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Underwater Hearing in the Loggerhead Turtle (*Caretta caretta*): A Comparison of Behavioral and Auditory Evoked Potential Audiograms

by

Kelly Jo Martin

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science
College of Marine Science
University of South Florida

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Date of Approval:
June 6, 2011

Keywords: bioacoustics, ear, auditory brainstem response, anthropogenic noise, sound, Testudines

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Dedication

For Montego - because nobody thought she could do it.

Acknowledgments

There are so many people that have made all of this work possible. First and foremost, I have to thank my coworker and friend, Sarah Alessi. Without her countless hours of assistance and dedication, willingness to do the impossible and endless supply of coffee, the project would never have been completed. She is the only other person who believed as strongly as I did that one turtle could actually do this. I also owe sincere gratitude to Joe Gaspard and Kim Dziuk, whose training assistance has more value than they will ever know. I also offer my sincerest appreciation to the many interns and volunteers who assisted me with this program on so many early mornings without being paid and without any complaints. Holly West deserves sincere thanks for continuing to train even when I was hours away. Lastly, I have to thank my graduate committee for supporting me and guiding me throughout the process. My knowledge of acoustics comes from the inspiration of my advisor, Dr. David Mann. Thank you so much for your patience. Dr. Gordon Bauer offered his patience and endless training expertise and never ceased to give up. Thank you so much for keeping us motivated and for keeping things simple – both for me and the turtles. Dr. Tony Tucker has inspired me to continue my quest to study and understand marine turtles throughout my career. Thank you for always keeping me thinking and providing me with so many motivational discussions. The work presented here would not be possible without the help of all of these amazing people.

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Abstract

Methods for collecting behavioral audiograms are often time consuming and require trained, captive subjects. It is more practical to measure hearing sensitivity using electrophysiological methods, such as auditory evoked potential (AEP) testing, in which electrodes measure action potentials in response to acoustic stimuli. These data can be collected in a matter of hours. However, results should be verified through behavioral testing. Current knowledge of marine turtle auditory abilities is based on a few electrophysiological tests. The purpose of this study was to collect and compare behavioral and auditory evoked potential audiograms in a captive adult loggerhead turtle (*Caretta caretta*). The behavioral audiogram was collected using a go/no-go modified staircase method utilizing 2-second pure-tone stimuli. AEP thresholds were measured underwater using subdermal electrodes placed beneath the frontoparietal scale, dorsal to the midbrain. Action potentials were measured in response to 50 ms tonal stimuli and averaged over a maximum of 1,000 responses. Evoked potential testing yielded thresholds from 100 - 1131 Hz with peak sensitivity at 200 and 400 Hz (110 dB re 1 µPa). Behavioral testing yielded thresholds from 50 - 800 Hz with peak sensitivity at 100 Hz (98 dB re 1 µPa). Behavioral thresholds averaged 8 dB lower than AEP thresholds from 100 to 400 Hz and 5 dB higher at 800 Hz. Results indicate that behavioral and evoked potential techniques are suitable for determining marine turtle hearing sensitivity. AEP testing is a good alternative when dealing with wild or untrained animals and when time is a critical factor.

Introduction

There are seven species of marine turtle globally, all threatened by increasing human presence in marine environments. The biggest threats facing loggerhead turtles are those related to commercial fisheries interactions, including trawl nets, longlines, and gillnets, as well as direct harvesting, vessel collisions, marine debris, pollution, predation, and the effects of beach development and erosion (NMFS and USFWS, 2008). The potential impact of anthropogenic noise has been studied more comprehensively in cetaceans and fish that rely heavily on sound detection and production for survival and communication, and whose survival is commercially important. In stark contrast, potential effects on marine turtles have been largely overlooked. There is a growing concern about the effects of increasing anthropogenic noise and the impact of potential behavioral changes and physical harm to marine mammals, species for which we have a much more thorough auditory understanding (Nowacek et al., 2007; NRC 2005; Richardson et al., 1995). Recently, emphasis has been placed on understanding the presence and increase of anthropogenic noises in fish habitats and recognizing potential effects (Popper and Hastings, 2009).

One major source of anthropogenic sound in the marine environment is the use of repetitive, high-intensity, low-frequency sound pulses during marine oil and gas exploration. Air guns used in these surveys are capable of producing high intensity sounds upwards of 250 dB re 1 μPa at 1 m (Richardson et al., 1995). Additional sources include vessel traffic, drilling, dredging, sonar, and explosions. In addition to anthropogenic sounds, biological sounds from wind, waves, seismic activity, storms, and marine animals contribute to the overall level of ocean noise. It is becoming apparent that there is a need to understand how all of these sources of marine sound could potentially impact marine turtle populations.

The impacts of anthropogenic noise in the marine environment are poorly understood in marine turtles partly because of limited studies addressing their auditory ability. Consequently, the auditory capabilities of marine turtles remain unclear and the functional role of hearing in their survival is unknown. The presence of high levels of anthropogenic noise overlapping with the assumed marine turtle hearing range has been documented in a key foraging habitat (Samuel et al., 2005). It is critical to understand what role sound plays in marine turtle survival and what effects anthropogenic noise may have on behavior.

Much of what is known about marine turtle hearing is inferred from results obtained in terrestrial and semi-aquatic turtles. Electrophysiological testing, in which electrodes are used to detect voltages generated by the brain in response to acoustic stimuli, supports findings that terrestrial turtles have low-frequency hearing (Adrian et al., 1938; Wever and Bray, 1931; Wever and Vernon, 1956a; Wever and Vernon, 1956b; Wever and Vernon, 1956c). Early observations of the marine turtle tympanic membrane left doubt that marine turtles were capable of hearing due to the thickness and rigidity of the membrane (DeBurlet, 1934). However, further anatomical investigations found that the marine turtle ear is capable of low-frequency aerial and bone conduction hearing (Wever, 1978; Lenhardt et al., 1985).

More recently, auditory evoked potential (AEP) testing of marine turtles has confirmed previous speculation that the ear is designed to detect low frequency stimuli. Cochlear potentials were measured in the juvenile green turtle (*Chelonia mydas*) to determine frequency sensitivities (Ridgway et al., 1969). Aerial stimuli ranging from 50 - 2,000 Hz and mechanical stimuli ranging from 30-700 Hz were tested on three specimens. Results indicated that the green turtle was capable of detecting low frequencies from 60 - 1,000 Hz with peak sensitivity ranging between 300 - 500 Hz. The hearing thresholds of juvenile and subadult loggerhead turtles (*Caretta caretta)* were measured by recording auditory brainstem responses elicited through vibrational clicks and tone bursts (Bartol et al., 1999). Results indicated frequency detection from 250 - 1,000 Hz with peak sensitivity at 250 Hz, which was the lowest frequency tested. Previous evoked potential work indicated that marine turtle hearing could extend beyond this to lower frequencies (Ridgway et al., 1969). Both of these studies were conducted with the animals removed from the water. The underwater frequency sensitivity of green turtles and Kemp's ridley turtles (*Lepidochelys kempii*) was measured with test subjects located at the water's surface with stimuli presented from an aerial speaker above the animal (Bartol and Ketten, 2006). Results indicated that green turtles were able to detect frequencies from 100 - 800 Hz with peak sensitivity at 200 - 700 Hz. Kemp's ridley turtles detected a narrower range of frequencies (100 - 500 Hz) with maximum sensitivity between 100 - 200 Hz.

Electrophysiological methods are often thought to underestimate the frequency range and threshold of the test subject (Katz, 1994). Electrophysiological testing does not provide a direct measure of hearing but allows the interpretation of electrical responses to estimate frequency range and sensitivity. It is essential to correlate the results of electrophysiological tests with those obtained through behavioral testing. Evidence indicates that terrestrial turtles are able to behaviorally respond to sound stimuli (Patterson, 1966; Andrews, 1915) and even learn more complex behaviors in response to directional sound cues (Lenhardt, 1981). Limited behavioral data exist for marine turtles but behavioral responses have been observed in free-swimming loggerhead turtles to seismic air guns in a closed canal (O'Hara and Wilcox, 1990). Behavioral responses in the form of bodily movement, head retraction, and limb extension were noted in response to vibrational stimuli in loggerhead and Kemp's ridley turtles in one of the first behaviorally based assessments of marine turtle hearing (Lenhardt et al., 1983). These responses diminished with habituation to the stimulus, emphasizing the need for an operantly conditioned, full behavioral audiogram. Due to the lengthy nature of behavioral testing and limited access to captive subjects, and the difficulty of training marine turtles, a full behavioral audiogram has not been collected.

While both behavioral and AEP methods have been proven useful in a wide variety of animals, each has benefits and drawbacks. Behavioral testing is time consuming and the number of individuals available for testing is often limited. Evoked potential testing is advantageous because the amount of time required to complete testing is minimal. This allows for a greater sample size and enables testing on wild and captive specimens. Evoked potential testing should not be fully relied upon until behavioral data are available to confirm the results. Evoked potential testing, once ground-truthed with behavioral data, is reliable, efficient and can be conducted with little discomfort to the test subject in a short period of time.

The objective of this study was to measure the underwater audiogram of the loggerhead turtle using both behavioral and evoked potential methods. Comparing behavioral and evoked potential audiograms from the same individual will help develop a better understanding of marine turtle hearing and ground-truth future results obtained through electrophysiological methods.

Methods

Subject

Behavioral and AEP data were collected from a single adult female loggerhead turtle. The 31-year old test subject weighed approximately 91 kg and was housed with a second adult female loggerhead of the same size and age at Mote Marine Laboratory in Sarasota, FL. The subject was fed a mixed diet of approximately 680 grams of squid and capelin in addition to vitamins and calcium supplements daily. Feeding took place twice daily and the subject typically consumed 60% of its food during behavioral testing sessions.

Prior to behavioral audiogram testing, the subject spent one year undergoing basic husbandry training. This training conditioned the animal to voluntarily participate in medical procedures, including blood sampling and weight measurements. This study was the first trained research project in which the subject participated.

Testing Location

Testing was conducted in the habitat in which the subject lived. The test subject was housed in a 66,000 liter, closed-system, concrete tank. The water temperature was maintained between 25-28ºC by a heating and cooling unit. The irregularly shaped tank had one large, acrylic viewing window (approximately 3 m x 1 m) and five small viewing windows (approximately .5 m x .5 m). Water depth was approximately 1.25 m throughout the entire enclosure. The tank bottom included large river rocks and a large

PVC tube used as an enrichment device in a rear portion of the enclosure, distant from the testing site. A training platform, from which testing was conducted, was suspended above the water. A small portion of the tank could be separated by a PVC gate to isolate the second turtle in the enclosure and exclude behavioral interference during testing. Testing was conducted before aquarium visiting hours to reduce the amount of noise and disruption. Pumps operating on the testing tank and adjacent turtle tanks were turned off during testing to reduce ambient noise. Filtration systems for the nearby manatee and dolphin tanks were left running during testing.

Training Apparatus and Equipment

Training was conducted in the center of the largest portion of the enclosure with approximately 3 m between the test subject and the nearest tank wall. The response apparatus and transducer were suspended separately from the training platform (Fig. 1). Two acrylic paddles hung from the end of the platform nearest the middle of the tank. The response paddle was positioned 0.75 m to the right of the station paddle. An LED light, potted in epoxy and clear PVC, was hung between the two paddles to indicate the start of a trial. A transducer (Clark Synthesis, Aquasonic AQ39) was suspended from the middle of the platform, 1 m in front of the turtle at a depth of .5 m. During sound presentation, the turtle was positioned directly in line with the transducer, with the ear approximately 25 cm below the water's surface. The trainer was positioned above the test subject on the platform.

Stimulus generation and recording were performed with hardware by Tucker-Davis Technologies (TDT) using SigGen and BioSig software (Tucker-Davis Technologies Inc., Alachua, FL).

Training and Data Collection

Training for the collection of the behavioral audiogram began in September 2007. Operant conditioning utilizing positive reinforcement was used to establish all behaviors for both research and husbandry purposes. Training took one year and data collection began in September 2008. A go/no-go paradigm was established through the conditioning of a response paddle press during tonal stimuli. During pre-trial periods and no-go trials, the turtle was trained to remain still with beak positioned directly in front of the station paddle. During go trials, the turtle indicated stimulus detection by moving to the response paddle. An LED light was used to indicate the start of a trial. An overhead light indicated an actively running session. The overhead light was turned off to indicate an incorrect response and cue the turtle to return to the station to start the next trial. This light remained off at all times outside of training sessions.

The experimental setup required a trainer positioned on the research platform above the apparatus to ensure the turtle was properly positioned in front of the station paddle and to reinforce the turtle for correct responses. The trainer wore headphones to prevent trainer bias or cueing. The trainer was blind to the order of go/no-go trials that were established through the use of Gellermann scales (Gellermann, 1933). On the opposite side of the tank, an assistant operated the TDT station to control stimulus presentation and to indicate correct or incorrect responses. The assistant indicated whether the subject was correct or incorrect using the overhead light. When left on, the subject was correct and received reinforcement. When turned off, the response was incorrect, no reinforcement was received, and the animal returned to the station paddle for the next trial.

The turtle was trained to approach the station paddle on command and remain stationary. Once positioned and still, the trainer cued the assistant for the start of a randomized go or no-go trial. The LED flashed for one second to indicate to the subject that the trial had started. During a go trial (tone trial), a two second tonal stimulus immediately followed the LED signal. If detected by the subject, she would swim to the response paddle and press it with her beak within 4 seconds of the termination of the tone. If correct, the subject received reinforcement and returned to the station paddle for the start of the next trial. If incorrect (subject did not press the response paddle), the overhead light was turned off for 6 seconds indicating an incorrect response. When the overhead light was turned on, the subject returned to the station paddle for the start of the next trial. For no-go trials (no-tone trials), the subject refrained from pressing the response paddle for 6 seconds following the LED to indicate the absence of signal detection. If correct, the subject received reinforcement and the next trial began. If incorrect (turtle pressed the response paddle), the overhead light was turned off for 6 seconds and this was scored as a false alarm.

Daily sessions were initiated with warm-up blocks to gauge subject motivation. Warm-up blocks consisted of four trials, two go trials and two no-go trials. Trial order was randomly assigned and the signal frequency and intensity were of known detectable level. The subject had to complete one warm-up block with at least 75% correct before testing began. If the first warm-up block was passed, testing could begin immediately. If it was not passed, an additional warm-up block was run with as many as four warm-up blocks taking place. If the fourth warm-up block was not passed, the day was considered for training purposes only, and the results were not counted towards threshold determination. Stimulus levels were kept constant throughout a ten-trial group deemed a “block.” Blocks were divided evenly into five go trials and five no-go trials. Order was assigned based on Gellermann scales (Gellermann, 1933). A block was considered passed if the subject successfully responded to 60% or more of the go trials and the false alarm rate remained at or below 40%. Each successful block resulted in a 6 dB attenuation of the stimulus intensity until a block was failed. Upon the first failed block, the signal was increased by 6 dB until the next passed block. Each positive-to-negative or negative-to-positive change in intensity was deemed a reversal. This modified staircase method (Schusterman and Balliet, 1971) continued in 6 dB steps, with one block serving as a step-size, until a minimum of 10 reversals was achieved. Blocks were excluded from data analysis for three conditions: (1) the false alarm rate for the block exceeded 40%, (2) an interruption occurred mid-block, i.e. severe weather, human interference in the exhibit, or loud outside noise, and (3) failure of warm-up trials resulting in the testing session being used for training purposes only.

Threshold Determination

Testing of each frequency was conducted over several weeks. Blocks resulting in reversals generally occurred over a period of days. Threshold was determined by averaging the intensities of blocks in which reversals occurred. Averages were calculated with an equal number of “failed” reversals – reversals in which the subject did not pass the necessary go trial criteria – and “passed” reversals – reversals which occurred when the subject passed the go trial criteria and intensity was decreased in the following block

(Fig. 2). These thresholds were calculated over 10-12 reversals. This process was repeated at 50, 100, 200, 400, 800, and 1131 Hz. Sound pressure levels (SPL) were measured and calibrated with a hydrophone (High Tech, Inc., HTI96) placed at the turtle's approximate tympanic location at the end of each test session. SPL recording during the test session was not possible due to the risk of damage from biting.

AEP Experimental Setup

Evoked potential testing was conducted in the same tank location used for behavioral testing. Two testing sessions were completed, one on 27 January 2009 and one on 24 July 2009. The January AEP testing session was conducted during the early stages of behavioral data collection and tested a wider range of frequencies. The July dataset was collected near the end of behavioral testing and was limited to the same frequencies tested during behavioral testing.

During testing, the subject was placed in a canvas medical stretcher and secured to a restraint board to minimize movement during testing. The subject offered negligible resistance during testing which minimized any noise detected in the response due to electrical impulses from muscle movement. Two animal handlers remained in the water with the animal to ensure that it remained positioned in front of the transducer, to assist if the animal became uncomfortable, and to ensure that the animal could easily lift its head to breathe. Two 27 gauge, 12 mm, subdermal, stainless steel electrodes (Rochester Electro-Medical Inc, Tampa, FL) were inserted just beneath the skin to a depth of approximately 3 mm. The recording electrode was placed anterior to the frontoparietal scale on the top of the head in a caudally-facing direction. The reference electrode was placed in the skin of the neck adjacent to the first marginal scute. The ground electrode was placed in the water near the subject. Exposed surfaces of the electrode that were not inserted into the skin were coated with enamel for insulation and the entire insertion point and electrode were sealed with petroleum jelly during testing to eliminate noise artifact.

AEP Signal Presentation

Stimulus generation was performed with the same Tucker-Davis workstation and Aquasonic AQ39 transducer used for behavioral testing. Signals were amplified with an American Audio VLP300 amplifier (American Audio, Los Angeles, CA). Auditory stimuli consisting of 50 ms, cosine-gated tone bursts with a 5 ms rise/fall time were presented at a rate of 11.0375 per second. Tone bursts were calibrated with an HTI 96-min hydrophone (sensitivity: -164±1 dBV/µPa from 2 Hz – 37 kHz) placed in the position of the subject's ear prior to the testing session. During January testing, the subject was tested at 50, 100, 200, 400, 600, 800, 1000, 1600, and 3200 Hz. During July testing, the subject was tested at 50,100, 200, 400, 800, 1131, and 1600 Hz.

Response Detection

Tones were presented up to 1000 times at each sound pressure level. Signals from the electrodes were passed through a digital biological amplifier (TDT DB4/HS4) and recorded over a 90.6 ms window using BioSigRP software at a sample rate of 24,412 Hz. Resulting evoked potentials were averaged for each frequency and SPL combination. To reduce testing time, if a clear evoked potential response was seen before 1000 averages, the program was advanced to the next SPL. Sound intensity was decreased in 6 dB steps for each frequency tested. Threshold was determined to be the level at which a clear signal was no longer detectable in the wave form spectra analysis and a peak was no longer seen in the Fourier transform frequency spectra plots (Fig. 3). Analysis was conducted using BioSig, Excel, and MATLAB software. Manual analysis was done because analysis of the signal-to-noise ratio did not allow accurate automated threshold measurements in most cases due to low frequency electrical noise present in most signals. Two independent observers analyzed each frequency and waveform plot to ensure threshold determination reliability.

To rule out artifacts, a deceased loggerhead was tested with the same equipment and software. Access to deceased turtles was limited to hatchlings from nearby nesting beaches that were provided by Mote Marine Laboratory's stranding and investigations program. A hatchling (4.5 cm straight carapace length) was tested at all frequencies to rule out artifact presence in signal responses of live turtles.

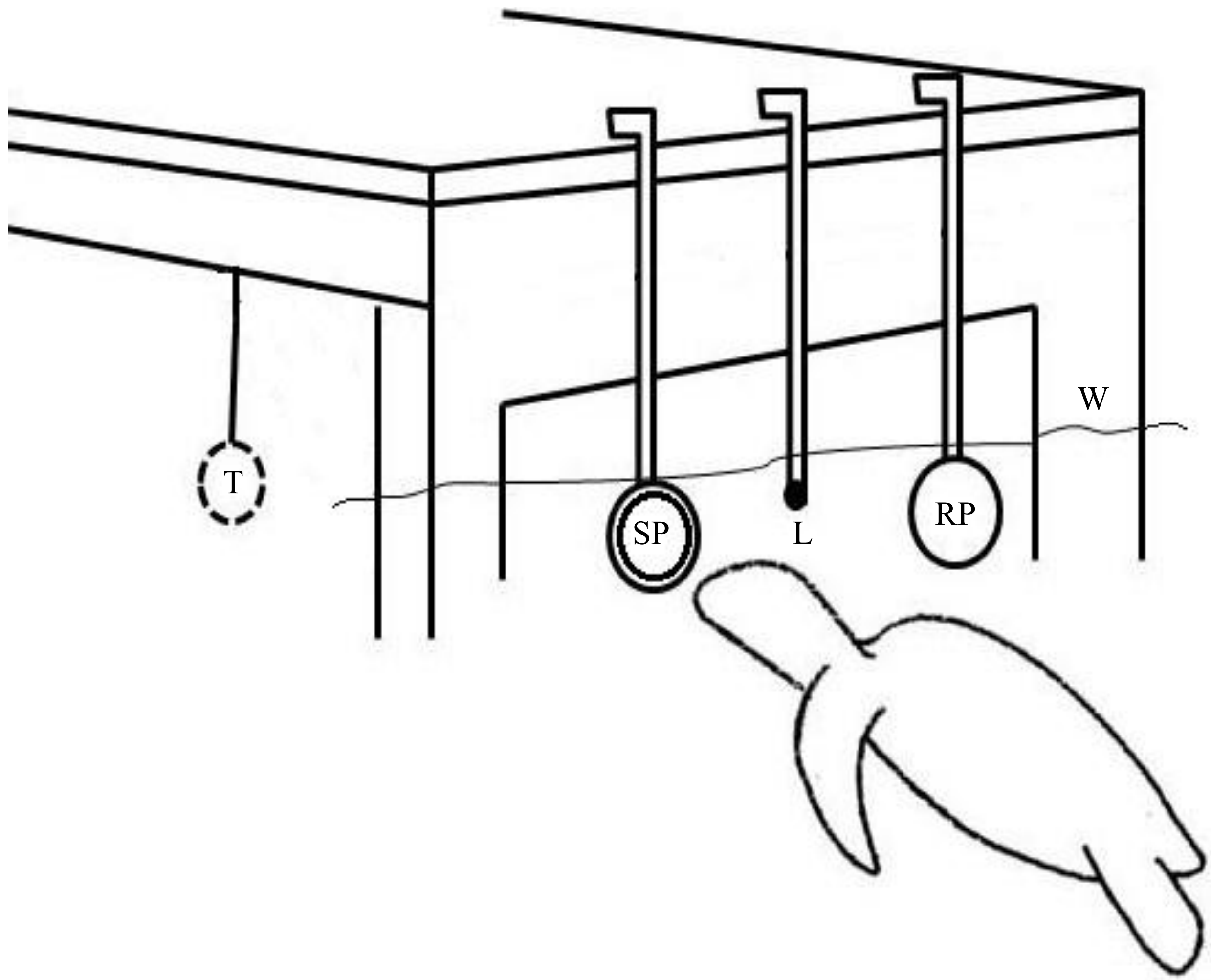

FIG. 1. Experimental design for the collection of behavioral audiogram data indicating locations of (T)-transducer located 1m from subject, (SP)-station paddle, (L)-indicator light, (RP)-response paddle and (W)-water level.

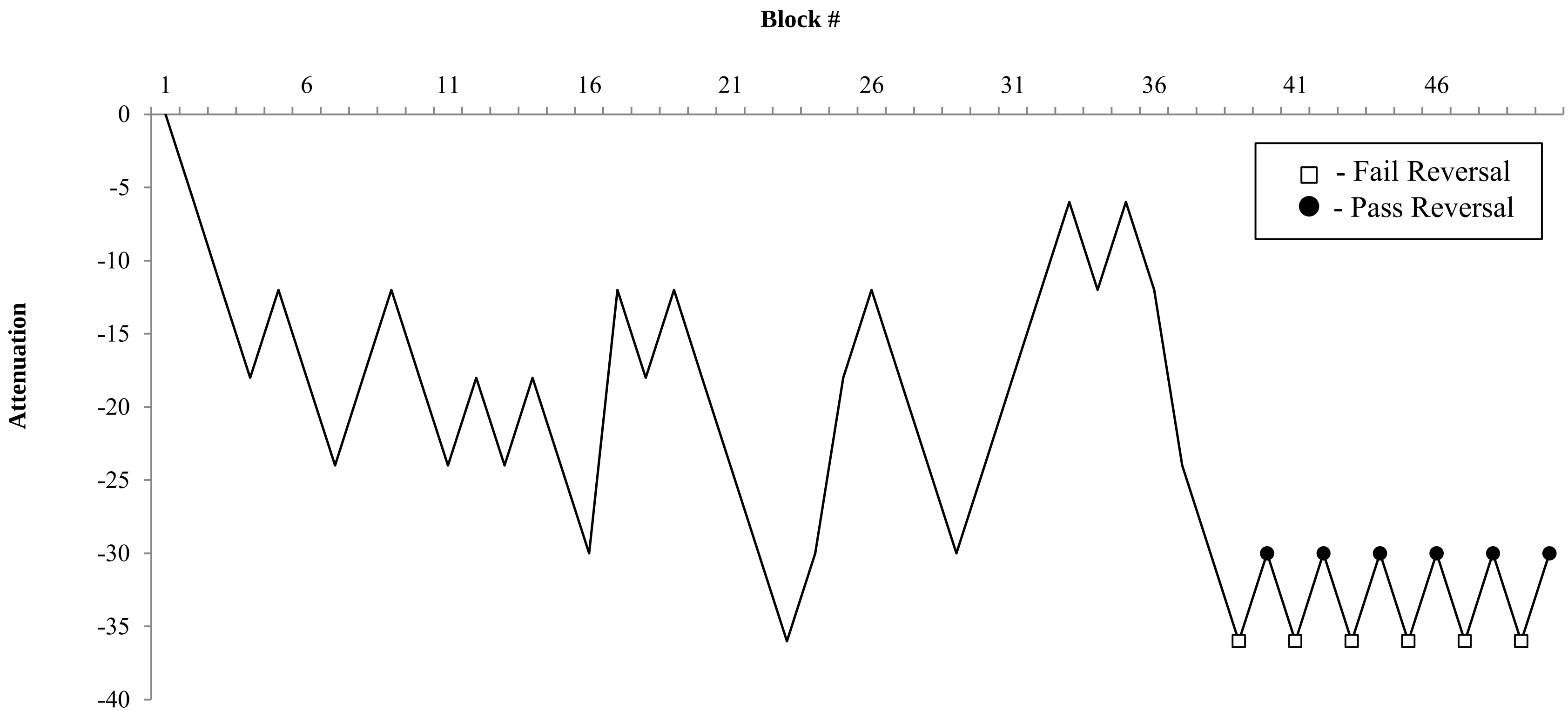

FIG. 2. Attenuations of all 10-trial blocks kept during behavioral collection of 400 Hz threshold, indicating the staircase used to determine threshold. A total of 12 reversals were obtained and used for threshold calculation. These data were collected between Aug. 1, 2008 and Sept. 14, 2008.

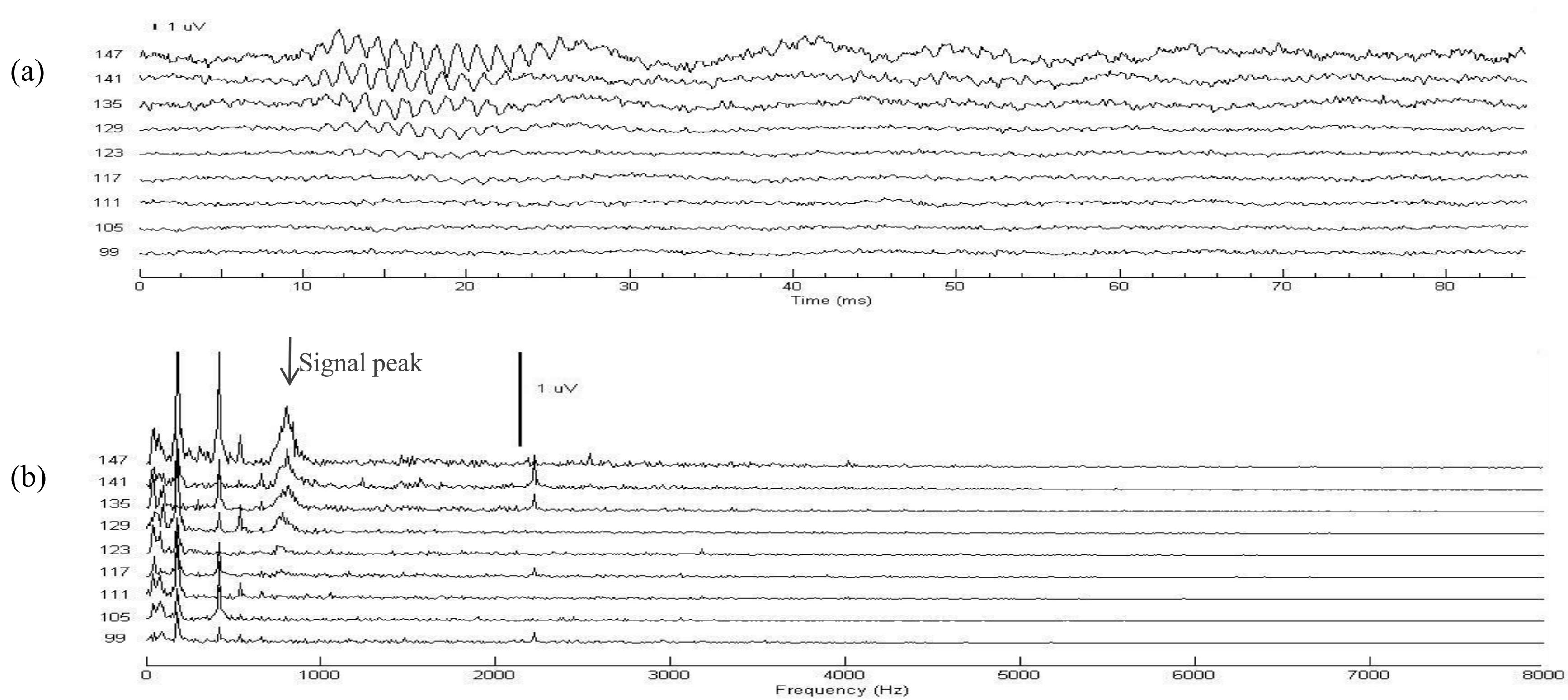

FIG. 3. AEP waveform (a) and frequency spectra (b) measurements at 400 Hz. SPLs are indicated next to each signal. Waveforms are presented as raw output. AEP signals in power spectra are indicated with an arrow. Other spectral peaks are due to electrical mains noise (multiples of 60 Hz).

Results

Behavioral Audiogram

Behavioral thresholds were collected over a period of one year. A total of six frequencies were tested and thresholds were determined for five frequencies. Each frequency took approximately 4 - 6 weeks with reversals occurring over a 10 - 15 day period. The subject responded to tones from 50 - 800 Hz. The amount of testing time and the amount of effort, calculated by the number of blocks kept, are indicated in Table 1 along with the calculated threshold for each frequency tested. Each threshold was calculated from 10 or 12 reversals. The resulting audiogram is a u-shaped curve with peak sensitivity of 98 dB re 1 μPa occurring at 100 Hz (Fig. 4). The range of best sensitivity was 100 - 400 Hz, with thresholds occurring within 10 dB re 1 μPa of peak sensitivity. Sensitivity decreased sharply above 400 Hz with an increase of 42 dB between 400 and 800 Hz and the subject was unable to detect any tones when tested at 1131 Hz at the loudest level possibly generated by the equipment (138 dB re 1 μPa). The threshold at 50 Hz (110 dB re 1 μPa) was slightly above peak sensitivity with a less dramatic decrease in sensitivity occurring at the lower frequency range than at the higher end.

To illustrate threshold determination, Table 2 summarizes data collected in all of the reversal blocks used to calculate the 400 Hz threshold and the calculated SPL of each reversal. Passed blocks are indicated as those in which the correct detection rate within the block is equal to or greater than 3 out of 5 (60%) correctly detected go trials and the false alarm rate is at or below 40%. A total of 436 blocks were used for testing all frequencies, of which 59.4% (259) were kept. The majority of discarded blocks were due to high false alarm rates or training blocks due to failed warm-ups. False alarm rate ranged from 14.3% (1131 Hz) to 24.5% (200 Hz) when averaged over all kept blocks for each frequency. Average false alarm rates during reversal blocks were 25% (50 Hz), 32% (100 Hz), 25% (200 Hz), 20% (400 Hz), and 18.3% (800 Hz). Variability in the presented SPL between each passed and failed block averaged 7 dB over all frequencies with the most amount of variability occurring at peak sensitivity (100 Hz, average variability of 9.7 dB).

AEP Audiograms

Evoked potential waveforms obtained from averaged responses to tonal stimuli were present at approximately 7 - 10 ms after tone initiation. Responses consisted of a waveform that was twice the presented frequency. The resulting thresholds from both auditory evoked potential testing sessions and their combined averages are listed in Table 3. Additionally, noise spectrum levels are listed for each testing session. There was minimal difference in calculated thresholds between the two sessions with a maximum difference of only 2 dB (Fig. 5). During the January testing session, peak sensitivity occurred at 200 Hz with a threshold of 110 dB re 1 μPa. During the July testing session, maximum sensitivity occurred at 200 and 400 Hz with a threshold of 109 dB re 1 μPa. No detectable threshold was observed at 50 Hz in either session. Average combined thresholds from both sessions were within 2 dB between 100 and 400 Hz with sensitivity decreasing by 25 dB between 400 and 600 Hz and decreasing to 143 dB re 1 μPa at 800 Hz. The highest detected frequencies were 1000 Hz during January testing and 1131 Hz during July testing. Testing was conducted beyond these frequencies but peaks and wave forms were only visible at the highest presented SPL. The signals at these high frequencies are attributed to artifact because they lack onset delay and they were also produced in control tests run in dead loggerhead turtles.

When testing the deceased loggerhead hatchling, signals were present at the highest presented SPLs at all frequencies tested with the exception of 50 Hz. These signals appeared at 1 - 2 ms after stimulus presentation and the waveforms directly resembled those of stimulus waveform. These signals quickly disappeared with a decrease in SPL.

Table 1. Summary of collection effort for behavioral data and resulting thresholds for each frequency tested.

Frequency (Hz)	Start Date	End Date	# Blocks	# Kept	% Kept	# Reversals	Threshold (dB re 1 μPa)
50	July 27, 2009	Aug. 24, 2009	73	50	68%	12	110
100	April 3, 2009	May 5, 2009	67	41	61%	10	98
200	Sept. 26, 2008	Nov. 24, 2008	157	80	51%	12	103
400	Aug. 1, 2008	Sept. 14, 2008	71	52	73%	12	106
800	May 12, 2009	June 8, 2009	61	29	48%	12	148
1131	July 22, 2009	July 24, 2009	7	7	100%	0	n/a

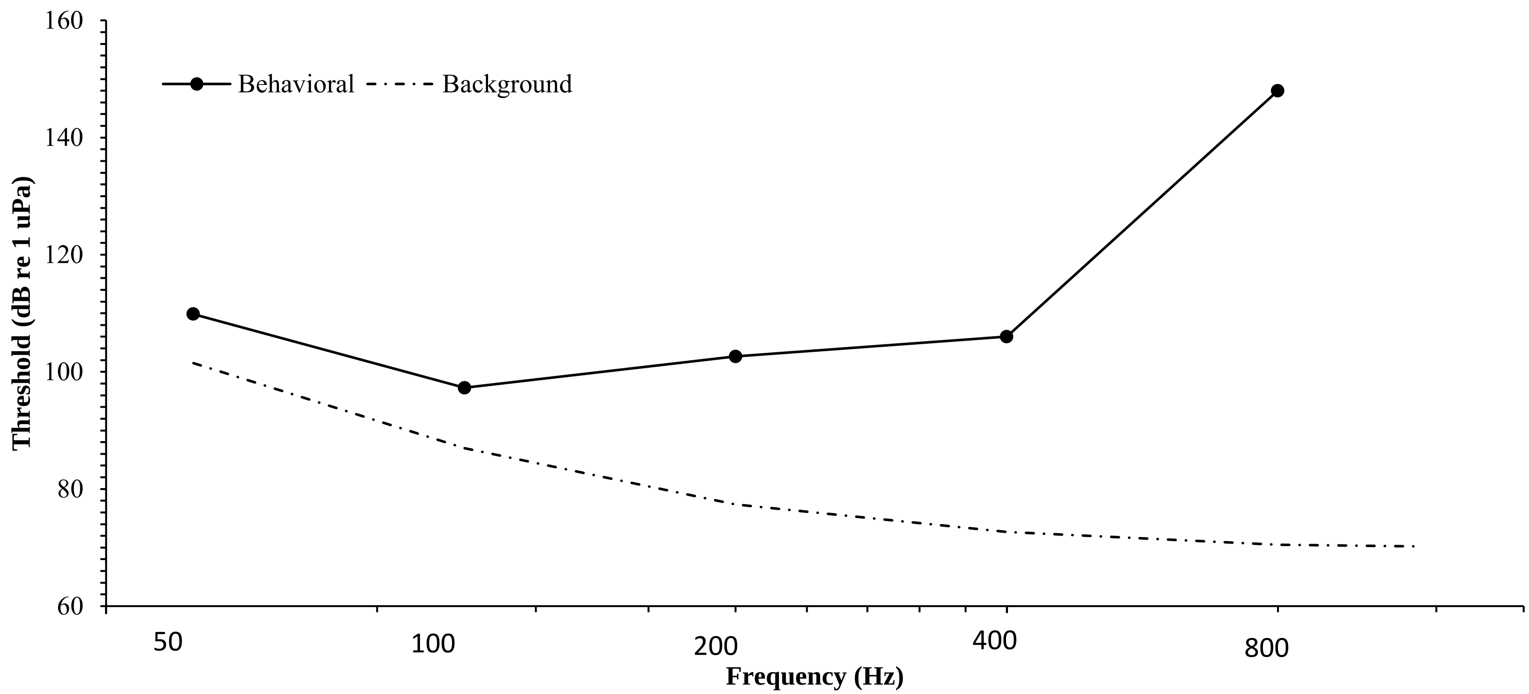

FIG. 4. Underwater behavioral audiogram and accompanying background noise. Background noise is presented as spectrum level (dB re 1 μPa^2/Hz).

Table 2. Data collected during reversal blocks at 400 Hz for behavioral audiogram. Five go trials and five no-go trials were presented in each block. The # correct out of five go trials is presented along with the false alarm rate (# incorrect out of five no-go trials). SPL was recorded at the end of each training session and some variability occurred even among similar intended presentation levels, likely due to tank characteristics and hydrophone placement.

Reversal	Date	Block of the Day	# Correct Go Trials	Pass/Fail	False Alarm Rate for Block	SPL
1	Sept. 3, 2008	2/4	1/5	Fail	20%	101
2	Sept. 3, 2008	3/4	3/5	Pass	0%	108
3	Sept. 3, 2008	4/4	2/5	Fail	20%	101
4	Sept. 4, 2008	1/4	5/5	Pass	40%	108
5	Sept. 4, 2008	2/4	2/5	Fail	0%	101
6	Sept. 4, 2008	3/4	4/5	Pass	20%	108
7	Sept. 5, 2008	2/4	2/5	Fail	40%	98
8	Sept. 5, 2008	4/4	4/5	Pass	40%	104
9	Sept. 13, 2008	2/4	2/5	Fail	20%	104
10	Sept. 13, 2008	3/4	4/5	Pass	20%	110
11	Sept. 13, 2008	4/4	0/5	Fail	20%	104
12	Sept. 14, 2008	1/1	4/5	Pass	0%	109
					Threshold:	106

Table 3. Thresholds and background noise spectrum level at each test frequency from two separate AEP testing sessions and combined mean values († - no signal detected. * - frequency not tested).

Frequency (Hz)	January 27 Threshold (dB re 1 μPa)	January 27 Background Noise (dB re 1 $\mu Pa^2/Hz$)	July 24 Threshold (dB re 1 μPa)	July 24 Background Noise (dB re 1 $\mu Pa^2/Hz$)	Mean Threshold (dB re 1 μPa)	Mean Background Noise (dB re 1 $\mu Pa^2/Hz$)
50	†	96	†	99	†	97
100	112	85	112	91	112	89
200	110	77	109	84	110	82
400	111	71	109	78	110	76
600	135	68	*	*	135	68
800	142	66	143	72	143	70
1000	138	64	*	*	138	64
1131	*	*	141	69	141	69
1600	146	64	146	66	152	65
3200	155	58	*	*	155	58

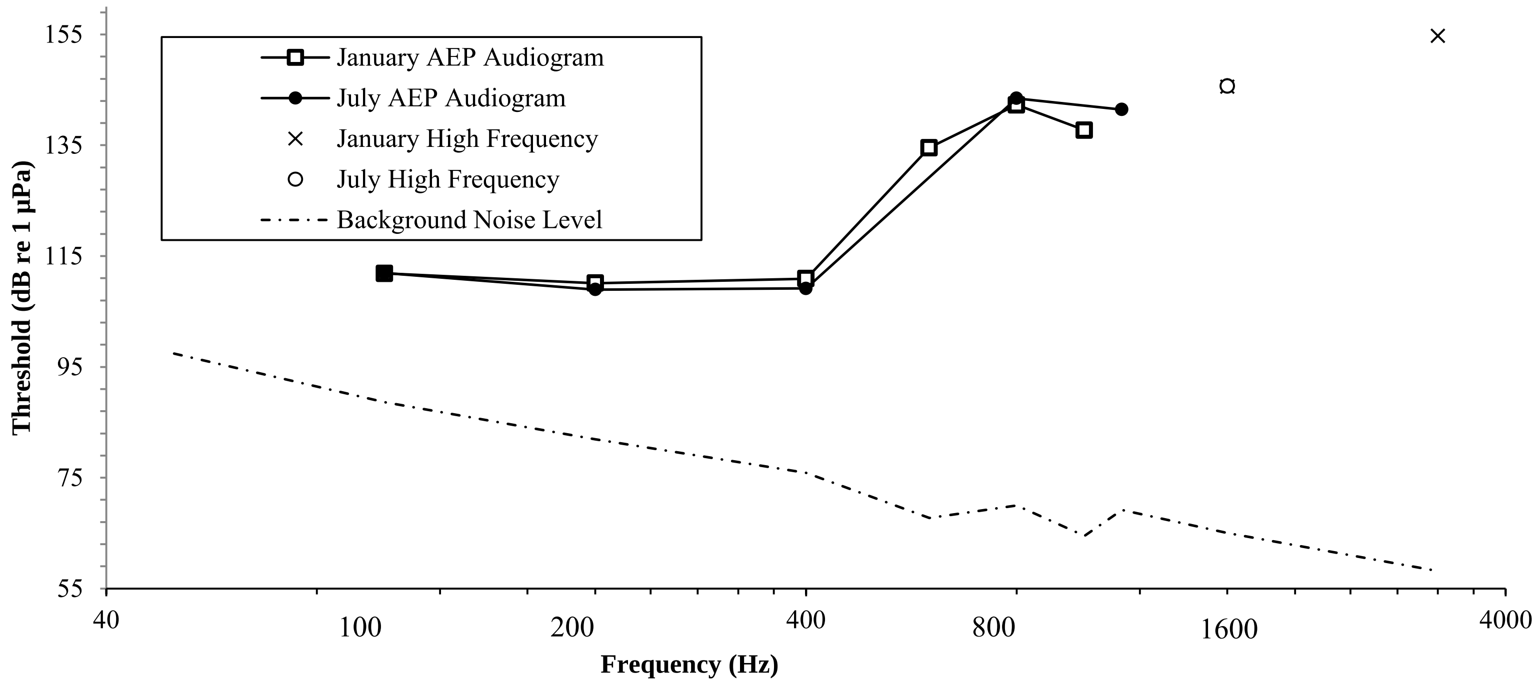

FIG. 5. Underwater AEP audiograms from two different testing sessions and average ambient noise spectrum level. During the January test session, measurements were taken at 50, 100, 200, 400, 600, 800, 1000, 1600, and 3200 Hz. During the July session, measurement were taken at 50, 100, 200, 400, 800, 1131, and 1600 Hz. Thresholds beyond 1000 Hz in January and 1131 Hz in July are believed to be due to artifact. Background noise levels are presented in spectrum level (dB re 1 $\mu Pa^2/Hz$).

Discussion

Behavioral Analysis

Initial training attempts with the test subject proved to be difficult. Husbandry training occurred for one year prior to the initiation of research training. Through this initial training, the subject was slow and inconsistent to perform learned behaviors. Only after months of repetition did behavioral consistency become evident and even after prolonged training, behavior was sometimes erratic and unpredictable. Such behavior is often seen in the conditioning of reptiles (Suboski, 1992). For this reason, we designed the modified staircase method for collecting behavioral data, using blocked trials to account for reversals. This allowed for the acceptance of some unpredictable behavior without creating an erratic staircase and widely varying reversals. The use of averaged false alarm rates and pass/fail rates over the ten-trial block provided consistent reversals.

In addition to difficulty with consistent behavior, the subject showed some seasonal behaviors that affected performance. Adult loggerhead turtles go through seasonal changes in behavior and appetite due to breeding cycles. Female loggerheads typically migrate from foraging grounds to breeding areas a few weeks to a few months prior to nesting season (Limpus et al., 1992; Schroeder et al., 2003). The subject was observed going through periods of appetite loss and lack of interest in training or other daily behaviors during the months of December to March regularly. A deterioration in performance was noted in October 2008 during testing of 200 Hz. Performance in testing sessions ceased completely in December and did not return until March 2009. Testing of

200 Hz took significantly more blocks than any other frequency, likely due to seasonal shifts in appetite and behavior.

False alarm rates ranged from 18.3% to 32%. High false alarm rates could result in biased thresholds. A tendency to respond in the absence of a signal could result in lower thresholds. In this case, the subject showed an equal tendency to respond when no signal was present as she did to refrain from responding to signals that were within her auditory range. Therefore, the high false alarm rate was balanced by similar behavior in signal-present trials. This unpredictable behavior was accounted for through the use of the modified staircase method.

Evoked Potential Analysis

Auditory evoked potential testing measures the small electrical impulses that are generated by the nerve cells in response to electrical signals from the stimulation of hair cells in the auditory system by an acoustical stimulus. In this study and studies of other species, including fish, a frequency doubling effect is seen when measuring evoked potentials (Casper and Mann, 2006a; Casper and Mann, 2006b; Egner and Mann, 2005). This is likely due to the orientation of hair cells within the ear so that certain cells fire on the compression phase of a sound wave and others fire on the rarefaction phase, resulting in a doubled response. These doubled responses were seen at all frequencies tested.

One of the difficulties associated with evoked potential testing is the subjective nature of threshold determination. Threshold determination at very low frequencies was made even more difficult by the high intensity of electrical background noise at low frequencies. Analysis of Fourier transformations of the signals indicated large peaks between 0-150 Hz making signal to noise ratio too low to detect a stimulus response at 50 Hz. Additionally, behavioral results indicate that the threshold at 50 Hz is 110 dB re 1 µPa. The loudest SPL that testing equipment could produce during evoked potential testing of 50 Hz was 119 dB re 1 µPa. Since AEP results indicated that thresholds at low frequencies are 6-7 dB higher than behavioral thresholds, it is possible that we were not able to present a signal with a SPL sufficient enough to produce an evoked response. We believe that with equipment adjustments, a threshold at 50 Hz could be determined through electrophysiological methods.

AEP testing was conducted at frequencies beyond the maximum hearing range determined by behavioral testing. During behavioral testing, the turtle showed no response to signals played at 1131 Hz at levels of 138 dB re 1 µPa. During evoked potential testing, a response was detected during the presentation of 1131 Hz at 141 dB re 1 µPa. Lower levels yielded no evoked potentials. Additional testing was conducted at 1600 Hz and 3200 Hz during the first session and 1600 Hz during the second session. During analysis of these frequencies, a visible waveform was apparent in the response at the highest presented SPL. These waveforms were attributed to artifact, likely from direct electrode detection of the electromagnetic field of the transducer. To validate this, signal onset delay was studied at all frequencies. At zero attenuation, evoked potential responses appeared between 7-10 ms after signal initiation for frequencies below 1600 Hz. At 1600 and 3200 Hz, response signals appeared between 1-2 ms after signal initiation. This 1 - 2 ms delay can be accounted for by the delay produced by the filters in the amplifier. In addition to validating true detections with response onset times, results were examined in a deceased turtle. A dead loggerhead hatchling was studied using the same methods as the adult test subject. Responses were present at the highest presented SPL for all frequencies with the exception of 50 Hz. These responses were visible between 1 - 2 ms after signal presentation. Responses in live turtles at lower frequencies did not occur until 7 - 10 ms after presentation. For these reasons, the responses at 1600 and 3200 Hz may be attributed to artifact and not actual detection.

Comparison of Results

Hearing abilities are better understood in marine mammals than in marine turtles. Access to multiple species of captive marine mammals and the relative ease in which they can be conditioned to participate in research tasks have allowed studies exploring the differences between behavioral and electrophysiological methods of auditory testing in the same individual (Finneran and Houser, 2006; Houser and Finneran, 2006; Schlundt et al., 2007; Szymanski et al., 1999; Wolski et al., 2003; Yuen et al., 2005). Each of these studies yielded a slight to significant difference in the results obtained from the two testing methods, with behavioral thresholds typically lower than auditory evoked potential thresholds, particularly at lower frequencies. When testing protocols are kept identical between the two methods, the difference in the calculated thresholds decreases (Houser and Finneran, 2006). With increasing utilization of more objective methods of determining thresholds during evoked potential testing, the gap between the two methods may decrease and evoked potential results may become more reliable.

Thresholds were determined through both AEP and behavioral methods at 100, 200, 400, and 800 Hz (Fig. 6). Thresholds differed by a maximum of 14 dB at 100 Hz and a minimum of 4 dB at 800 Hz. A threshold was not detected at 50 Hz during auditory evoked potential testing but behavioral data indicated a threshold of 110 dB re 1 μPa.

Evoked potential testing elicited responses at higher frequencies while behavioral responses indicated a complete lack of detection beyond 800 Hz. Audiogram curves followed similar shapes for both methods of testing, with the behavioral results showing more of the traditional u-shaped curve. Both curves showed substantial decreases in sensitivity at frequencies higher than 400 Hz.

Behavioral thresholds were lower than evoked potential thresholds between 100 and 400 Hz and higher than evoked potential thresholds at 800 Hz. These differences may be caused by multiple factors. Natural variability in responses and behavior due to the unpredictable nature of marine turtles could have led to these differences. In addition, there was potential variability in perceived signal SPL due to slight movements by the subject during testing and variations in the acoustic environment attributed to proximity to the surface, a naturally reflective surface. Movement by the subject during testing likely resulted in minimal variation in perceived SPL because the subject remained very still during testing. Another possibility is that during evoked potential testing, at low frequencies, the background electrical noise is naturally higher, making detection of small evoked potentials more difficult. However, differences of only 4 - 14 dB between behavioral and electrophysiological means still establishes confidence that evoked potential testing serves as a cost-effective and time-saving method of determining hearing abilities.

Noise Spectrum Level and Masking

There is a strong possibility that noise masking occurred during low frequency testing, resulting in higher thresholds. Critical ratio data are lacking in turtles and must be inferred from other species. Critical masking ratios, defined as the difference between the sound pressure level of the tone at threshold and the spectrum level of masking noise, are poorly defined in reptiles. Critical masking ratios have been measured in the bullfrog (*Rana catesbeiana*) (Megela Simmons, 1988). The critical masking ratio of the bullfrog when measured aerially at 100 Hz is 29 dB (Megela Simmons, 1988). Thresholds measured in the loggerhead were 98 dB determined behaviorally and 112 dB determined through evoked potential testing. Noise spectrum levels were 87 and 89 dB respectively, resulting in a difference of 10 and 23 dB. If critical ratios are similar to those of the bullfrog, masking is likely occurring. At 50 Hz, the behaviorally determined threshold was 110 dB, only 8 dB above the noise spectrum level of 102 dB. During evoked potential testing, the average SPL of the 50 Hz signal was 118 dB with a background spectrum level between 96 and 99 dB. It is possible that masking was occurring during both methods of testing, and it is crucial to develop a better understanding of critical masking ratios in marine turtles. It is possible that critical masking ratios are much lower in marine turtles than in the bullfrog. We recommend conducting future testing in a quieter environment in which outside noise could be further reduced during testing. Samuel et al. (2004) measured noise levels in a known loggerhead, Kemp's ridley and green turtle habitat in New York during peak levels of human activity and low periods of activity. Results indicated SPLs as high as 113 dB re 1 μPa between 200 - 700 Hz during peak activity. When recorded overnight, with naturally lower levels of human activity, noise levels were still around 83 dB re 1 μPa. These results indicate a strong likelihood that marine turtle hearing abilities are being masked by high levels of anthropogenic noise. This is especially true in shallow, coastal areas where noise levels, particularly those in the low-frequency range, are higher than in open-ocean areas due to coastal construction, a higher number of commercial and recreational vessels, and the presence of seismic exploration. These coastal areas provide key habitats for juvenile and subadult turtles as well as nesting and mating adults.

Although the true function of hearing in marine turtles remains poorly understood, it is possible that aural sensing may assist in danger avoidance, beach location, or navigation. If masking is occurring due to the presence of high levels of anthropogenic noise in the low frequency range, natural behavior and survival may be affected. In addition, sources of sound with high SPLs, such as those associated with seismic surveys, may result in permanent or temporary damage to the ear. Bowles et al. (1997) observed temporary threshold shifts (TTS) in desert tortoises (*Gopherus agassizi)* exposed to 10 repeated simulated sonic booms separated by 3 s (145 dB SPL). Aerial sonic booms share similar sound properties to the impulses used in underwater seismic surveys. The effects of these intense SPLs and the potential for damage are not well understood in the marine turtle ear but it is reasonable to assume that damage, in the form of TTS or permanent hearing loss, could occur with high energy sound sources. In addition to physical damage to the ear, the presence of anthropogenic sound could result in behavioral alterations, energy loss, and masking of important sound detection.

Summary

Results from this study indicate that loggerhead turtles detect low frequency sound with a functional hearing range of 50 - 1131 Hz and peak sensitivity between 100 - 400 Hz. These results are consistent with previous work done in other marine and terrestrial turtles and establish the first underwater audiogram of a loggerhead turtle. Behavioral results confirm that auditory evoked potential measurements function as good indicators of marine turtle hearing. Threshold calculations differed by an average of 8 dB between the two methods. Frequency ranges detected by the loggerhead were similar to those found in green turtles and Kemp's ridley turtles by Bartol and Ketten (2006). Green turtles had a frequency range of 100 – 800 Hz with peak sensitivity occurring between 200 - 400 Hz in one group tested and 600 - 700 Hz in another group. Kemp's ridley turtles had a frequency range of 100 - 500 Hz with highest sensitivity between 100 and 200 Hz. Bartol et al. (1999) examined hearing ranges in loggerhead turtles with the animal tested in air. Frequency ranges in that study were 250 - 750 Hz. Peak sensitivity was at 250 Hz, the lowest frequency tested. Threshold levels are difficult to compare because the study utilized a mechanical vibrator attached directly to the tympanum to relay stimuli through the bone and the results of vibrational testing (presented in dB re 1 g rms) cannot be easily compared with results presented in SPL. However, the frequency ranges are similar to those found in the current study. Similarly, Ridgway et al. (1969) found green turtle sensitivity to be 60 - 1000 Hz with a peak between 300 and 500 Hz. Each of these studies indicates that marine turtles have hearing abilities that detect low frequency sound with a narrow band of greatest sensitivity. The current study confirms the results of previous electrophysiological tests through behavioral test comparisons. It would be beneficial to collect behavioral audiograms in other marine turtle species. However, given the similarity between evoked potential results and auditory system anatomy among the species, results from this study indicate that behavioral and evoked potential results should show close correlation in these species as well.

The current understanding of marine turtle auditory abilities is based on results from morphological findings and electrophysiological results that have until now, not been substantiated with behavioral results. This study showed that an audiogram collected through operantly conditioned behavioral methods was similar to that measured with auditory evoked potential testing, which can be conducted in just a few hours on an untrained animal. The thresholds calculated in this study could have potentially been masked as a result of tank noise and other sources of background noise, resulting in a threshold above the actual audible level. However, it is likely that similar levels of background noise would be seen in a natural coastal environment with increased levels of low-frequency noise from wind, waves, marine vessels, construction, and other natural and anthropogenic sources. Additional testing with increased sample sizes as well as additional age classes may be needed to account for any ontogenetic changes in auditory function, behavior or habitat usage. While behavioral testing methods provide accurate measures of threshold determination, evoked potential testing is beneficial when time is a factor and multiple individuals need to be tested. With continued improvement in threshold estimations through electrophysiological testing, a more rapid and thorough understanding of marine turtle auditory abilities will aid in future assessments of the impacts of anthropogenic noise and the function of sound in the marine turtle environment.

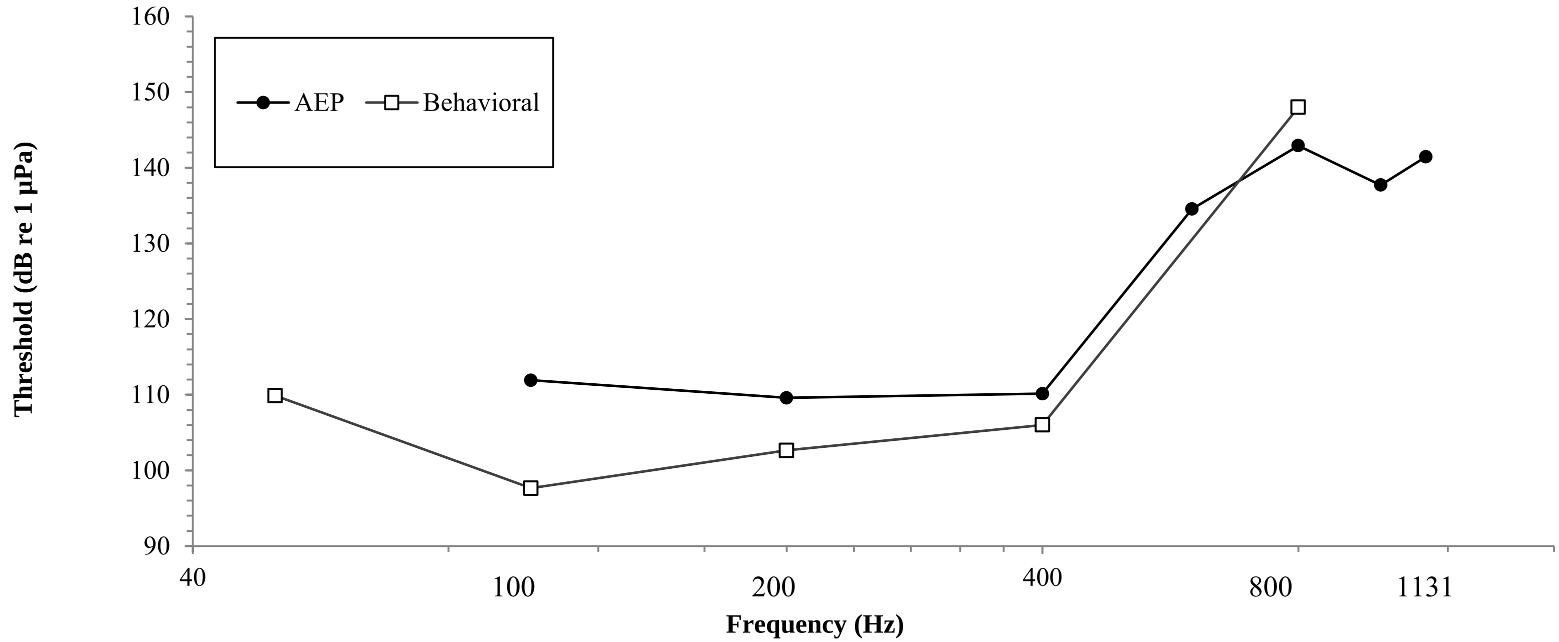

FIG. 6. Comparison of underwater behavioral and mean AEP audiograms. AEP threshold values are averaged over two testing sessions.

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