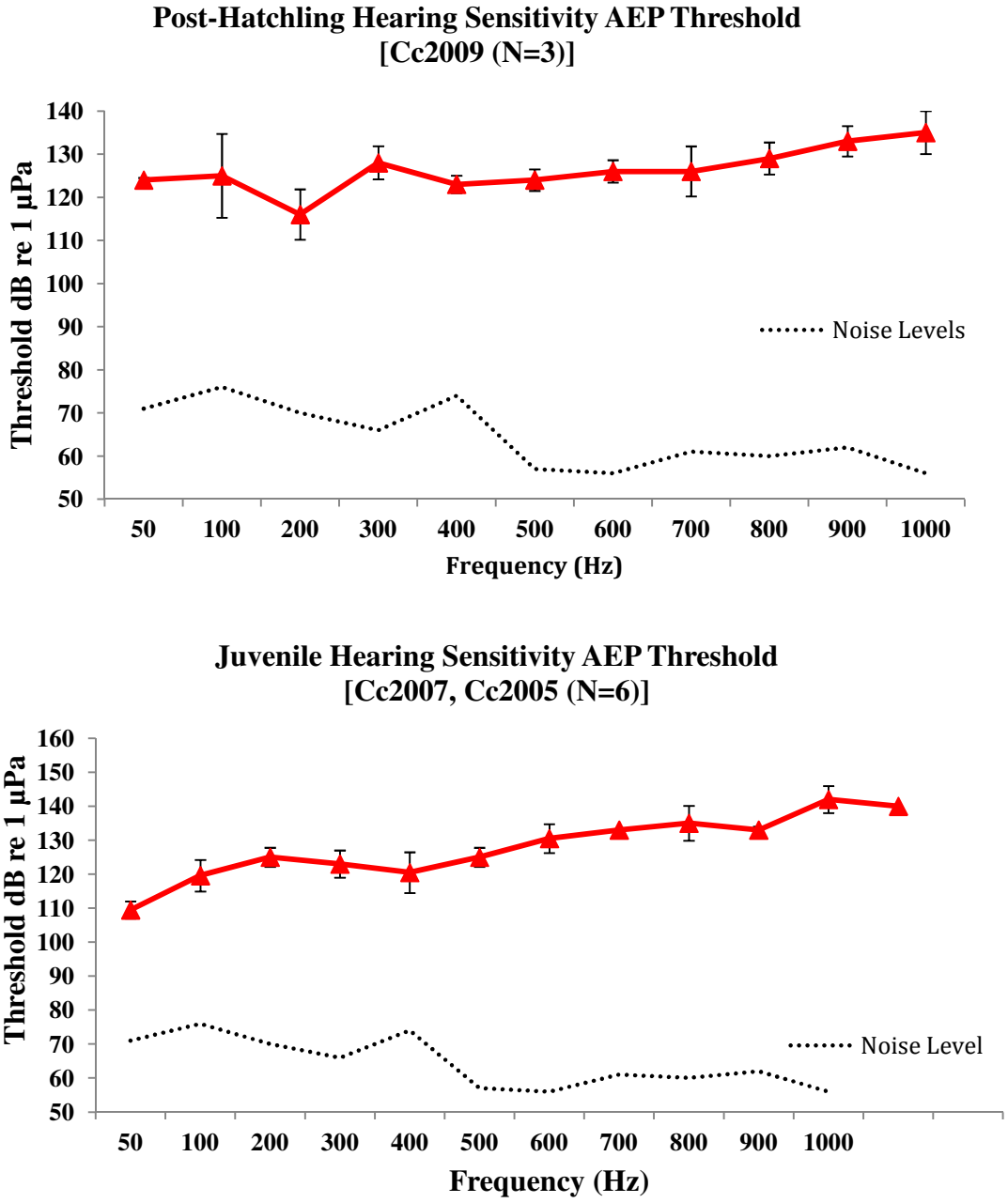


Figure 14. *Mean AEP audiograms for post-hatchlings (Cc2009) (top) and juveniles (Cc2005, Cc2007)(bottom). Error bars denote ±1 S.E. of the mean. The black dotted line represents ambient noise conditions in the experimental tank.*

Integration of Behavioral and Physiological Data

Finding 1: Both post-hatchlings and juveniles had significantly higher AEP-derived than behavior-derived auditory thresholds.

Behavior-derived auditory thresholds were consistently lower than AEP-derived auditory thresholds (paired two-tailed t-test (post-hatchlings): $df = 9$, $t_{crit} = 2.262$, $t_{stat} = 12.65$, $P < 0.0001$; paired two-tailed t-test (juveniles): $df = 10$, $t_{crit} = 2.228$, $t_{stat} = 5.566$, $P < 0.0002$) (Fig. 15). These results indicate that AEPs underestimate actual thresholds, and behavior-based work is more sensitive for determining hearing sensitivity.

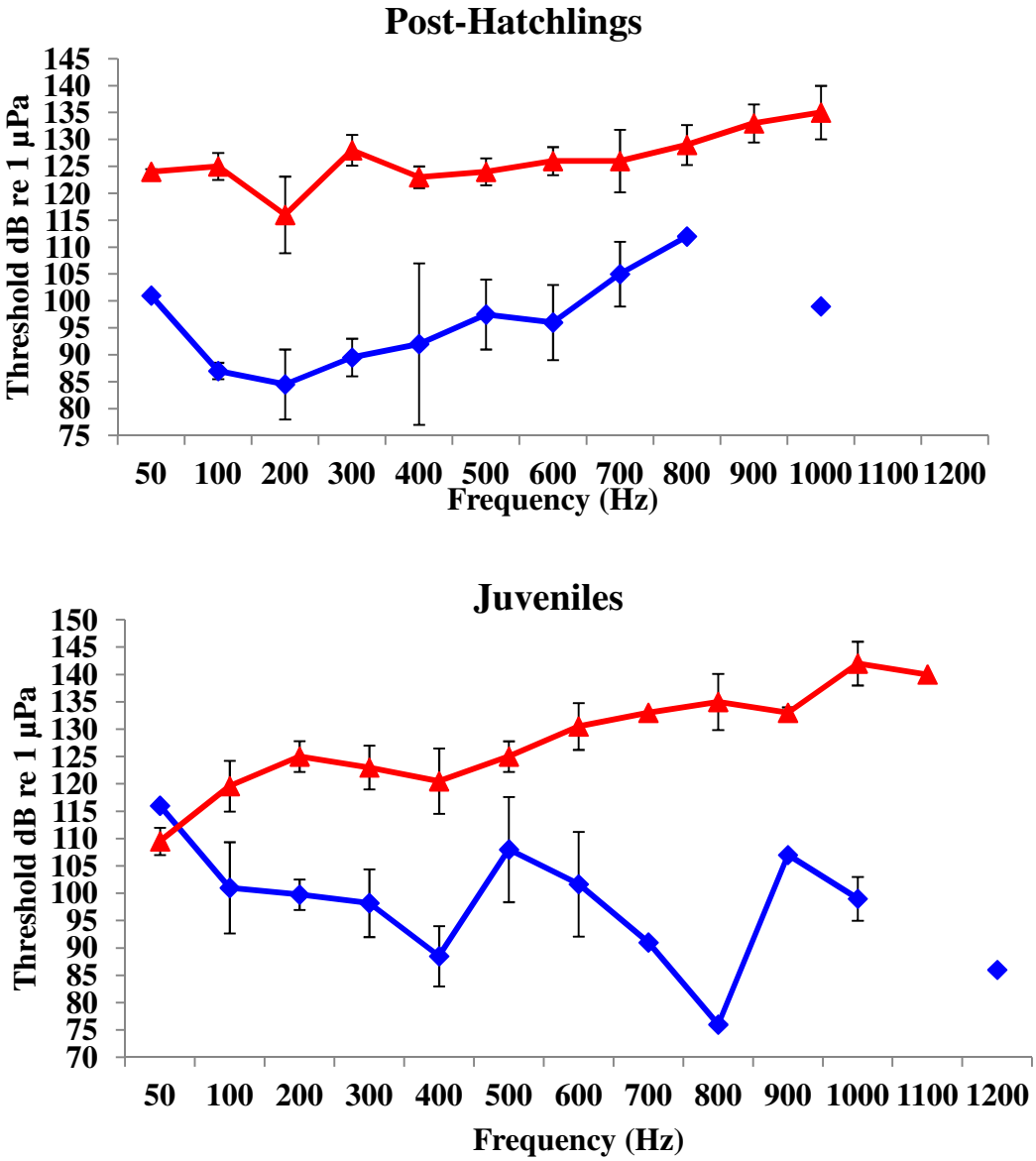


Figure 15. *AEP audiograms (red curves) and behavior audiograms (blue curves) for post-hatchlings (Cc2009) (top) and juveniles (Cc2005, Cc2007)(bottom). Error bars denote ±1 S.E. of the mean.*

Finding 2: The hearing frequency range detected in both behavior and AEP experiments were consistent and behavior/AEP peak sensitivities were similar in post-hatchlings.

The hearing frequency range measured for post-hatchlings in behavior audiograms (50-1000 Hz) was similar to that measured in AEP audiograms (50 – 1000 Hz) (Fig. 15). Similarly, the frequency range measured for juveniles in behavior audiograms (50-1200 Hz) was similar to that measured in AEP audiograms (50 – 1100 Hz). Both AEP and behavioral data revealed that maximum hearing sensitivity occurs at 200 Hz in post-hatchlings. In juveniles, there were differences in peak sensitivities between the two methods, with AEPs showing greatest sensitivity at 50 Hz and behavior studies showing greatest sensitivity at 800 Hz (Fig. 15).

Findings for Tank Mapping

Finding 1: Particle motion diminished rapidly from the sound source and was low at behavioral thresholds.

Particle motions, i.e., particle acceleration and particle velocity, were measured along three mutually perpendicular axes (Fig. 16). Particle accelerations (m s^{-2}) and particle velocity (m s^{-1}) generally diminished rapidly from the speaker source to either the location of the ear during AEP experiments (70-90 cm from speaker source) or location of the observer key during behavioral experiments ($\geq$30 cm from speaker source) (Figs. 17-20). For example, at 500 Hz, particle acceleration and velocity along the z-axis decreased >96% from the source to the head position for Cc2005 turtles, i.e., 70 cm (6.03 m s^{-2} to 0.217 m s^{-2} and 0.002 m s^{-1} to 0.000069 m s^{-1}) in AEP experiments with no attenuation in the TDT system. In behavioral experiments, particle acceleration and velocity along the z-axis decreased >90% from the source to the observer key at 500 Hz for Cc2009 turtles (6.03 m s^{-2} to 0.56 m s^{-2} and 0.002 m s^{-1} to 0.00018 m s^{-1}) with no attenuation in the TDT system. This decreasing trend in particle motion held for most axes and frequency ranges, irrespective of whether head or observer key locations were considered. The greatest drop in particle accelerations and velocities occurred along the x- and z-axes, with the lowest drops occurring along the y-axis where the lowest particle motions were produced by the J9 transducer (Figs. 17-20).

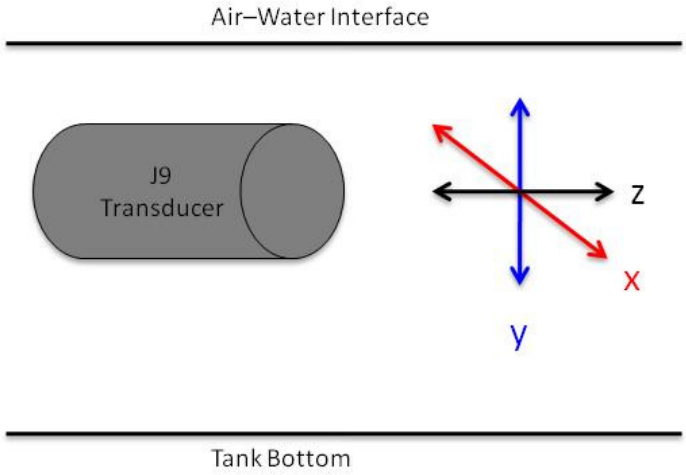


Figure 16. *Axes considered in tank mapping. The x-axis and y-axis are orthogonal to the longitudinal axis of the J9 while the z-axis is parallel to the longitudinal axis of J9. The air-water interface and tank bottom are also depicted.*

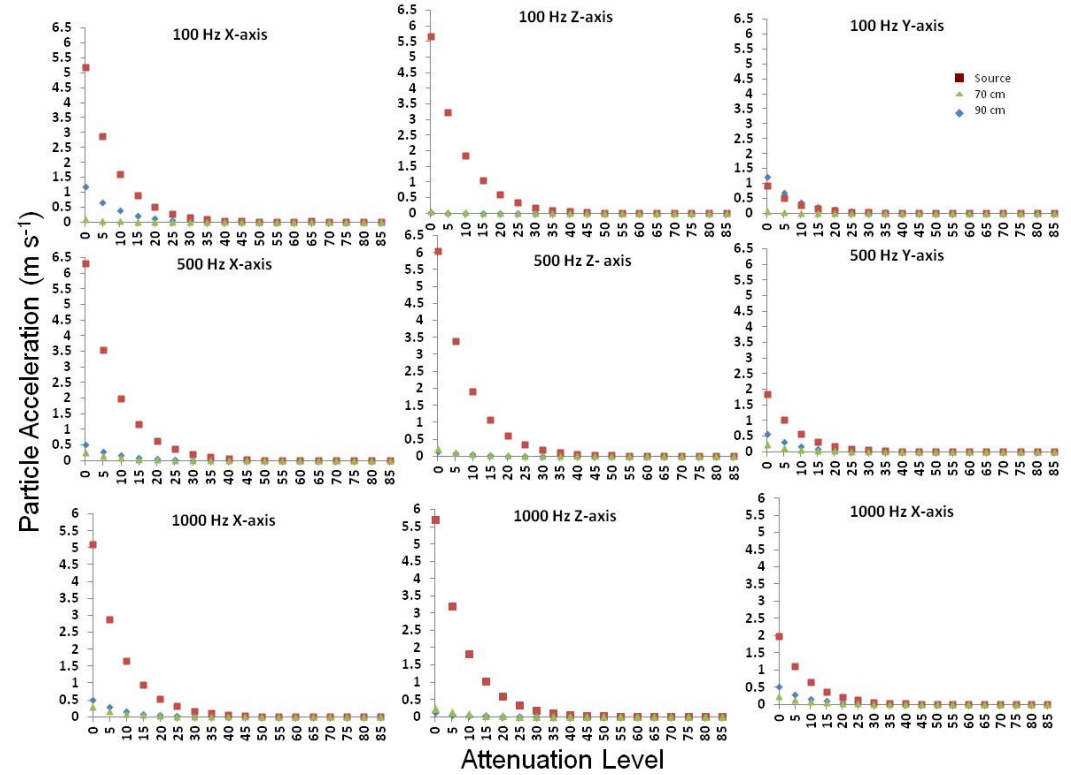


Figure 17. *Particle accelerations (m s^{-2}) along the x-, y-, and z-axes for sound presentations of 100 Hz (top row), 500 Hz (middle row), and 1000 Hz (bottom row) for the different attenuation levels used with the TDT stimulus delivery system during AEP experiments. Source = J9 speaker location, 70 cm = head location for Cc2005 turtles, 90 cm = head location for Cc2009 turtles. Data for the head position of Cc2007 turtles are not shown but the position was intermediate between the Cc2005 and Cc2009 turtles (80 cm).*

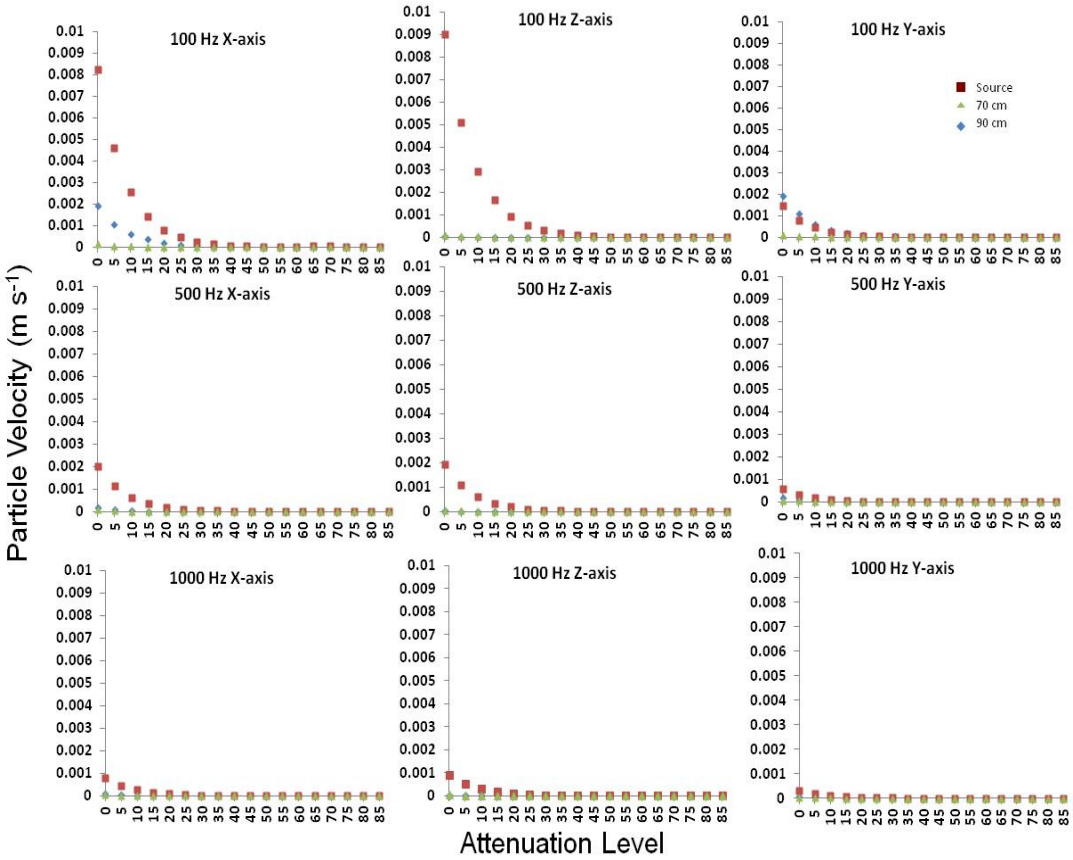


Figure 18. *Particle velocities (m s^{-1}) along the x-, y-, and z-axes for sound presentations of 100 Hz (top row), 500 Hz (middle row), and 1000 Hz (bottom row) for the different attenuation levels used with the TDT stimulus delivery system during AEP experiments. Source = J9 speaker location, 70 cm = head location for Cc2005 turtles, 90 cm = head location for Cc2009 turtles. Data for the head position of Cc2007 turtles are not shown but the position was intermediate between the Cc2005 and Cc2009 turtles (80 cm).*

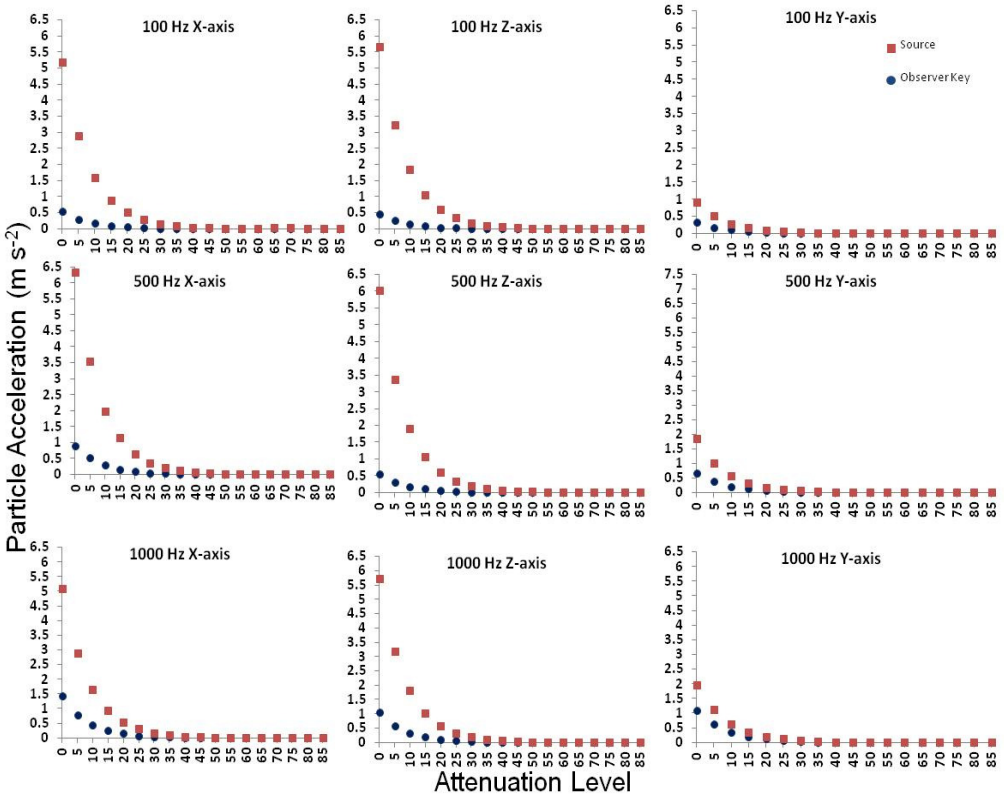


Figure 19. *Particle accelerations (m s^{-2}) along the x-, y-, and z-axes for sound presentations of 100 Hz (top row), 500 Hz (middle row), and 1000 Hz (bottom row) for the different attenuation levels used with the TDT stimulus delivery system during behavioral experiments. Source = J9 speaker location, observer ring = position of observer ring during Cc2007 and Cc2009 trials. Data for the observer ring during Cc2005 turtles are not shown but the ring was located farther from the J9 and thus accelerations were significantly lower than those shown.*

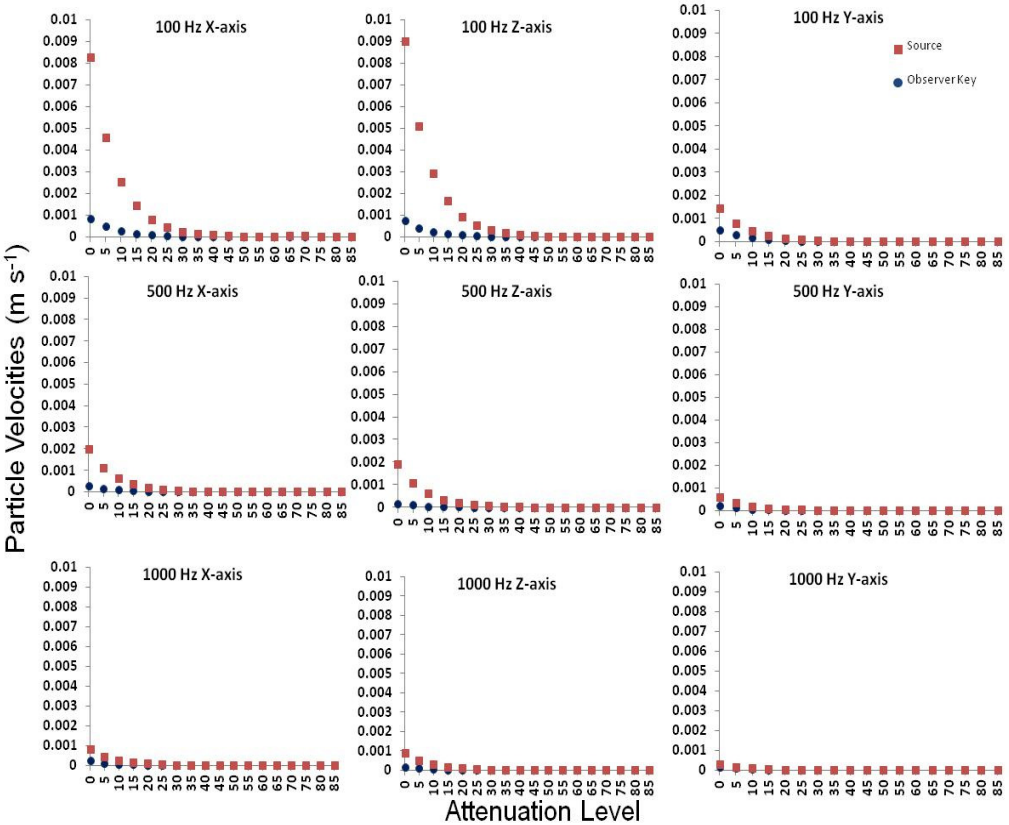


Figure 20. *Particle velocities (m s^{-1}) along the x-, y-, and z-axes for sound presentations of 100 Hz (top row), 500 Hz (middle row), and 1000 Hz (bottom row) for the different attenuation levels used with the TDT stimulus delivery system during behavioral experiments. Source = J9 speaker location, observer ring = position of observer ring during Cc2007 and Cc2009 trials. Data for the observer ring during Cc2005 turtles are not shown but the ring was located farther from the J9 and thus accelerations were significantly lower than those shown.*

One notable exception to the decreasing trend in particle accelerations and velocities occurred at frequencies of 50-100 Hz along the y-axis, where, in some cases, particle accelerations at the Cc2009 head location exceeded those at the source (Fig. 17). This pattern was likely a product of complex sound wave reverberations and reflections in the tank as well as interactions with the air-water interface, which can serve as a reflector of sound waves. In many cases, particle accelerations and velocities were higher 90 cm from the J9 compared to 70 cm (Fig. 17), which again is likely a product of complex wave patterns produced in the experimental tank.

Particle velocities and particle accelerations were consistently less than 0.002 m s^{-1} and 1.5 m s^{-2} for all axes, frequencies, and attenuation levels for both head and observer key locations. At threshold, particle accelerations and velocities were low, especially for behavioral trials. At AEP-derived thresholds, particle velocities ranged from 3.228 x 10^{-6} to 7.625 x 10^{-5} m s^{-1} and particle accelerations ranged from 0.0112 to 0.2875 m s^{-2} (Tables 5-6). Particle motions were even lower at behavior-derived thresholds, with particle velocities ranging from 1.386 x 10^{-8} to 5.351 x 10^{-6} m s^{-1} and particle accelerations ranging from 0.0002 to 0.0095 m s^{-2} (Tables 7-8).

Table 5. *SPL, particle velocity, and particle acceleration at AEP thresholds for Cc2009 sea turtles at sound presentation levels of 100, 500, and 1000 Hz.*

Freq. (Hz)	**SPL (dB re 1 µPa)**		**Velocity (m s^{-1})**			**Acceleration (m s^{-2})**	
		x-axis	**z-axis**	**y-axis**	**x-axis**	**z-axis**	**y-axis**
100	125	7.625 x 10^{-5}	3.466 x 10^{-5}	6.932 x 10^{-5}	.1126	.1170	.0261
500	124	1.525 x 10^{-5}	1.802 x 10^{-5}	8.318 x 10^{-6}	.0566	.0566	.0261
1000	135	2.495 x 10^{-5}	2.634 x 10^{-5}	2.010 x 10^{-5}	.1568	.1656	.1263

Table 6. *SPL, particle velocity, and particle acceleration at AEP thresholds for Cc2007 and Cc2005 sea turtles at sound presentation levels of 100, 500, and 1000 Hz.*

Freq. (Hz)	**SPL (dB re 1 µPa)**		**Velocity (m s^{-1})**			**Acceleration (m s^{-2})**	
		x-axis	**z-axis**	**y-axis**	**x-axis**	**z-axis**	**y-axis**
100	119.6	4.159 x 10^{-5}	1.789 x 10^{-5}	3.466 x 10^{-5}	.0160	.0112	.0129
500	125	1.525 x 10^{-5}	1.248 x 10^{-5}	3.228 x 10^{-6}	.0479	.0392	.0392
1000	142	4.583 x 10^{-5}	3.812 x 10^{-5}	3.951 x 10^{-5}	.2875	.2395	.2482

Table 7. *SPL, particle velocity, and particle acceleration at behavior thresholds for Cc2009 sea turtles at sound presentation levels of 100, 500, and 1000 Hz.*

Freq. (Hz)	**SPL (dB re 1 µPa)**		**Velocity (m s^{-1})**			**Acceleration (m s^{-2})**	
		x-axis	**z-axis**	**y-axis**	**x-axis**	**z-axis**	**y-axis**
100	87	3.813 x 10^{-7}	5.596 x 10^{-7}	1.386 x 10^{-8}	.0002	.0004	.0003
500	97.5	4.783 x 10^{-7}	3.826 x 10^{-7}	5.684 x 10^{-7}	.0015	.0012	.0018
1000	99	4.166 x 10^{-7}	3.105 x 10^{-7}	5.864 x 10^{-7}	.0026	.0020	.0037

Table 8. *SPL, particle velocity, and particle acceleration at behavior thresholds for Cc2007 and Cc2009 sea turtles at sound presentation levels of 100, 500, and 1000 Hz.*

Freq. (Hz)	**SPL (dB re 1 µPa)**		**Velocity (m s^{-1})**			**Acceleration (m s^{-2})**	
		x-axis	**z-axis**	**y-axis**	**x-axis**	**z-axis**	**y-axis**
100	101	5.351 x 10^{-6}	2.981 x 10^{-6}	4.450 x 10^{-6}	.0034	.0018	.0028
500	108	3.036 x 10^{-6}	2.105 x 10^{-6}	1.885 x 10^{-6}	.0095	.0066	.0059
1000	99	4.166 x 10^{-7}	3.105 x 10^{-7}	5.864 x 10^{-7}	.0026	.0020	.0037

Findings 2: SPL levels diminished less with distance than particle motion and were closely correlated with attenuation steps throughout the tank.

SPL levels (dB re 1 µPa) were consistent along the 3 axes measured and also decreased from the source to either the turtle's head location (AEP experiments) or observer rings (behavioral experiments) but not nearly to the level of particle accelerations (Figs. 21, 22). As expected, the observed decrease in SPL was linear over much of the attenuation range and was closely correlated with the 5 dB re 1 µPa attenuation steps used, indicating that attenuation changes in the TDT system result in similar attenuation changes at the turtle's head (electrophysiology trials) and observer key (behavioral trials). The observed regions of curvilinearity at higher attenuation levels at the source, site of turtle's head, and observer key are a product of the speaker not being able to produce low SPL levels at certain frequencies, i.e., limitations of the J9 transducer.

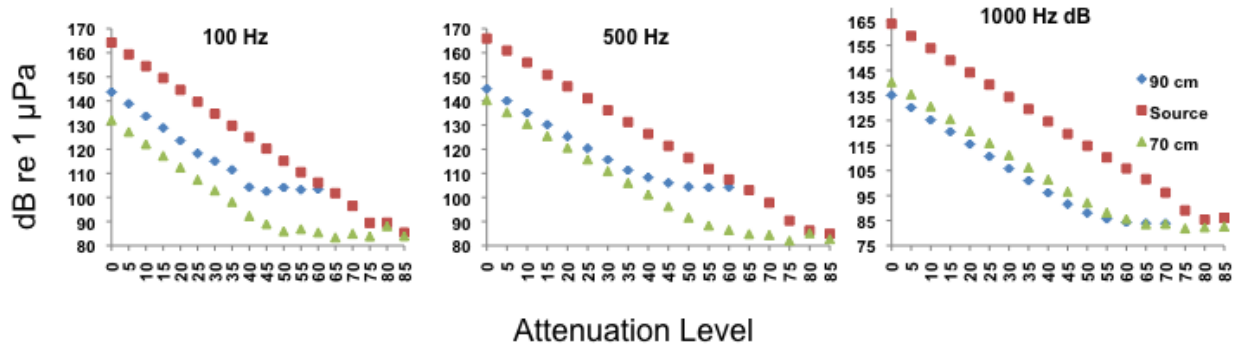


Figure 21*. Sound pressure levels (dB re 1 µPa) for sound presentations of 100 Hz (left), 500 Hz (middle), and 1000 Hz (right) for the different attenuation levels used with the TDT stimulus delivery system during AEP experiments (levels did not vary significantly with axis). Source = J9 speaker location, 70 cm = head location for Cc2005 turtles, 90 cm = head location for Cc2009 turtles. Data for the head position of Cc2007 turtles are not shown but the position was intermediate between the Cc2005 and Cc2009 turtles (80 cm).*

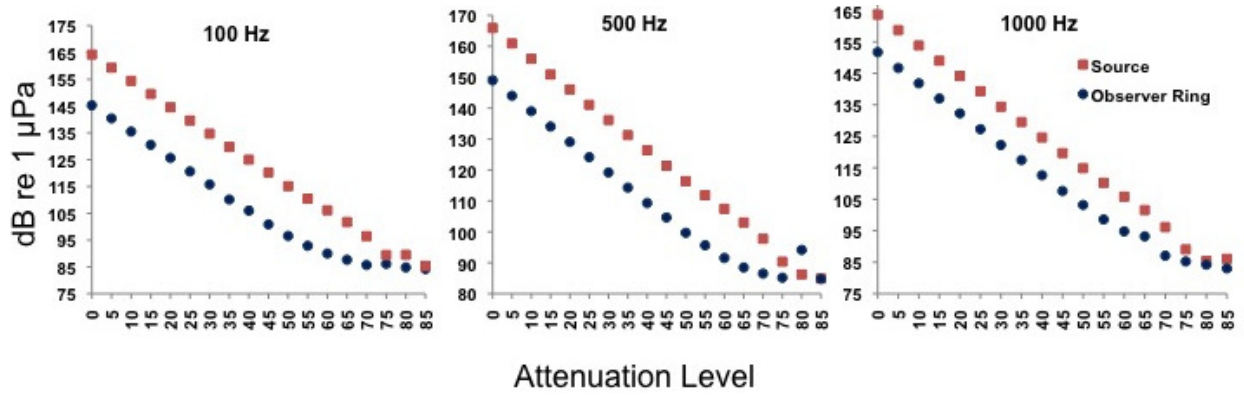


Figure 22. *Sound pressure levels (dB re 1 µPa) for sound presentations of 100 Hz (left), 500 Hz (middle), and 1000 Hz (right) for the different attenuation levels used with the TDT stimulus delivery system during behavioral experiments (levels did not vary significantly with axis). Source = J9 speaker location, observer ring = position of observer ring during Cc2007 and Cc2009 trials. Data for the observer ring during Cc2005 turtles are not shown.*

Discussion

Although current electrophysiological studies provide some insight into the auditory capabilities of sea turtles, they do not correlate electrophysiological results with behavioral responses or provide information on hearing capabilities throughout ontogeny. For mammals and many other animals, behavioral audiograms have been the standard for defining hearing range and sensitivity because behavioral audiograms determine the lowest detectable sound that will elicit a response from the animal. In this study, we employed an integrative approach, whereby we directly compared electrophysiological and behavioral audiograms. Behavioral audiograms were constructed from eight turtles over a period of three years, with training taking between 3-6 months on average before trials could begin. Once the turtles were trained, we found consistent responses during trials. The resultant audiograms illustrate that both size classes of loggerheads, post-hatchlings and juveniles, respond to sounds between 50 and 1200 Hz. Overall, post hatchling turtles (Cc2009) responded with the greatest sensitivity at 200 Hz (84.5 dB re 1 µPa), with sensitivity decreasing above and below 200 Hz. Juveniles (Cc2007 and Cc2005) responded with the greatest sensitivity at two peaks: 700/800 Hz (91 dB re 1 µPa/76 dB re 1 µPa), and 400 Hz (88.5 dB re 1 µPa). Though there was some variability in the most sensitive frequency (200 Hz for post-hatchlings and 800 Hz for juveniles), there was no overall statistical difference between the two groups. From these data we can confidently conclude that these animals respond only to low frequencies (<1200 Hz) and at levels of 85-117 db re 1 µPa.

From these same size classes, we were also able to collect underwater AEPs with a speaker some distance (70 -90 cm) from the animal. As is the case with fishes and squid, we observed consistent, clearly definable AEP FFT peaks at twice the stimulus frequency (Egner and Mann, 2005; Fay, 1974, Mooney et al., 2010). This doubling frequency occurs because of responses from two groups of hair cells oriented in opposite directions; one group responds to the pressure peak of the sinusoidal sound wave (forward movement of fluid in ear) and the other group responds to the pressure trough (rearward movement of fluid in ear). Unlike previous electrophysiological studies, we did not find a significant difference in range or threshold levels between post-hatchling and juvenile loggerhead sea turtles; both size classes responded to frequencies between 50 and 1000 Hz. Post-hatchlings (Cc2009) responded with the greatest sensitivity at 200 Hz (116 dB re 1 µPa), with lowest sensitivity at 1000 Hz (135 dB re 1 µPa).

Juveniles responded with the greatest sensitivity at 50 Hz (110 dB re 1 µPa), with lowest sensitivity at 1000 Hz (142 dB re 1 µPa).

One critical finding of this study is that thresholds are significantly higher (less sensitive) for AEPs 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 30 dB 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 (Richardson, 1995; Yuen, 2005; Fay, 1988). While behavioral approaches produce the most sensitive thresholds, collecting behavioral data is not always practical when compared to AEP data collection. In the case of sea turtles, behavioral trials require considerable training (3-6 mos.), maintenance of animals in captivity for extended periods, and large tank facilities for larger life history stages. Moreover, the capture of sea turtles is unpredictable and long-term husbandry permits, which are required for behavioral studies, are extremely difficult to obtain. In contrast, AEP data collection is rapid (1-3 hours), repeatable, and field portable, eliminating many of the husbandry challenges of behavioral studies. Furthermore, AEPs can be collected from injured/sick animals that could not otherwise perform training exercises.

While it is understandable why AEPs are generally the preferred method for assessing hearing in sea turtles, one important interpretation of our results is that AEP thresholds should not be used as the standard for setting sound exposure levels in the field. Behavioral experiments provide a more sensitive approach for defining threshold and are a better indicator of absolute threshold levels. While behavioral experiments are indeed more sensitive than AEP experiments, 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 frequency sensitivities. Moreover, there was no major difference in hearing frequency range or sensitivity thresholds between post-hatchlings and juveniles in either behavior or electrophysiology tests. While it is not advisable to extrapolate these results to other life history stages, our results to date indicate that there may not be a large difference in hearing capabilities across ontogeny.

Currently, there is emphasis on the consideration of both the pressure and particle motion components of sound in marine hearing studies (Fay and Popper, 1999; Casper and Mann, 2006; Horodysky et al., 2008; Mooney et al., 2010;). In fish, both the pressure and particle motion components of sound can be very important as pressure can be detected with swim bladders (if they are present) and particle motion can be detected by inducing whole body movements relative to the otoliths of the inner ear. Sea turtles do not have small hard structures (otoliths) floating on a bed of hair cells in their inner ears, and a mechanism for detection of the particle motion component of the sound field has not been found, i.e., there is no evidence that sea turtles possess an accelerometer-based system for encoding particle motions outside the body to the movement of hair cells in the inner ear system for purposes of sound detection. While some stress is potentially applied and distributed through the tectorial membrane during particle accelerations, which could cause some deflection and potentially trigger a response, the effect is assuredly very small given the density of the material. Thus, pressure and not particle motion is most likely driving the observed thresholds.

While our study was not explicitly designed to tease out whether sea turtles are sensing the pressure or particle motion components of sound, our mapping results provide further support that our observed thresholds are driven by the pressure component of sound. First, there is significant drop-off in particle acceleration and velocity levels with distance from the J9

transducer (>90% for AEP sampling locations and observer key locations at no attenuation), reducing the impact of the particle motion component of sound in our study. In contrast, declines in SPL levels with distance were significantly less and largely linear. Second, the particle motions at threshold are low when compared to particle motion detection thresholds in fish, particularly for our behavioral thresholds. At 100 Hz, our peak particle velocities for our AEP and behavioral thresholds are on the order of 10^{-5} m/s and 10^{-8} m/s - 10^{-6} m/s, respectively, which are near the *maximum* sensitivity limit of fishes with well-defined otolith inner ear systems at 100 Hz, such as Pacific fat sleepers (threshold = 1 x 10^{-7} m/s), bamboo sharks (threshold = 1 x 10^{-6} m/s), and sergeant major damselfish (1 x 10^{-5} m/s) (Mann et al., 2007; Egner and Mann, 2005; Casper and Mann, 2007). Moreover, the fish particle velocities are underestimates of true particle velocities. The particle velocity measurements for the fish experiments above were performed using a geophone or a vibraphone in front of a speaker to mimic the motions of the fish body, with the actual particle motions in the water (which is what we measured in this project using pressure measurements and equations from acoustics that relate pressure and velocity) being considerably higher. The geophone does not follow the fluid particle motion induced by a sound wave precisely, especially at higher frequencies, unless it is perfectly neutrally buoyant and much smaller than the sound wavelength. Consequently a comparison of actual particle motions would likely reveal that the sensitivity limits of fish are consistently higher than our particle motions at threshold. Third, the displacement of sensory hairs at our threshold levels is so small that it is hard to conceive that they could be detected with the sea turtle ear system. For example, if we assume that the velocity oscillations are perfectly sinusoidal, the *peak* displacement experienced by a fluid particle in a 500 Hz oscillating field (middle of the sea turtle sensitivity range) with a peak threshold velocity of 4.78 x 10^{-7} m s^{-1} (see Table 7) is 0.15 nm (displacement = peak velocity / frequency in rad s^{-1}). Assuming that (a) the turtle body tracks with the fluid velocity (i.e., fluid particle motion) and (b) the tectorial membrane remains fixed relative to the turtle's body and hair cells, then the *maximum* displacement between the tectorial membrane and hair cells would be 0.15 nm (about the diameter of an atom). The actual displacement between the tectorial membrane and hair cells, of course, would be much smaller than this because assumptions (a) and (b) are invalid, as the turtle is not neutrally buoyant (nor significantly smaller than the sound wavelengths) and the tectorial membrane is not fixed because it closely matches the density of the surrounding tissue. Even if the assumptions did hold, the hair cells, which are generally 5 – 10 microns in length, would need to detect a displacement on the order of 0.1 nm or 1/50,000 – 1/100,000 the length of the hair, which seems highly improbable.

Based on ear morphology, the observed particle motions, the fact that sea turtles are negatively buoyant and do not mimic fluid particle motion, and the small displacement of hair cells, it is reasonable to conclude that sea turtles are most likely detecting pressure and not particle motion at threshold.

This comprehensive assessment of loggerhead sea turtle hearing is useful in the evaluation of potential impacts of anthropogenic noises. We know very little about the extent that sea turtles use their auditory environment. In the inshore environment, where juvenile and adult sea turtles generally reside, the ambient environment is noisier than the open ocean environment of the hatchlings; this inshore environment is dominated by low frequency sound (Hawkins and Myrberg, 1983) and in highly trafficked areas, virtually constant low frequency noises from shipping, recreational boating, seismic surveys, and pile driving compound the potential for acoustic impact (Hildebrand, 2005). Data from this project help define the hearing frequency range and threshold of two ontogenetic stages of loggerhead sea turtles and provide a cautionary note, namely that AEPs should not set the standard for sound limits as they are less sensitive than behavioral approaches. While this study provides some important data for

management agencies, more research on the behavioral and physiological responses to sounds needs to be conducted on sea turtles before we can set appropriate noise exposure criteria for reduced fitness, injury and death. Currently there are few data on hearing loss/damage, hair cell regeneration, and masking for sea turtles. Controlled experiments in the natural environment need to be conducted to document and classify reactions to sound as either nuisance (i.e. causing the animal to move away, changing the animals' behavior to another acceptable consequence) or injurious (i.e. preventing the animal from completing essential behavior). The results of these research studies promise to provide new insights into the hearing ability and response to sound for sea turtles and a quantitative base for assessing the potential impact of human-made sound sources on multiple species of sea turtles across habitats and developmental stages.

Project Participants

Soraya Moein Bartol (Virginia Wesleyan College) and Ian K. Bartol (Old Dominion University) were the principal investigators on this grant and were responsible for program oversight, technical assistance, mentorship of project personnel, and scientific input. Ashley Lavender, a doctoral candidate at Old Dominion University, worked on the grant for the entire performance period and collected all of the data for this project. These data will serve as the foundation for her dissertation research for a doctoral degree within the Ecological Sciences Program at Old Dominion University. Over the course of this project, several paid undergraduates and volunteers played an important role in assisting the research team during data collection sessions at the NOAA Fisheries Service Galveston Laboratory and subsequent data processing sessions at Old Dominion University and Virginia Wesleyan College. The individuals most involved in these activities were: Tomeka Bandy, Katie Costello, Stephanie Couture, Chris Cotto, Darrin Dukes, Kim Janusaitis, Suzie Lazarowitz, Angela Mojica Osorio, Mark Prinz, Misty Snider, Jeanine Stewart, and Brent Winters. These students acquired skills in the maintenance of seawater systems, animal husbandry, electronics, behavioral training, physiological auditory research, video analysis, and Matlab processing. Most of these students assisted during experimental runs and acquired a working knowledge of behavioral and electrophysiological techniques used with marine turtles. Most importantly, all of these students gained exposure to the day-to-day operations of a biological research lab and developed insight into what biological research is like.

Meetings, Presentations, and Manuscripts

Data from this project were presented at several regional, national, and international conferences. These presentations are included below.

Lavender, A.L., S.M. Bartol, and Bartol, I.K. 2011. A two-method approach for investigating the hearing capabilities of loggerhead sea turtles *Caretta caretta*. Annual Symposium on Sea Turtle Biology and Conservation, San Diego, CA, April 12-15, 2011.

Lavender, A.L., S.M. Bartol, and I.K. Bartol. 2010. Hearing capabilities of loggerhead sea turtles (*Caretta caretta*) throughout Ontogeny. Second International Conference of the Effects of Noise on Aquatic Life, Cork, Ireland, August 15-20, 2010.

Lavender, A.L. 2010. Hearing capabilities of loggerhead sea turtles (*Caretta caretta*) throughout

ontogeny. Biology Graduate Student Organization Spring Symposium, Old Dominion University, Norfolk, Virginia.

As a result of the impact of Hurricane Ike on the Galveston Sea Turtle Facility during year 1 of this grant, the extended period required to train sea turtles, and integrated nature of this project, completing data collection and analysis at the end of the grant performance period was unavoidable. As a consequence, several manuscripts are in the preparation stages, with an anticipated submission to *The Journal of Experimental Biology* by the end of this year. Below we have listed the manuscripts that are either in press or preparation.

Lavender, A.L., S.M. Bartol, and I.K. Bartol. (in press). Hearing capabilities of loggerhead sea turtles (*Caretta caretta*) throughout Ontogeny. In *Proceedings of the Second International Conference of the Effects of Noise on Aquatic Life.* To appear in the journal *Bioacoustics*.

Lavender, A.L. , S.M. Bartol, and I.K. Bartol. (in prep). Electrophysiological and behavioral assessment of hearing in post-hatchling sea turtles *Caretta caretta*.

Lavender, A.L. , S.M. Bartol, and I.K. Bartol. (in prep). Electrophysiological and behavioral assessment of hearing in juvenile sea turtles *Caretta caretta*.

Lavender, A.L. , S.M. Bartol, and I.K. Bartol. (in prep). An integrative approach to assessing hearing in sea turtles: challenges and importance of correlating behavioral research with electrophysiological approaches.

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In addition to the financial support of JIP, we wish to thank a multitude of individuals for their assistance throughout this project. Obviously, this work could not have been completed without the efforts of our graduate student Ashley Lavender, who collected all of the data for this project and spent three field seasons at the NOAA Fisheries Service Galveston Laboratory. Ben Higgins, Director of the NOAA Fisheries Service Galveston Laboratory, was incredibly helpful, providing not only access to all of the sea turtles used in this study but also infrastructural support, scientific input, and resources for this project. Members of the Protected Species Branch staff at the NOAA Galveston Lab were also integral to the success of this project. We were fortunate to have a number of undergraduate students and volunteers helping us during our busy field seasons, including Tomeka Bandy, Katie Costello, Stephanie Couture, Chris Cotto, Darrin Dukes, Kim Janusaitis, Suzie Lazarowitz, Angela Mojica Osorio, Mark Prinz, Misty Snider, Jeanine Stewart, and Brent Winters.

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