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14. ABSTRACT

A custom auditory evoked potential (AEP) system was used to assess the feasibility of rapidly testing the hearing of bottlenose dolphins by tracking the magnitude of the envelope following response (EFR). Tests were conducted in-air (N=4) and on submerged dolphins (N=3) for which behavioral audiograms had been obtained in San Diego Bay or a quiet above ground pool. For in-air AEP measurements, differences between AEP and pool behavioral thresholds increased with threshold magnitude and ranged from 0 to +18 dB. For underwater AEP measurements, differences between AEP and pool behavioral thresholds varied from -10 to9 dB. After benchmarking the AEP approach, AEP thresholds were obtained from 42 dolphins housed at the Navy Marine Mammal Program. Animals ranged from 4 to 47 years of age and consisted of 28 males and 14 females. Consistent with other mammalian systems, the range and sensitivity of hearing declined with age with onset typically occurring between the ages of 20 and 30. Males generally exhibited hearing loss at a younger age than female dolphins. The AEP system was subsequently applied to the northern elephant seal (*Mirounga angustirostris*) to determine how to adapt AEP approaches to larger animals with less robust auditory systems

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Final Technical Report for N00014-04-1-0455: Auditory evoked potentials for the evaluation of hearing sensitivity in Navy dolphins

Modification P00002: Assessment of hearing sensitivity in adult male elephant seals

Abstract

A custom auditory evoked potential (AEP) system was used to assess the feasibility of rapidly testing the hearing of bottlenose dolphins by tracking the magnitude of the envelope following response (EFR). Tests were conducted in-air (N=4) and on submerged dolphins (N=3) for which behavioral audiograms had been obtained in San Diego Bay or a quiet above ground pool. For in-air AEP measurements, differences between AEP and pool behavioral thresholds increased with threshold magnitude and ranged from 0 to +18 dB. For underwater AEP measurements, differences between AEP and pool behavioral thresholds varied from -10 to9 dB. After benchmarking the AEP approach, AEP thresholds were obtained from 42 dolphins housed at the Navy Marine Mammal Program. Animals ranged from 4 to 47 years of age and consisted of 28 males and 14 females. Consistent with other mammalian systems, the range and sensitivity of hearing declined with age with onset typically occurring between the ages of 20 and 30. Males generally exhibited hearing loss at a younger age than female dolphins. The AEP system was subsequently applied to the northern elephant seal (*Mirounga angustirostris*) to determine how to adapt AEP approaches to larger animals with less robust auditory systems.

Introduction

Grant N00014-04-1-0455 was provided to Biomimetica to (1) develop a portable eletrophysiological system capable of recording auditory evoked potentials (AEP) in Navy dolphins, (2) evaluate stimulus presentation and AEP recording techniques for audiogram determination, (3) benchmark audiograms determined with AEP techniques against behaviorally determined audiograms, and (4) use the system to evaluate the hearing sensitivity of all available Navy Marine Mammal System (MMS) dolphins. The system was to be used to screen Navy dolphins for hearing deficits and data were to be mined to identify relationships between age, gender, and hearing sensitivity. The grant was augmented based upon successful application of AEP techniques in bottlenose dolphin to apply developed AEP technology to the assessment of hearing sensitivity in adult male elephant seals within their natural habitat. The goal of this research augmentation was to provide the first measures of hearing sensitivity on adult males of this species and modify AEP approaches for the testing of hearing in these larger species with less robust auditory systems.

Note* - Many of the details of methodology, results, and interpretation of the results can be found in papers published from this research. PDF files of these papers are attached to this report.

Auditory evoked potentials for the evaluation of hearing sensitivity in Navy dolphins

Materials and Methods

Benchmarking AEP methods in bottlenose dolphins and hearing survey of the Navy Marine Mammal Program's (NMMP) dolphin population

The subjects used for in-air AEP testing were four bottlenose dolphins: BLU (female, 39-y, 200 kg), BUG (male, 23-y, 190 kg), BUS (male, 24-y, 180 kg) and WEN (male, 21-y, 210 kg). Three bottlenose dolphins were used for underwater AEP measurements and included BEN (male, 41-y, 324 kg), BLU (female, 39-y, 200 kg), and WEN (male, 21-y, 210 kg). All subjects had previously participated in cooperative psychophysical tasks, including auditory detection tasks, and all had behavioral audiograms available for comparison of AEP and behavioral thresholds. Following benchmarking of the AEP system in both in-air and underwater threshold estimation, the procedure was conducted on 42 of the NMMP's dolphin population. Dolphins ranged in age from 4-47 and consisted of 28 males and 14 female dolphins.

Figure 1 is a block diagram of the AEP measurement system. A personal computer (PC) with a multifunction data acquisition card (National Instruments PCI-MIO-16E-1) was used to generate sound stimuli and digitize the evoked responses. Sound stimuli were attenuated (Tucker-Davis Technologies PA-5), bandpass filtered (Krohn-Hite 3C module,

1 to 150 kHz), and amplified (Hafler P1000) before being presented to the subject. The attenuator featured a USB interface that allowed the PC to programmatically change the attenuation.

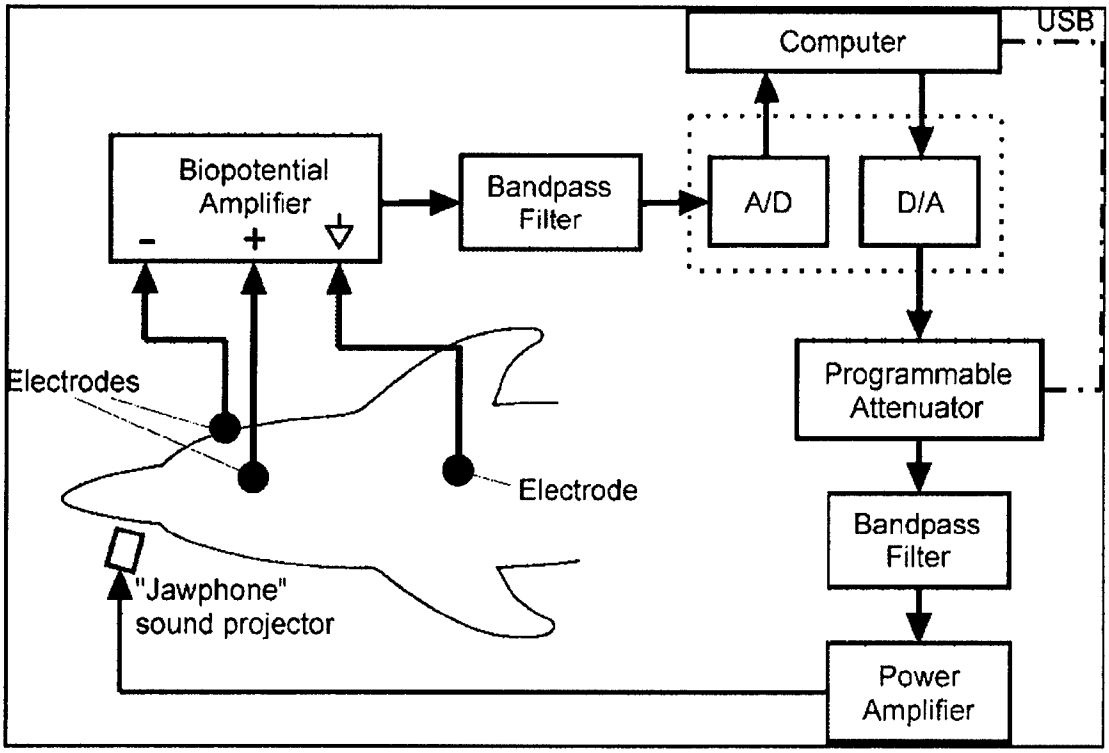

FIG. 1. AEP measurement system configuration.

Sounds were presented to the subjects using piezoelectric sound projectors embedded in silicon rubber (Rhodia V-1065) suction cups. The suction cup-embedded projectors have been labeled "jawphones" in prior literature (e.g., (Brill *et al.*, 2001). The jawphone was placed on the subject's lower jaw, in the pan region, a site which has been previously demonstrated to be an important pathway for high frequency sound reception in dolphins (Brill *et al.*, 1988; Møhl *et al.*, 1999). Jawphones were calibrated by measuring underwater RMS sound pressure levels (SPLs) at a distance of 15 cm from the jawphone. The 15-cm distance between jawphone and calibration hydrophone was based on the estimated distance between the jawphone attachment point and the auditory bullae. This distance was measured from a computed tomography (CT) scan of WEN (Houser *et al.*, 2004). SPLs were measured using a calibrated hydrophone (B&K 8105) and charge amplifier (B&K 2692 or 2635).

Two types of surface electrodes were used to detect evoked potentials: disposable electrodes consisting of a flexible, conductive, self-adhesive Ag/Ag-Cl pad (Ambu Neuroline 710 series) and re-usable 6 and 10 mm diameter gold cup electrodes (e.g., Grass FH-E6G series) embedded in 25 mm diameter silicon suction cups. Electrode placement sites were dried using gauze pads and/or alcohol swabs prior to placement. Disposable electrodes were subsequently covered with waterproof bandages (Nexcare

Absolute Waterproof) to prevent contact with water periodically sprayed over the dolphin. Re-usable electrodes were protected from contact with water by the suction cup itself. A three-electrode configuration was used: the active (+) electrode was placed near the vertex, approximately 10 cm posterior of the blowhole and offset approximately 2 cm contralateral of the ear being tested; the reference (-) electrode was placed contralateral of the ear being tested, just posterior to the external auditory meatus; and the ground (common) electrode was placed on the subject's back near the dorsal fin. When using re-usable suction cup electrodes, the exact position of the active electrode varied depending on the shape of the subject's head; i.e., in some cases, curvature of the head prevented attachment of the suction cup electrodes at the desired site behind the blowhole. The electrode signals were amplified and filtered using a biopotential amplifier (Grass IP-511). The biopotential amplifier output was proportional to the difference in the voltage between the active and reference electrodes. The biopotential amplifier gain was fixed at 100 000. High and low-pass filters varied from 100 to 300 Hz and 3 to 10 kHz, respectively, depending on the stimulus modulation frequency and measurement sampling rate. Additional filtering (Krohn-Hite 3C, bandpass from 0.1 to 5 kHz) was sometimes used, depending on signal quality. The resulting signal was digitized by the PCI-MIO-16E-1 at either 15 or 20 kHz.

Sound stimuli consisted of sinusoidal amplitude modulated (SAM) tones, resulting in steady-state, periodic AEPs with a fundamental at the modulation frequency. An evoked response such as this that is phase-locked to the amplitude modulation rate is sometimes called the envelope following response (EFR) or auditory steady-state response (ASSR; (Dolphin & Mountain, 1993; Rance *et al.*, 1993; Dolphin *et al.*, 1995). Eleven carrier frequencies, from 10 to 150 kHz were tested. These frequencies were chosen to cover the effective range of bottlenose dolphin echolocation (Au, 1993). The modulation rate was 1 kHz. Modulation rate transfer function (MRTF) measurements have shown this rate to produce a strong evoked response in *T. truncatus* (Dolphin *et al.*, 1995; Supin & Popov, 2000; Finneran & Houser, 2004).

For in-air tests, BLU and WEN were tested using both intermittent and continuous SAM tones while BUG and BUS were tested only with continuous SAM tones. For intermittent presentations, tone durations varied from 12 to 15 ms (the majority were 13 ms), with a 1 ms cosine envelope rise and fall. Intermittent stimuli were presented at a rate of approximately 50/s. Continuous stimuli were presented for approximately 10 s. All dolphins tested underwater were tested using intermittent SAM tones with a 1 ms rise/fall time. Tone durations varied from 13 to 23 ms for SAM tones presented in SD Bay and were presented at rates from ~34/s to ~53/s. Tone durations varied from 23 to 32 ms for SAM tones presented in the pool and were presented at a rate of ~26/s to 35/s, except at 5 kHz. Because of difficulties in obtaining evoked signals adequate for analysis at this carrier frequency, the stimulus duration was extended to 62 ms with a tone presentation rate of ~12/s.

Evoked responses were differentially amplified and filtered using a Grass IP-511 biopotential amplifier. The amplifier gain was fixed at 100 000. The high and low-pass filters were set at 300 Hz and 3 kHz, respectively, except for measurements at 5 kHz

where the high pass filter was set at 100 Hz. Additional filtering (Krohn-Hite 3C, 100 Hz and 5 kHz) was sometimes used, depending on signal quality. The resulting signal was digitized (National Instruments PCI-MIO-16E-1) at either 15 or 20 kHz. Signals greater than 20 μV were rejected from analysis. For each frequency tested with an intermittent stimulus, 500 epochs were recorded. For in-air tests, frequency analysis was performed on 10-13 ms epochs; for underwater tests, frequency analysis was performed on 21-30 ms epochs, except for measurements at 5 kHz where the epoch duration was set to either 30 or 60 ms. For continuous stimuli, 19 ms epochs were analyzed. The portions of the evoked response corresponding to the stimulus rise and fall were not included in the frequency analysis. To avoid spectral leakage in the frequency domain, durations for frequency analysis were constrained to integral multiples of 1 ms (1 kHz modulation rate) or 2 ms (500 Hz modulation rate).

The magnitude-squared coherence (MSC) was calculated during each AEP measurement and used to objectively determine if the measured AEP component at the modulation frequency was statistically different from noise (Dobie & Wilson, 1989; Dobie, 1993; Dobie & Wilson, 1996). MSC is a ratio of the power (at a single frequency) contained in the "grand" coherent average to the average of the powers within individual "segments" or "subaverages" of the total data stream. The MSC provides a ratio of the signal power to signal-plus-noise power and varies from zero (all noise) to one (all signal). The MSC calculation used 20 subaverages, with the number of original epochs contributing to the subaverages dependent on the total number of records collected. Critical values for MSC, using $\alpha = 0.01$, were obtained from (Amos & Koopmans, 1963) and (Brillinger, 1978). If the calculated MSC was greater than the critical value, the AEP at the modulation frequency was considered to be detected. This process of objective response detection provided a yes/no result for each AEP measurement and permitted adaptive procedures for adjusting stimulus levels (e.g., modified staircase technique). All MSC calculations were done on-line during test procedures to permit the use of adaptive search techniques during data collection.

Data collection began with a stimulus SPL of 80 dB re 1 μPa. An automated staircase technique was used to adjust stimulus levels to quickly reach threshold. Stimulus levels were decreased after each detected response (a "hit") and increased after each measurement without a detected response (a "miss"). The amount the stimulus was raised or lowered (the step size) started at 30 dB and was reduced after each "reversal" — a transition from a hit to a miss or a miss to a hit. The step size was multiplied by 0.4 after each miss-hit reversal and 0.6 after a hit-miss reversal. Unequal multipliers were used to avoid repeated testing at the same levels, provide a more continuous distribution of stimulus levels tested, and to increase the chance of obtaining a hit on the next trial. Multiple samples were not taken at the individual stimulus levels to avoid an uneven sampling of stimuli and ensure that time was available for sampling at all of the frequencies of interest. The staircase was terminated when the step size was reduced below 4 dB.

Considering only responses that were detected, a linear regression was then performed on the AEP amplitude versus stimulus SPL data. Detected responses with amplitudes greater

than 400 nV were excluded from the regression analysis. Testing was concluded if the regression r^2 value from a minimum of four detected responses reached 0.9. If the minimum r^2 value was not obtained, additional measurements were conducted at SPLs selected to fill the gaps within the AEP amplitude versus stimulus SPL data. With each additional detected response, another regression was performed on the data series. This process was repeated until the criterion r^2 was met or until a maximum of eight detections at different stimulus SPLs was made. When necessary, additional stimulus levels were tested to ensure sufficient data for post-hoc estimates of hearing threshold were obtained.

Each ear of WEN and BLU was tested in-air at least three times. BUG and BUS were only tested once [except BUG's right ear which was tested twice on the same day at 40 kHz]. For hearing tests conducted in SD Bay, all frequencies were tested 3 – 7 times for both ears of each test subject. For pool recordings, all frequencies were tested 4 – 5 times for both of BLU's ears. The exception for pool testing was at 100 kHz, where testing was performed once on the left ear and twice on the right ear. The decision to reduce the number of trials at 100 kHz was due to BLU's insensitivity at this frequency.

Hearing thresholds were estimated for each frequency using a linear regression technique. The regression was first performed on the four detected responses with the lowest amplitudes. If the regression r^2 was less than 0.9, additional points obtained at consecutively higher stimulus levels were added to the regression analysis until the r^2 criterion of 0.9 was met, or an obvious plateau was reached in the AEP amplitude data. The threshold was estimated by extrapolating the regression line to the 0-V level and determining the SPL for the zero-crossing. Occasionally, only three points could be included in the regression because of the appearance of a plateau in the amplitude response curve that occurred at relatively low stimulus levels. If a minimum of a three point regression could not be performed at a particular frequency, estimates for that frequency were not performed.

Results

Benchmark of in-air AEP methods

Three of the four subjects (BLU, BUG, BUS, and WEN) showed varying degrees of high-frequency hearing loss, with upper cutoff frequencies ranging from approximately 80 kHz (BUG) to 30 kHz (BUS). The subject WEN demonstrated a full range of hearing with an upper frequency cutoff of approximately 140 kHz. Low-frequency thresholds in SD Bay were elevated, presumably from masking caused by the relatively high ambient noise levels.

Evoked responses could not be detected above 100 kHz at the highest SPLs the jawphones could generate for the subject BLU. No significant differences between threshold estimates using intermittent or continuous tones were observed for BLU ($p > 0.05$); for the remaining analyses, intermittent and continuous data were pooled to produce a single mean threshold at each frequency for each ear. All threshold estimates reveal poor high frequency hearing, with AEP and pool behavioral threshold estimates

rising at about 47 dB/octave above 30 kHz. Below 40 kHz, a clear separation existed between the behavioral thresholds measured in the pool and SD Bay, most likely from the ambient noise differences between the two environments. Overall, AEP threshold estimates tended to be higher than the behavioral estimates. Differences between thresholds (AEP minus behavioral) increased with threshold magnitude and ranged from 0 to +18 dB, with a mean of 8 dB. AEP thresholds were strongly correlated with behavioral thresholds across the range of hearing ($r = 0.98$) with a regression equation slope of 1.1. The mean AEP thresholds from both ears ranged from 3 to 15 dB above the pool behavioral thresholds.

Both BUG and BUS exhibited poor high-frequency hearing, with thresholds increasing above 60 and 20 kHz, respectively. In contrast, WEN's thresholds remained relatively low across the expected functional range for dolphins and did not begin increasing until exceeding 130 kHz. For all three subjects, AEP threshold estimates and behavioral estimates agree closely as to the upper cutoff frequency beyond which thresholds increased sharply.

Behavioral audiograms for WEN, BUS and BUG were obtained in SD Bay. AEP threshold estimates below 40 kHz were generally lower than behavioral estimates, probably because the behavioral thresholds were masked by ambient noise in the bay at these frequencies. At high frequencies, AEP estimates tended to be higher than behavioral estimates, especially in WEN, the only subject with relatively low thresholds at high frequencies. Mean AEP thresholds (average of both ears) were within -26 to +20 dB of the SD Bay behavioral thresholds for all four subjects. AEP threshold estimates for individual ears ranged from -28 to +22 dB. Excluding comparisons at 40 kHz and below, where masking likely affected threshold measurements, mean AEP thresholds (average of both ears) were within -20 to +20 dB of the SD Bay behavioral thresholds for all four subjects, with a mean difference of +6 dB.

Benchmark of underwater AEP methods

AEP responses could not be detected above 100 kHz for subject BLU at the highest SPL the jawphone could generate. The pattern of hearing sensitivity with frequency was similar regardless of whether AEP estimates or behavioral estimates of threshold are used. The AEP threshold estimates tended to be lower than the behavioral estimates and there was a strong correlation between estimates ($r = 0.93$). When the sensitivity of each ear was considered independently, differences between estimates (AEP threshold estimates minus behavioral thresholds) ranged from -18 to +22 dB re 1 µPa. The mean underestimate of threshold was 7 dB while the mean overestimate of threshold was 11 dB. When the sensitivities of the ears were averaged, differences between estimates ranged from -16 to +20 dB and the mean underestimate and overestimate of threshold was -5 and +12 dB, respectively.

At frequencies below 60 kHz in the subject WEN, AEP threshold estimates were consistently underestimated relative to behavioral thresholds obtained in SD Bay. Conversely, above 60 kHz, AEP threshold estimates were overestimated. Considering each ear independently, differences between estimates ranged from -28 to +23 dB, the

mean underestimate of threshold was 16 dB, and the mean overestimate of threshold was 13 dB. The correlation between threshold estimates was 0.50. When the sensitivities of the ears were averaged, differences between estimates ranged from -20 to +20 dB and the mean underestimate and overestimate of threshold was -14 and +14 dB, respectively.

In contrast to BLU and WEN, AEP thresholds for BEN were generally higher than behavioral estimates. Behavioral thresholds for BEN were much higher than either WEN or BLU from 15 to 30 kHz. Differences between estimates ranged from -20 to +29 dB. The mean underestimate of threshold was 8 dB while the mean overestimate of threshold was 13 dB. The correlation between threshold estimates was 0.90. When the sensitivities of the ears were averaged, differences between estimates ranged from -8 to +21 dB and the mean underestimate and overestimate of threshold was -7 and +12 dB, respectively.

Agreement between mean behavioral and AEP estimates (i.e., average of the left and right ears) were best at 60 and 80 kHz when all measurements made in SD Bay were considered. All AEP predictions of threshold were lower than behavioral thresholds at 50 and 10 kHz, whereas AEP estimates of threshold were both lower and higher than behavioral thresholds at frequencies from 20 to 40 kHz. Considering data collected from all three of the subjects, the average underestimate of threshold was 11 dB and the average overestimate was 13 dB.

BLU was the only animal for which pool collections of AEP and behavioral data were made, thus providing a single comparison for a quiet environment. Threshold estimates derived from AEP data collected in the pool were consistently higher than behavioral thresholds. Considering each ear independently, differences between estimates ranged from -6 to +27 dB, with the greatest deviations occurring at the highest frequencies tested. The mean underestimate of threshold was 3 dB while the mean overestimate of threshold was 12 dB. When the average threshold was determined for both ears, differences between estimates ranged from -3 to +25 dB. Because equivalent testing for each ear occurred across the range of hearing, the mean overestimate and underestimate for the averaged threshold were the same as that obtained when each ear was considered independently.

Population-level audiometry

The average age of subjects was 23.8 yr for males and 25.4 yr for females. The youngest and oldest males tested were 4 and 41 yr, respectively, and the youngest and oldest females were 12 and 47 yr, respectively. Bilateral testing of the ears was obtained for all but 5 of the subjects ($N = 38$ for the left ear, $N = 41$ for the right ear; these subjects are denoted by the subscript "d" in Table I).

Nine of the 42 animals qualified as having baseline hearing. These animals ranged in age from 4-27 yr. All dolphins over the age of 27 had some degree of hearing loss when compared to the baseline audiogram. Six of the animals tested had upper frequency limits of hearing (F_L) between 100 and 140 kHz and 16 of them had F_L between 50 and 100 kHz. Of the remaining animals, 7 had F_L below 50 kHz , 2 demonstrated aberrant audiograms, and 2 were considered to have profound hearing loss across the range of

frequencies tested. The high frequency roll-off in sensitivity generally occurred across less than one octave.

In general, younger dolphins had a better range of hearing than older dolphins and less variability in mean thresholds than older animals. As animals increased in age there was an overall trend for a reduction in sensitivity at progressively higher frequencies. The F_L generally appear to decline between the ages of 20 and 30 yr, although some animals older than 30 yr had a frequency range of hearing in excess of 120 kHz, and some animals younger than 20 yr showed hearing loss. ANCOVA utilizing sex as a fixed factor and age as the covariate showed a significant impact of sex on the relationship between age and F_L. For a covariate mean of 24.3 yr, significant differences existed between the mean F_L of females (113 kHz) and males (81 kHz).

The aberrant audiograms of two male dolphins were characterized by notches below F_L. The notches are reductions in sensitivity that occur between higher and lower frequencies to which the animal is more sensitive. The aberrant audiogram of a 41-yr male was characterized by a notch in hearing sensitivity at 20 and 30 kHz. The audiogram of this male shows a similar pattern of hearing sensitivity across the range of hearing to that of his male offspring, 15 years younger. The upper cutoff frequencies for the two animals differ by ~10 kHz.

Animals with profound hearing loss produced no detectable EFR across a broad range of frequencies. For both of these animals, a 41 yr female and a 26 yr male, all detected evoked responses were in excess of 130 dB re 1 µPa. The inability to detect evoked responses always occurred at test frequencies below 50 kHz.

Modification P00002: Assessment of hearing sensitivity in adult male elephant seals

Materials and Methods

Audiometric evaluations were conducted on elephant seals at the Año Nuevo State Reserve, CA, USA. All elephant seals selected for the audiometric testing were either between 1.3-1.8 yrs of age (age estimates were based upon the time of year the seals are on land, size, and lack of secondary sexual characteristics), sub-adult or adult males. Tests were conducted between the spring of 2005 and the summer of 2006 and were performed on thirteen subjects.

A rugged notebook computer with a multifunction data acquisition card (National Instruments PCI-6251) was used to generate stimuli at a 2 MHz update rate and with 16-bit resolution. Sounds were lowpass filtered (20 kHz, Krohn-Hite 3C module) and passed through a custom attenuator before delivery to the headphones. Sounds were presented to the subjects using either TDH-39 (Telephonics Corp.) or Bose 2 Acoustic Noise Cancelling (Bose Corp.) headphones. Headphones were placed over the external meatus of the subjects. At the start of each session, the stimuli (clicks, tone pips, and SAM

tones) were calibrated with an Etymotic probe microphone (sensitivity of 50 mV/Pa). Pk-pk sound pressure was measured for clicks while the root mean-squared (rms) sound pressure was measured for tone pips and SAM tones. Clicks consisted of either 100 or 200 μs rectangular waveforms with no rise or fall time. Tone pips consisted of five cycles: a two cycle linear rise time, 1 cycle at full amplitude, and a two cycle linear fall time (2-1-2 pip). The duration of the tone pips depended on the frequency of the tone. SAM tones were generated with 1 ms cosine rise and fall times and were amplitude modulated at variable frequencies. Unless noted otherwise, the polarity of the stimulus was sequentially alternated in order to cancel out any artifact introduced into the AEP recordings.

Subcutaneous stainless steel needle electrodes (Neuroline, 1.7 cm needle, 50 or 100 cm lead wires) were used for the detection of evoked potentials. For all seals, the non-inverting (+) electrode was inserted on the dorsal midline of the head, equidistant from the left and right external ears, or 2 cm in front of this position on the midline. The maximum amplitude of the evoked response varied from seal to seal but was maximal within these limits, as was determined from prior exploratory tests. The inverting electrode (-) was placed 5 cm below and 7 cm behind either the right or the left external meatus. The ground was placed on the back of the seal, approximately at the longitudinal insertion of the pectoral flippers. Once inserted, an impedance check was made to ensure that the impedance difference across all of the electrodes was less than 5 kΩ. Electrode signals were differentially amplified and filtered using a biopotential amplifier (Grass ICP-511). The biopotential amplifier gain was fixed at 100 000. Unless otherwise noted, high and low-pass filters varied from 100 to 300 Hz and 100 Hz to 1 kHz, depending on the stimulus modulation frequency and measurement sampling rate. The resulting signal was digitized using the PCI-MIO-16E-1 or PCI-6251. Recording sampling rates, recording durations and stimulus durations varied as a function of the test being conducted (see below).

The following methods section describes the approaches that yielded useful results.

Click evoked Response

A 1.8 yr female elephant seal (10MAR06A) was immobilized at Año Nuevo on 10 March 2006 to determine the waveform characteristics of click evoked responses. Clicks were presented at a rate of ~39 Hz, the evoked response recording window was 26 ms, responses were digitized at 10 kHz, and 4000 averages were collected for each stimulus presentation. Two stimulus presentations were performed using a click pk-pk level of 126 dB re 20 μPa. The artifact rejection level was set at 8 μV and the high and low pass filters were set at 100 Hz and 1 kHz, respectively. Three adult male elephant seals were immobilized to compare the characteristics of the click evoked response to that obtained in the yearling. Similar procedures to those described above were applied with the exception that 8000 averages were collected for the click evoked response.

Tone Pips and Tone Pip Trains

A 1.8 yr male seal (15AUG06A) was immobilized on 15 August 2006 for the study of tone pip evoked responses. Multiple variations on the stimulus presentation were performed to determine the characteristics of the tone pip evoked response and the

feasibility of using tone pip trains to estimate auditory sensitivity. Testing parameters consisted of the following:

I. Single 2-1-2 pips were generated using at 2 kHz center frequency (output duration of 2.5 ms). Tone pips were presented at a rate of 33 Hz; the evoked response recording window was set to 30 ms, responses were digitized at 10 kHz, and 2000 averages were collected for the stimulus presentation. The stimulus level was set at 100 dB re 20 μPa and the artifact rejection level was set at 8 μV.

II. A series of 10 tone pips were used to create a tone pip train. Tone pips were 2-1-2 pips with center frequencies of either 2 or 4 kHz (output durations of 2.5 and 1.2 ms, respectively). Trains were presented such that there was a 10 ms delay between the onsets of successive tone pips. Evoked response recordings were 100 ms in duration and 4000 averages were acquired for each stimulus presentation. Band-pass filters were set at 0.1-1 kHz and evoked responses were digitized at either 2 or 6 kHz. The artifact rejection level was set at 8 μV for all stimulus presentations. For the 2 kHz tone pips, the stimulus level was set at 100 dB re 20 μPa for the initial stimulus presentation and decreased by 10 dB for the second presentation. Stimulus level was then sequentially reduced by 5 dB on consecutive presentations. For the 4 kHz tone pips, the stimulus level was set at 95 dB re 20 μPa for the initial stimulus presentation and decreased by 10 dB for the second presentation. Stimulus level was then sequentially reduced by 5 dB on consecutive presentations. A total of eight stimulus presentations were conducted with the 2 kHz tone pips and total of ten were conducted with the 4 kHz tone pips.

III. Three series of pip trains with different stimulus presentation rates were used to investigate the rate following response (RFR), the relationship between the evoked response amplitude and the rate at which stimuli are presented. Each tone pip train consisted of a series of 2-1-2 pips with a 2 kHz center frequency. The repetition rate of the tone pips was varied at 200, 300 and 400 Hz and recording windows were set at 55, 38 and 30 ms. All tone pips were presented at 100 dB re 20 μPa and 2000 averages were acquired for each stimulus presentation.

Modulation Rate Transfer Function (MRTF) and the Envelope Following Response (EFR)

A 1.8 yr male seal (16AUG06B) was immobilized on 16 August 2006 to investigate the MRTF of the northern elephant seal. A 100 ms duration SAM tone with a 4 kHz carrier was used to test the evoked response amplitude as a result of SAM tone modulation rate. Modulation rates of 80-1000 Hz were used. SAM tones were presented at stimulus levels of 113 dB re 20 μPa. The bioamplifier high and low pass filters were set at 0.03–1 kHz (80 Hz modulation rate), 0.1–1 kHz (100–900 Hz modulation rate), or 0.1–3 kHz (1000 Hz modulation rate). The data acquisition scan rate was either 2 kHz (80–900 Hz modulation rate) or 6 kHz (1000 Hz). The measured AEP amplitudes and phase angles were corrected for the frequency response of the bioamplifier filters and the 6 ms latency between the stimulus onset and the analysis window start.

A 4 kHz carrier SAM tone with a 200 Hz modulation rate was used as a stimulus to determine the feasibility of tracking the amplitude of the EFR as a possible means of using the method to estimate auditory thresholds. Stimuli were 100 ms in duration and the response recording window for each epoch was set to ~111 ms. A total of 4000 averages were collected for each presentation of a particular stimulus level. Stimuli were presented at 113 dB re: 20 μPa and reduced on successive stimulus presentations by 10 dB; a total of 7 stimulus presentations were made covering a stimulus level range of 53-113 dB. Bioamplifier high and low pass filters were set at 100 Hz and 1 kHz, respectively. Evoked responses were recorded with a scan rate of 2 kHz.

Magnitude-squared coherence (MSC) was calculated during each measurement and used to objectively determine if the measured AEP component at the modulation frequency was statistically different from noise (Dobie, 1993; Dobie & Wilson, 1989; Dobie & Wilson, 1996). MSC is a ratio of the power (at a single frequency) contained in the "grand" coherent average to the average of the powers within individual "segments" or "subaverages" of the total data stream. The MSC provides a ratio of the signal power to signal-plus-noise power and varies from zero (all noise) to one (all signal). The MSC calculation used 20 subaverages. Critical values for MSC, using $\alpha = 0.01$, were obtained from Amos and Koopmans (1963) and Brillinger (1978). If the calculated MSC was greater than the critical value, the AEP at the modulation frequency was considered to be detected (see Finneran et al., this issue, for discussion of the MSC relative to other objective response techniques). This process of objective response detection provided a "yes/no" result for each AEP measurement and permitted adaptive procedures for adjusting stimulus levels (e.g., modified staircase technique).

Following data collection, a linear regression was applied to all of the detected responses. The SPL value corresponding to the 0 V crossing of the regression line was then used as an extrapolated threshold value for the frequency tested. Similar processes have been used in the estimation of hearing sensitivity in humans (Campbell et al., 1977) and odontocete cetaceans (Finneran & Houser, 2006; Houser & Finneran, 2006; Popov et al., 2005; Supin et al., 2001; Yuen et al., 2005).

Results

Numerous approaches were attempted on young and adult elephant seals to assess the techniques with the most promise in future AEP work on elephant seals. Only the techniques that yielded promising results are presented here.

Click evoked Response

The click evoked waveform of the yearling northern elephant seal is characterized by three early, positive peaks ~ 2.6, 4.4 and 6.1 ms following stimulus onset. A minor positive peak, or ripple, was also observed at ~5.4 ms following stimulus onset. A pronounced negative peak, the most notable characteristic of the click evoked waveform, occurred 7.2 ms following stimulus onset. The numbering of the click evoked waveforms used for humans is not instituted here since there is no indication that a direct correspondence in waveforms should exist. Therefore, the dominant peaks are identified

in order as P1, P2, P3 and N4. Pk-pk amplitude of the waveform, corresponding to the difference between P3 and N4 amplitudes, averaged 264 nV.

The click evoked responses obtained from adult males were qualitatively similar to that obtained in the yearling seal. The dominant peaks were notable (P2, P3 and N4), but all responses were attenuated by ~100 nV relative to the average pk-pk amplitudes observed in the yearling. Increased physiological noise observed in the adult males, and reduced evoked response amplitude, resulted in a need for additional averaging (approximately twice as many averages as needed in the yearling).

Tone Pips and Tone Pip Trains
The evoked waveform elicited by the tone pip is grossly similar to that evoked by the 200 μs click with the P2, P3 and N4 peaks being observable at comparable latencies. Pk-pk amplitude of the waveform generated by individual tone pips was 211 nV. In waveforms produced by 2 and 4 kHz pip trains, periodicity is noted not only at the fundamental repetition rate of the stimulus (100 Hz), but also at harmonics of the repetition rate. Whereas the 200 Hz harmonic is obvious in the 2 kHz tone pip trains, both the 200 and 300 Hz harmonics are noted in the 4 kHz tone pip train, even though the fundamental repetition rate was the same for both series. As the stimulus level decreased, the amplitude of the evoked response also generally decreased at both 100 and 200 Hz. The response curves at 100 and 200 Hz, for both 2 and 4 kHz tone pips, showed marked nonlinearities; however, at the lower stimulus levels of the 4 kHz tone pip train, the response curves approached a linear decline in amplitude with decreasing stimulus level.

RFR amplitudes were 21.2, 17.8 and 4.0 nV for stimulus presentation rates of 200, 300 and 400 Hz, respectively. The 100 Hz rate utilized previously produced an RFR amplitude of 36.0 nV, thus eliciting the maximal response of the presentation rates tested. The evoked response waveform was well defined for both the 200 and 300 Hz presentation rates, but diminished in quality at 400 Hz.

MRTF and the EFR
EFR amplitudes diminished with increases in the modulation rate (80 Hz t 1 kHz) and were similar to that observed for the limited number of RFR responses tested. Responses were detected across the range of modulation frequencies tested and the maximum amplitude corresponded to a modulation frequency of 80 Hz. However, the range of EFR amplitudes was relatively narrow (3-38.6 nV) across the range of amplitude modulation frequencies tested. For the phase data, a linear regression was performed over the modulation frequency range 80-800 Hz where the data points exhibited good linearity (r2 = 0.99). The group delay Td:

$$T_d = \frac{\Delta\theta / \Delta f_m}{2\pi}$$

was calculated from slope of the regression line, $\Delta\theta/\Delta f_m$, where the slope is in units of rad/Hz and Td is expressed in seconds. The group delay calculated from the regression line slope was 2.9 ms.

For the 4 kHz SAM, the amplitude of the evoked response at the modulation frequency declined with stimulus level. As in the RFR tests, spectral peaks were notable not only at the modulation frequency (200 Hz), but also at harmonics of 400 and 600 Hz. For threshold estimates, the EFR amplitude at the modulation frequency was regressed against the stimulus level. Extrapolation to the 0 V crossing of the regression line relating EFR amplitude to stimulus level yielded a threshold estimate for the 4 kHz SAM of 45 dB re: 20 μPa.

Summary

The use of AEPs for estimating hearing sensitivity in bottlenose dolphins has been quantified. The results demonstrate that the variability between behavioral and AEP thresholds is on the order of that observed in human studies and that it is an effective means for rapidly testing the hearing sensitivity of dolphins. The application AEPs to a dolphin population provided the first population-level assessment of hearing sensitivity for any group of marine mammals. The results of that study demonstrated that age and sex related reductions in hearing sensitivity and range exist in dolphins, and is comparable to that observed in humans and other mammals. Further synthesis of the results of the studies described above is best presented in the peer reviewed literature in which they are described. The PDF files for these manuscripts are provided as part of this report.

Initial attempts to use AEPs to study elephant seal hearing delivered promising results. Clicks, tone pips and SAM tones all proved useful at providing information on auditory sensitivity. As the size of the animals increased from yearling to adult (a difference of ~2000 kg), the utility of the more frequency specific stimuli declined. Although threshold estimates were obtainable using the EFR, it is likely that a combination of click, tone pip and SAM tones will need to be utilized to cover the range of hearing of the elephant seal and accommodate the range of size within this species.

Additional studies not directly related to the objectives of this grant were leveraged off of the developed equipment and the availability of test subjects. Specifically, a study was conducted to quantify the relationship between behavioral and AEP thresholds when the stimulus presentation was held constant between the two and conducted at the same time. The goal of the study was to determine to what extent differences in stimuli used in behavioral and AEP procedures caused differences in threshold estimates. The result of that study demonstrated that congruence of stimuli substantially reduced the differences in AEP and behavioral thresholds. This study is currently under consideration for publication (see below).

Publications resulting from the grant support

Published or in press

Houser, D. S. and Finneran, J. J. 2006. **Variation in the hearing sensitivity of a dolphin population obtained through the use of evoked potential audiometry.** Journal of the Acoustical Society of America. **120**(6): 4090-4099.

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Houser, D. S., Crocker, D. E. and Finneran, J. J. In Preparation. **Click evoked responses of the adult male northern elephant seal.**

Schlundt, C. E., Finneran, J. J. and Houser, D. S. Submitted. **Simultaneously measured behavioral and electrophysiological hearing thresholds in a bottlenose dolphin (*Tursiops truncatus*).** Journal of Comparative Physiology A.

Presentations

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Houser, D. S. and Finneran, J. J. 2005. **Auditory evoked potentials (AEP) methods for population-level assessment of hearing sensitivity in bottlenose dolphins.** 149th Meeting of the Acoustical Society of America, Vancouver, Canada. May 16 – 20.

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Invited Lectures

Stranded animal evoked potential audiometry for the conservation of marine mammals. Southeast Region Marine Mammal Stranding Network Biennial Conference. Panama City, Florida; May 3 – 5.

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A comparison of underwater hearing sensitivity in bottlenose dolphins (*Tursiops truncatus*) determined by electrophysiological and behavioral methods

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Variable stimulus presentation methods are used in auditory evoked potential (AEP) estimates of cetacean hearing sensitivity, each of which might affect stimulus reception and hearing threshold estimates. This study quantifies differences in underwater hearing thresholds obtained by AEP and behavioral means. For AEP estimates, a transducer embedded in a suction cup (jawphone) was coupled to the dolphin's lower jaw for stimulus presentation. Underwater AEP thresholds were obtained for three dolphins in San Diego Bay and for one dolphin in a quiet pool. Thresholds were estimated from the envelope following response at carrier frequencies ranging from 10 to 150 kHz. One animal, with an atypical audiogram, demonstrated significantly greater hearing loss in the right ear than in the left. Across test conditions, the range and average difference between AEP and behavioral threshold estimates were consistent with published comparisons between underwater behavioral and in-air AEP thresholds. AEP thresholds for one animal obtained in-air and in a quiet pool demonstrated a range of differences of −10 to 9 dB (mean=3 dB). Results suggest that for the frequencies tested, the presentation of sound stimuli through a jawphone, underwater and in-air, results in acceptable differences to AEP threshold estimates. © *2006 Acoustical Society of America.* [DOI: 10.1121/1.2229286]

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I. INTRODUCTION

Auditory evoked potential (AEP) techniques currently provide the best opportunity for successfully responding to National Research Council (NRC) recommendations to obtain population-level audiograms for marine mammals and estimate the hearing sensitivity of rare or hard to maintain marine mammals. Specifically, the NRC stated that "population-level audiograms of many individuals are necessary for establishing baseline hearing capabilities and normal hearing loss in marine mammals" and that there should be coordinated efforts to "obtain audiometric data from stranded or ensnared whales" (NRC, 1994; 2000). Hearing information remains to be obtained on most marine mammal species and the natural variation in hearing sensitivity within a population (i.e., as a function of age and/or sex) is unknown for all marine mammal species.

A number of AEP techniques exist for measuring auditory thresholds. These include the measurement of auditory brainstem responses (ABRs) resulting from exposure to clicks, tone pips, and sinusoidal amplitude modulated (SAM) tones. Clicks have broad spectral content and are suitable for testing hearing sensitivity across a range of frequencies simultaneously. Tone pips are short bursts of a tone, typically less than a few ms, which improve frequency specificity of the evoked response because of a narrower spectral content (compared to a click). However, the technique gaining favor for use with small odontocetes is the recording of the envelope following response (EFR). This technique often uses SAM tones as stimuli in order to elicit rhythmic ABRs. The magnitude of the EFR depends on the SAM tone carrier frequency and sound pressure level, as well as the amplitude modulation rate and the depth of modulation. Studies on several odontocete species have demonstrated optimal amplitude modulation rates and depths for detection of the EFR (Dolphin *et al.*, 1995; Supin and Popov, 1995, 2000; Mooney and Nachtigall, 2006). The rhythmic nature of the EFR makes it amenable to analysis in the frequency domain and provides greater frequency specificity than do tests measuring evoked responses to either clicks or tone pips. The EFR has been used to estimate the hearing sensitivity of several marine mammal species (Dolphin, 2000; Klishin *et al.*, 2000; Andre *et al.*, 2003; Nachtigall *et al.*, 2005; Popov *et al.*, 2005; Yuen *et al.*, 2005), but quantification of differences between behavioral and EFR thresholds obtained within the same animal has only recently been performed (Yuen *et al.*, 2005; Finneran and Houser, 2006). Prior to broad acceptance of the EFR technique for use in estimating hearing sensitivity in marine mammals, appropriate quantification of the differences that exist between more commonly accepted behavioral approaches to estimating hearing sensitivity and those determined with EFR should be performed.

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The conditions under which marine mammals are available for study influence how SAM stimuli are presented or how EFRs are recorded. For example, a stranded delphinid on the beach may require use of a "jawphone" (suction cup embedded sound projector) for stimulus presentation (Brill *et al.*, 2001; Finneran and Houser, 2006), where a rehabilitating or trained delphinid tested underwater in a pool may allow direct field presentation of acoustic stimuli (Nachtigall *et al.*, 2005). The differences in threshold estimates from alternative methods are currently not quantified, but may vary as a function of equipment used (e.g., jawphone versus direct field) or the sound propagation characteristics of the testing environment (e.g., stimuli emitted underwater versus those emitted in air). Threshold estimates resulting from differing approaches to EFR data collection should be cautiously applied and placed in the proper context of how the data were collected. Ultimately, variation in threshold estimates resulting from these different approaches should be quantified to facilitate comparisons in threshold estimation across studies.

Finneran and Houser (2006) quantified differences in EFR and behavioral thresholds for the same dolphins and at the same frequencies. The electrophysiological approach was used with four animals, all of which were out of the water and quietly resting on a padded mat. Behavioral estimates of hearing sensitivity were obtained underwater, either in San Diego Bay (SD Bay) or in a quiet pool. Compared to behavioral thresholds obtained in SD Bay at unmasked frequencies (>40 kHz), AEP estimates were on average 6 dB higher. Comparison across the range of hearing for a single animal whose behavioral estimates were obtained in a quiet pool indicated that AEP threshold estimates were from 0 to 18 dB higher, with the greatest differences occurring at the lowest (10 kHz) and highest frequencies (80 and 100 kHz) at which that individual was tested. These differences must necessarily be placed in context of the data collection design used in that study. Dolphins were in air during the procedure and stimuli were presented to them via a jawphone coupled to the pan region of the lower jaw. Differences between behavioral and EFR threshold estimates obtained in this manner may differ from those obtained underwater or in which stimulus presentation occurred in the direct field.

The jawphone has been used for stimulus presentation in the mapping of the sensitivity of the dolphins' lower jaw to click-like signals (Møhl *et al.*, 1999). It has also been used to preferentially stimulate one ear over the other during behavioral audiometry conducted with the animal underwater (Brill *et al.*, 2001). The results of Brill *et al.* suggested that behavioral audiograms obtained from an animal with the jawphone compared favorably to hearing sensitivity measured in the direct field. The study presented here extends the use of the jawphone for presenting SAM stimuli and applying the EFR technique to submerged animals. EFRs were recorded at multiple carrier frequencies and analyzed to estimate hearing thresholds. The results are compared to behavioral thresholds within the same dolphins to determine the differences between the two approaches. EFR-derived threshold estimates obtained on submerged dolphins are further compared to estimates on the same dolphins in air (Finneran and Houser, 2006) to examine how threshold predictions obtained with EFRs varied as a function of the medium in which the data are collected.

II. METHODS

A. Subjects

Three bottlenose dolphins were used: BEN (male, 41 yr, 324 kg), BLU (female, 39 yr, 200 kg), and WEN (male, 21 yr, 210 kg). Subjects were housed in floating netted enclosures, 9×9 to 12×24 m, located in SD Bay, CA. The study followed a protocol approved by the Institutional Animal Care and Use Committee of the Biosciences Division, SSC San Diego, and followed all applicable U.S. Department of Defense guidelines for the use and treatment of animals in science.

B. AEP measurements

1. Hardware

All AEP measurements were conducted underwater. BEN, BLU, and WEN were tested in SD Bay; BLU was also tested in an above-ground vinyl-walled pool. Representative ambient noise levels for the pool and SD Bay test sites are provided in Finneran *et al.* (2005). Within SD Bay, pressure spectral densities were approximately 75 dB *re*:1 μPa^2/Hz at 5 kHz and declined to approximately 63 dB *re*:1 μPa^2/Hz as frequency increased to 50 kHz. Noise levels in the pool were approximately 20 dB below ambient noise levels in SD Bay.

The equipment used to generate sound stimuli and acquire data in SD Bay has been previously detailed (Finneran and Houser, 2006). Briefly, a multifunction data acquisition card (National Instruments PCI-MIO-16E-1) was used to generate sound stimuli and digitize the evoked responses via a personal computer. Sound stimuli were attenuated (Tucker-Davis Technologies PA-5), bandpass filtered (Krohn-Hite 3C module, 1–150 kHz), and amplified (Hafler P1000) before being projected to the dolphin. Stimuli were presented to the subjects via a jawphone consisting of a piezoelectric sound projector embedded in a Rhodia V-1065 silicon rubber suction cup (Brill *et al.*, 2001; Finneran and Houser, 2006). The jawphone was coupled to the dolphin's lower jaw over the pan region, a site which is an important entryway to the auditory system for high frequency sounds (Bullock *et al.*, 1968; McCormick *et al.*, 1970, 1980; Brill *et al.*, 1988; Møhl *et al.*, 1999). A diagram of the system, reproduced from Finneran and Houser (2006), is provided in Fig. 1.

Two jawphones were used in this study: JP4 was fabricated using a commercially available sound projector (ITC 1042) while JP5 was constructed using a custom piezoelectric sphere. JP4 was used for all subjects tested in SD Bay and JP5 was used for BLU when she was tested in the pool. Jawphones were calibrated by measuring underwater rms sound pressure levels at a distance of 15 cm from the jawphone (Finneran and Houser, 2006). The 15-cm distance was based on the measured distance between the jawphone attachment point and the ipsilateral auditory bulla as determined from a computed tomography (CT) scan of WEN (Houser *et al.*, 2004). Sound pressure levels were measured

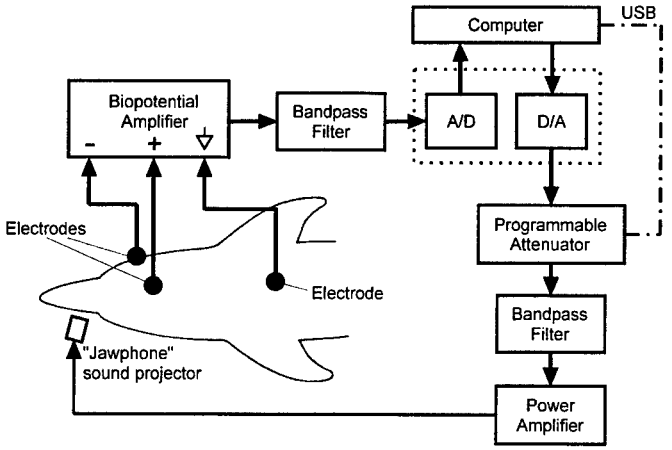

FIG. 1. AEP measurement system configuration (reproduced from Finneran and Houser, 2006).

using a calibrated hydrophone (B&K 8105) and charge amplifier (B&K 2692 or 2635). Figure 2 shows the resulting transmitting voltage responses (TVR). Note that the TVR differs from that of a free transducer; the response of the transducer is damped by its being embedded in the silicon rubber. The straight lines represent linear regressions of TVR versus log-frequency performed over a range of 5–100 kHz for JP4 and 5–40 kHz for JP5 (the frequency range over which each TVR was approximately linear with log-frequency). Regression slopes were 12.5 and 12.3 dB/octave and r^2 values were 0.95 and 0.99 for JP4 and JP5, respectively.

Gold cup electrodes (6 and 10 mm diameter) embedded in 25-mm-diam Rhodia V-1065 silicon rubber suction cups were used as surface electrodes. Prior to electrode placement, the dolphin positioned the region of its body where the electrode was to be placed out of the water and the attachment site was dried using gauze pads. The noninverting electrode (+) was placed near the vertex of the dolphin's head, approximately 10 cm posterior of the blowhole and offset ~2 cm contralateral of the ear being tested. The exact position of the (+) electrode varied depending on the shape of the subject's head. Curvature of the head sometimes prevented adequate sealing of the suction cups at the preferred attachment site and the suction cup had to be moved slightly to

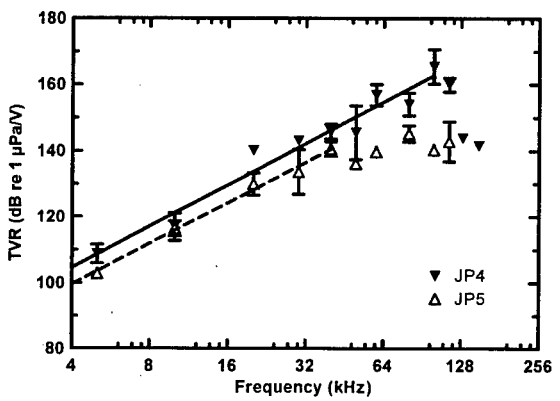

FIG. 2. Transmitting voltage response (TVR) from jawphones JP4 and JP5 measured 15 cm from the transducer. The lines represent linear regressions of TVR vs log-frequency.

ensure an adequate seal. The inverting electrode (–) was placed contralateral to the ear being tested, and just behind the external auditory meatus. A ground (common) electrode was placed either on the subject's back near the dorsal fin or on the dorsal fin itself. Subjects were positioned near the surface so that the (+) and ground electrodes were normally above the waterline.

2. Stimuli

SAM tones were used as stimuli to generate the EFRs. Carrier frequencies ranged from 5 kHz (pool only) or 10 kHz up to 150 kHz. Modulation depth was 100%. Modulation rates were 1 kHz for all carrier frequencies except 5 kHz, where the modulation rate was lowered to 500 Hz to keep the stimulus bandwidth within the anticipated width of the auditory filter centered at the carrier frequency (Au and Moore, 1990; Finneran *et al.*, 2002). Prior research on bottlenose dolphins has indicated that modulation rates from approximately 500 to 600 and 1000 to 1200 Hz evoke strong responses (Dolphin *et al.*, 1995; Supin and Popov, 1995; Popov and Supin, 1998).

All dolphins were tested using intermittent SAM tones with 1 ms rise/fall times. Interstimulus intervals (quiet period between stimuli) were ≥6 ms. Preliminary data showed no significant changes in EFR amplitudes for ISIs ≥6 ms. The specific durations depended on the need to balance the speed achieved with shorter stimuli versus the better frequency resolution and lower noise levels achieved with longer stimuli. Initial tests in SD Bay used durations of 13 ms; this was increased to 23 ms for the majority of tests. Tests in the pool used durations of 23 and 32 ms except at 5 kHz, where the stimulus duration was extended to 62 ms.

3. Evoked responses and response detection

Evoked responses were differentially amplified and filtered using a Grass IP-511 biopotential amplifier. The amplifier gain was fixed at 100 000. The high- and low-pass filters were set at 300 Hz and 3 kHz, respectively, except for measurements at 5 kHz where the high-pass filter was set at 100 Hz. Additional filtering (Krohn-Hite 3C, 100 Hz and 5 kHz) was sometimes used, depending on signal quality. The resulting signal was digitized (National Instruments PCI-MIO-16E-1) at either 15 or 20 kHz. Signals greater than 20 μV were rejected from analysis. For each frequency tested, 500 epochs were recorded. Frequency analysis was performed on 11–30 ms epochs, except for measurements at 5 kHz where the epoch duration was set to either 21 or 60 ms. The portions of the evoked response corresponding to the stimulus rise and fall were not included in the frequency analysis. To avoid spectral leakage in the frequency domain, durations for frequency analysis were constrained to integral multiples of 1 ms (1 kHz modulation rate) or 2 ms (500 Hz modulation rate).

Magnitude-squared coherence (MSC) was calculated from the total collection of epochs obtained for each frequency and stimulus level tested. The MSC was used to assess whether the spectral component at the modulation frequency was statistically different from noise (Dobie and

Wilson, 1989; Dobie, 1993; Dobie and Wilson, 1996). The MSC calculation used 20 subaverages and critical values for MSC, using α=0.01, were obtained from Amos and Koopmans (1963) and Brillinger (1978). Evoked responses with MSC values larger than the critical MSC (a function of α and the number of subaverages) were considered to be detected.

4. Data collection

Data collection began with a stimulus SPL of 80 dB re 1 μPa. The same automated modified staircase technique described in Finneran and Houser (2006) was used to adjust stimulus levels to quickly reach threshold. The staircase was terminated when the step size was reduced below 4 dB. A linear regression was then performed on the AEP amplitude versus stimulus SPL data. Data included in the regression were all detected responses except those exceeding 400 nV. If the regression value (r^2) from a minimum of four detected responses reached 0.9, the test was concluded. If the minimum r^2 value was not obtained, additional measurements and regression analyses were conducted until the criterion r^2 was met or a maximum of eight detections was made.

For hearing tests conducted in SD Bay, all frequencies were tested three to seven times for both ears of each test subject. For pool recordings, all frequencies were tested four to five times for both of BLU's ears. The exception for pool testing was at 100 kHz, where testing was performed once on the left ear and twice on the right ear. The decision to reduce the number of trials at 100 kHz was due to BLU's insensitivity at this frequency.

5. Threshold estimation

Hearing thresholds were estimated for each frequency using a linear regression technique (Supin *et al.*, 2001). The regression was first performed on the four detected responses with the lowest amplitudes. If the regression r^2 was less than 0.9, additional points obtained at consecutively higher stimulus levels were added to the regression analysis until the r^2 criterion of 0.9 was met, or an obvious plateau was reached in the AEP amplitude data. The threshold was estimated by extrapolating the regression line to the 0 V level and determining the SPL for the zero-crossing. Occasionally, only three points could be included in the regression because of the appearance of a plateau in the amplitude response curve that occurred at relatively low stimulus levels. If a minimum of a three point regression could not be performed at a particular frequency, estimates for that frequency were not performed.

C. Behavioral measurements

Behavioral thresholds were measured underwater in SD Bay (all subjects) and the above-ground pool (BLU only). Subjects were trained to whistle in response to test tones and to stay quiet otherwise. Stimulus levels were adjusted using a modified up/down staircase technique with 500 ms pure tone stimuli. Details of the behavioral test methods for WEN and BLU may be found in Finneran and Houser (2006). Behavioral thresholds for BEN were acquired as part of a prior study on noise-induced temporary threshold shifts (Finneran *et al.*, 2005). Testing for WEN was performed in context of a single interval experiment. The behavioral threshold procedures for BEN and BLU were based on the Method of Free Response (MFR; Egan *et al.*, 1961).

D. Analysis

T-tests for independent samples (α=0.05) were used to compare AEP threshold estimates obtained from stimulation of the left and right ears. Statistical analyses were performed with the software package STATISTICA©(StatSoft, Inc.).

III. RESULTS

Figure 3(a) shows examples of the EFR wave form obtained from BLU, while submerged, in response to an intermittent 50 kHz SAM tone. Responses are easily observed in the wave forms at high stimulus levels. As the stimulus SPL is lowered, the magnitude of the response decreases until it is eventually indistinguishable from noise in the time domain. Figure 3(b) shows the frequency spectra of the wave forms from Fig. 3(a). The feature of interest for detection of the EFR is the spectral peak at 1 kHz, which corresponds to the amplitude modulation frequency of the SAM tone.

Figure 4 shows the underwater AEP audiogram and behavioral audiogram for BLU derived from measurements made in SD Bay. AEP responses could not be detected above 100 kHz at the highest SPL the jawphone could generate. As previously demonstrated for BLU, sensitivity above 30 kHz becomes progressively poorer with a reduction in sensitivity of ~47 dB/octave (Finneran and Houser, 2006). The pattern of hearing sensitivity with frequency is similar regardless of whether AEP estimates or behavioral estimates of threshold are used. The AEP threshold estimates tended to be lower than the behavioral estimates and there was a strong correlation between estimates (r=0.93). When the sensitivity of each ear was considered independently, differences between estimates (AEP threshold estimates minus behavioral thresholds) ranged from −18 to +22 dB re 1 μPa. The mean underestimate of threshold was 7 dB while the mean overestimate of threshold was 11 dB. When the sensitivities of the ears were averaged, differences between estimates ranged from −16 to +20 dB and the mean underestimate and overestimate of threshold was −5 and +12 dB, respectively. Differences between the left and right ear AEP audiograms were insignificant across all frequencies tested, except at 50 kHz (t=3.8, p=0.02). At this frequency the left ear threshold was ~4 dB higher than that obtained for the right ear.

Figure 5 compares underwater AEP threshold estimates for WEN to behavioral thresholds measured in SD Bay. At frequencies below 60 kHz, AEP threshold estimates consistently underestimated behavioral thresholds obtained in SD Bay. Conversely, above 60 kHz, AEP threshold estimates overestimated behavioral thresholds. Considering each ear independently, differences between estimates ranged from −28 to +23 dB, the mean underestimate of threshold was 16 dB, and the mean overestimate of threshold was 13 dB. The correlation between threshold estimates was 0.50. When the sensitivities of the ears were averaged, differences be-

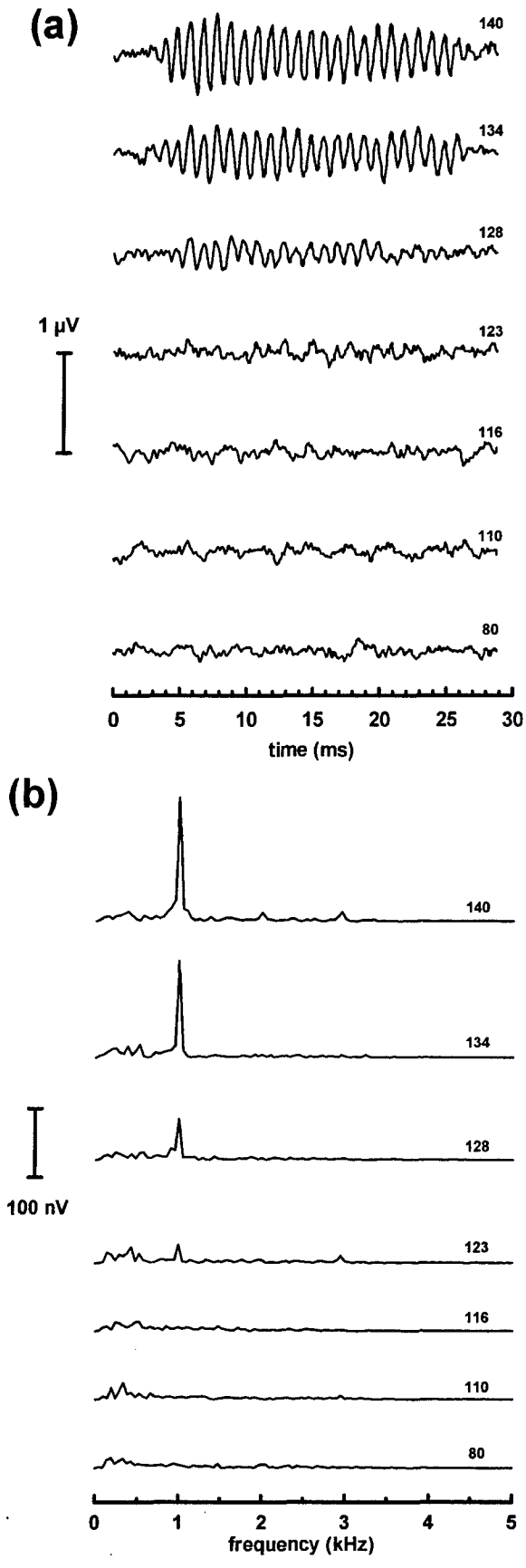

FIG. 3. EFR (a) wave forms and (b) frequency spectra measured at different stimulus SPLs for BLU while she was submerged. The modulation rate of the SAM tone was 1 kHz and the carrier frequency was 50 kHz. Time $t=0$ corresponds to the stimulus onset. The frequency spectra were calculated from a 10 ms analysis window. The numbers next to each series indicate the stimulus SPL (dB re 1 μPa).

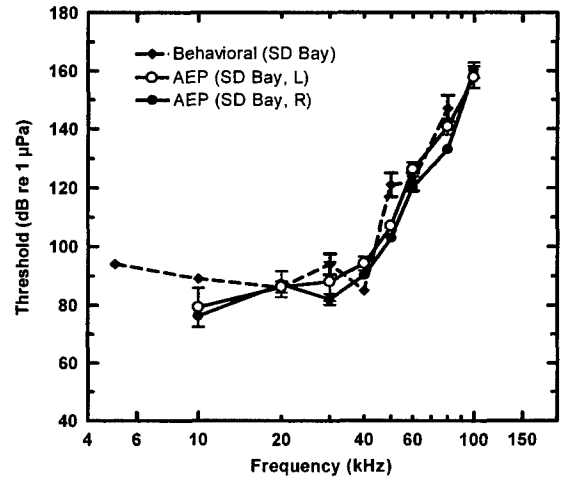

FIG. 4. Comparison of EFR threshold estimates obtained from left (L) and right (R) ears to behavioral thresholds for subject BLU. All EFR tests and behavioral tests were performed in SD Bay. Symbols represent mean values; error bars show the mean ± one standard deviation.

tween estimates ranged from −20 to +20 dB and the mean underestimate and overestimate of threshold was −14 and +14 dB, respectively. Differences between the left and right ear AEP audiograms were insignificant across all frequencies tested, except at 115 kHz ($t=-5.2$, $p<0.01$). At this frequency the right ear threshold was ~6 dB higher than that obtained for the left ear.

Figure 6 compares AEP threshold estimates for BEN to his behavioral thresholds measured in SD Bay. In contrast to BLU and WEN, AEP thresholds were generally higher than behavioral estimates. Behavioral thresholds for BEN were much higher than either WEN or BLU from 15 to 30 kHz (note the notch at these frequencies in Fig. 6). Differences between estimates ranged from −20 to +29 dB. The mean underestimate of threshold was 8 dB while the mean overestimate of threshold was 13 dB. The correlation between threshold estimates was 0.90. When the sensitivities of the ears were averaged, differences between estimates ranged from −8 to +21 dB and the mean underestimate and overes-

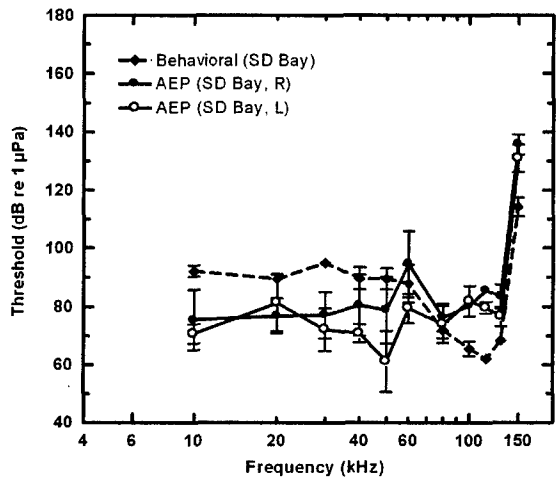

FIG. 5. Comparison of EFR threshold estimates obtained from left (L) and right (R) ears to behavioral thresholds for subject WEN. All EFR tests and behavioral tests were performed in SD Bay. Symbols represent mean values; error bars show the mean ± one standard deviation.

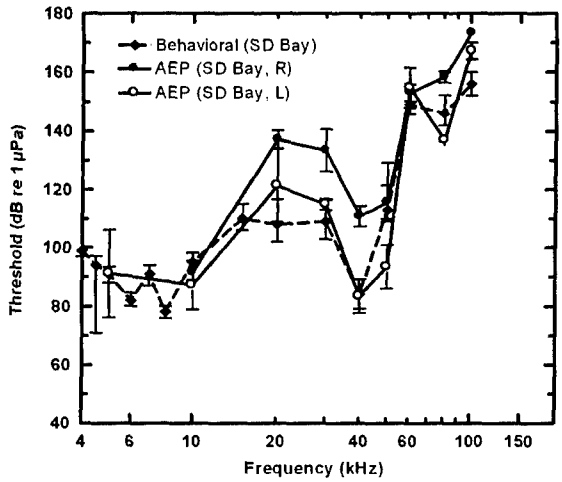

FIG. 6. Comparison of EFR threshold estimates obtained from left (L) and right (R) ears to behavioral thresholds for subject BEN. All EFR tests and behavioral tests were performed in SD Bay. Symbols represent mean values; error bars show the mean ± one standard deviation.

timate of threshold was −7 and +12 dB, respectively. AEP threshold estimates obtained for the right ear of BEN were significantly higher than those obtained from the left for frequencies of 20 ($t=-2.7$, $p=0.02$), 30 ($t=-5.7$, $p<0.01$), 40 ($t=-6.2$, $p<0.01$), 50 ($t=-3.4$, $p=0.02$), 80 ($t=-19.7$, $p<0.01$), and 100 kHz ($t=-3.6$, $p=0.02$). Thresholds were 15–34 dB higher when testing the right ear, except at 100 kHz where it was 6 dB higher. Differences at 10 and 60 kHz were not significantly different. Overall, AEP threshold estimates for the left ear were nearer to behavioral thresholds than were those obtained from the right ear.

Agreement between mean behavioral and AEP estimates (i.e., average of the left and right ears) were best at 60 and 80 kHz when all measurements made in SD Bay were considered (Fig. 7). All AEP predictions of threshold were lower than behavioral thresholds at 50 and 10 kHz, whereas AEP estimates of threshold were both lower and higher than behavioral thresholds at frequencies from 20 to 40 kHz. Con-

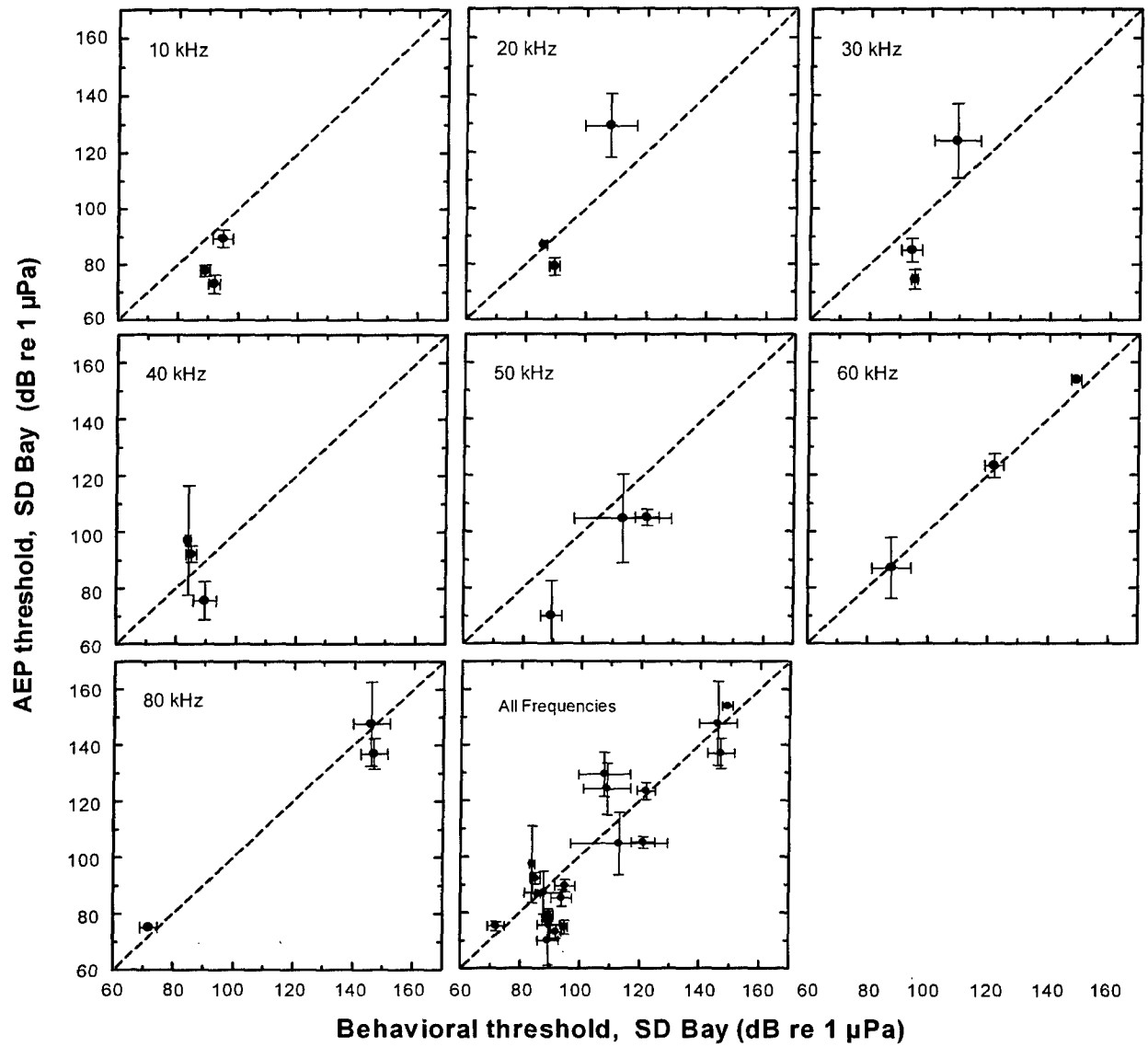

FIG. 7. EFR threshold estimates vs behavioral thresholds for all measurements made in SD Bay. Each panel, except the bottom center panel, shows data from all subjects for a single frequency. The bottom, center panel shows data from all of the subjects and for all of the frequencies tested. The dotted line indicates where perfect agreement (equivalence) between the threshold predictions would occur. Vertical error bars denote the standard deviation of AEP threshold estimates. Horizontal error bars denote the standard deviation of behavioral thresholds.

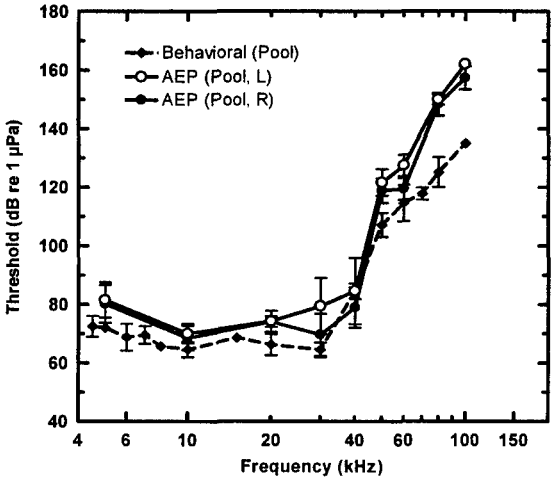

FIG. 8. Comparison of EFR threshold estimates obtained from left (L) and right (R) ears to behavioral thresholds for BLU. The EFR tests and behavioral tests were performed in an above ground pool. Symbols represent mean values; error bars show the mean ± one standard deviation.

sidering data collected from all three of the subjects, the average underestimate of threshold was 11 dB and the average overestimate was 13 dB.

Figure 8 demonstrates the relationship between AEP threshold estimates and behavioral thresholds collected in a pool for BLU. This was the only animal for which pool collections were made, thus providing a single comparison for a quiet environment. Threshold estimates derived from AEP data collected in the pool were consistently higher than behavioral thresholds. Considering each ear independently, differences between estimates ranged from −6 to +27 dB, with the greatest deviations occurring at the highest frequencies tested. The mean underestimate of threshold was 3 dB while the mean overestimate of threshold was 13 dB. When the average threshold was determined for both ears, differences between estimates ranged from −3 to +25 dB. Because equivalent testing for each ear occurred across the range of hearing, the mean overestimate and underestimate for the averaged threshold were the same as that obtained when each ear was considered independently. Differences between the left and right ear AEP audiograms were insignificant across all frequencies tested, except at 60 kHz (t=3.2, p=0.01). At this frequency the left ear threshold was ~8 dB higher than that obtained for the right ear.

IV. DISCUSSION

A. AEP and behavioral thresholds

1. San Diego Bay

Behavioral thresholds for BLU and WEN in SD Bay, and those for BLU in the pool, have been previously reported and discussed (Finneran and Houser, 2006). When only comparisons in San Diego Bay are considered, differences between underwater behavioral thresholds and underwater AEP thresholds were more variable for WEN than for BLU. The relatively high ambient noise levels in SD Bay likely mask hearing up to at least 40 kHz, as previously suggested (Finneran and Houser, 2006); however, it is unlikely that this alone explains the differences in variability since the masking effect should have been consistent between the animals. The variances in behavioral data were generally small compared to variances observed in the AEP data, suggesting that the variability lies within the AEP thresholds and not the behavioral thresholds. The behavioral thresholds for BLU were obtained via the MFR, whereas those of WEN were obtained in a single interval experiment (Finneran *et al.*, 2005; Finneran and Houser, 2006). Behavioral thresholds at low frequencies (where thresholds were masked by ambient noise) were similar, suggesting that the two methods produced comparable results. Sensitivities measured for BLU and WEN were dramatically different at high frequencies where BLU exhibited hearing loss. Whether reduced sensitivity results in greater consistency between AEP and behavioral thresholds is unknown for dolphins. It is possible that the variability in ambient noise levels, from biological sources and vessel traffic, had a more dramatic effect on WEN's thresholds than BLU's, since BLU possessed higher thresholds and test stimuli would have been larger relative to the background noise.

The audiogram of BEN is atypical in that it contains a notch, or reduction in sensitivity, between 10 and 40 kHz. The etiology of this hearing anomaly is unknown and it cannot be determined whether the underlying cause of the notch is also related to differences between the left and right ears. BEN exhibited large left/right differences, with thresholds for the right ear substantially higher (up to 34 dB) than the behavioral results. In contrast, thresholds were similar for both the left and right ears of BLU and WEN for all but one of the frequencies tested. Asymmetry in hearing sensitivity has previously been observed in another bottlenose dolphin (HEP) whose sensitivity was tested through the use of a jawphone (Brill *et al.*, 2001). Right ear thresholds of this subject were up to ~30 dB greater than those of the left and a bilateral reduction in sensitivity was observed above 50 kHz, similar to that observed in BEN. Both BEN and HEP are older males, >35 yr old, and it is possible that the underlying factors contributing to left and right differences in auditory sensitivity are augmented by presbycusis. Unlike WEN and BLU, whose behavioral thresholds were measured concurrently or within weeks of the AEP thresholds, the behavioral audiogram of BEN was obtained several years prior to his AEP audiogram. Given the age of BEN (>40 yr), it is reasonable to suspect that his hearing sensitivity might have continued to decline with aging, thus contributing to the observed differences in AEP and behavioral thresholds. Alternatively, the behavioral audiogram may be more reflective of the more sensitive of the two ears. In this case, the appropriate comparison would be to the AEP threshold from BEN's left ear (the more sensitive ear). The resulting differences between the behavioral and AEP thresholds from the left ear are not significant at most frequencies. The possibility that the audiogram is reflective of the more sensitive ear deserves more research as it could provide insight into the accommodation of hearing loss in odontocete species.

2. Pool

Because of the likelihood for masking to have occurred in SD Bay measurements, the best comparisons of underwa-

ter AEP and behavioral thresholds come from those obtained in the pool environment for BLU. The range of differences between underwater AEP and behavioral thresholds obtained in the pool was approximately 15 dB greater than that observed when in-air AEP threshold estimates were compared to the pool data (Finneran and Houser, 2006). However, the trend was the same between the two studies with the AEP-estimated thresholds typically overestimating the behavioral thresholds.

B. Methodological issues

Prior estimates of hearing sensitivity in delphinid cetaceans obtained via EFRs utilized different methodologies. For example, Yuen *et al.* (2005) performed EFR assessments of hearing sensitivity with their subject, a false killer whale (*Pseudorca crassidens*), underwater in the direct field of the sound source. Other studies have utilized similar procedures (Nachtigall *et al.*, 2005; Popov *et al.*, 2005). In contrast, Finneran and Houser (2006) used EFRs to estimate hearing sensitivity in four bottlenose dolphins by coupling the stimulus to the lower jaw with a suction cup while the animals rested out of the water on a foam mat. Cook *et al.* (2006) used a similar jawphone technique for underwater EFR measurements in a beaked whale. The different manner of stimulus presentation implemented in these studies might result in the use of different acoustic paths to the ear, thus altering the received characteristics of presumably similar stimuli.

Comparability of AEP results when the jawphone is used for stimulus delivery underwater versus in air is of potential concern because the jawphone is not jacketed with sound absorbing material. Thus, the stimulus would have a more omni-directional radiation underwater and could potentially enter the ear via pathways that are otherwise isolated during in-air testing. Using the average threshold of the two ears and considering only the measurements made in SD Bay, the range of differences between underwater AEP threshold estimates and underwater behavioral thresholds obtained in this study (−20 to 21 dB) compared similarly to those observed between in-air AEP threshold estimates and underwater behavioral thresholds (−26 to 20 dB; Finneran and Houser, 2006). The average difference and standard deviation of the differences were also comparable (3±13 dB vs −2±13 dB, respectively) suggesting that the variations were largely unaffected by the medium in which the animals were tested (i.e., water versus air).

Figure 9 demonstrates the relationship between AEP threshold estimates obtained underwater, in the pool, to AEP threshold estimates previously obtained for BLU in air (Finneran and Houser, 2006). The procedures between the two studies were the same, except that the current study presented stimuli and recorded evoked potentials with the subject underwater, and the durations for intermittent stimuli were extended several tens of milliseconds to compensate for electrical leakage to the water. Differences between the in-air and underwater AEP thresholds, calculated as in-air estimates minus those obtained in the pool, ranged from −10 to 9 dB, with a mean difference of 3 dB. This comparison provides the most direct assessment of how medium differences and the potential excitation of different auditory pathways

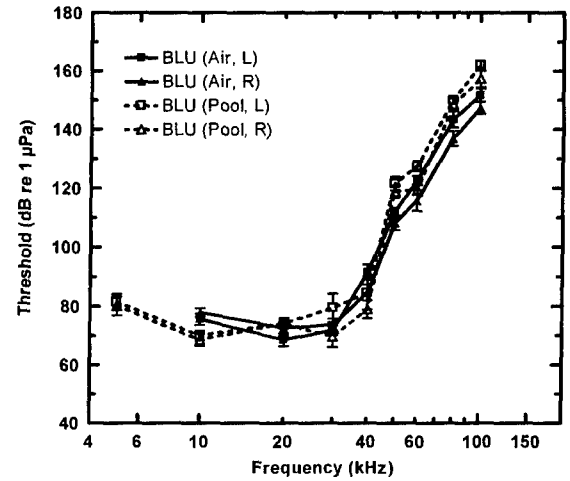

FIG. 9. Comparison of EFR threshold estimates for BLU obtained underwater and in-air. In-air EFR threshold estimates are from Finneran and Houser (2006).

may affect AEP threshold estimates obtained with jawphones. The range of threshold differences between the two data collection efforts and the small mean difference suggests that the two approaches do not result in substantially different threshold estimates. In conjunction with previously discussed comparisons, it appears that greater sound radiation from the jawphone during submersion is not a substantial concern when considering frequencies involved in echolocation and that the primary acoustic pathway at these frequencies is via the lower jaw (Bullock *et al.*, 1968; McCormick *et al.*, 1970, 1980; Brill *et al.*, 1988; Møhl *et al.*, 1999). Similar conclusions may not be valid for lower frequencies, such as those used in whistle communication, because the longer wavelength could be too long to utilize the jaw fat pathway or could diffract around the animal and possibly reach the auditory system through alternative acoustic pathways (Au, 1993).

The suitability of jawphone-measured thresholds as predictors of free field or direct field underwater thresholds is somewhat controversial. There has been no universally accepted technique for calibrating jawphones and the effects of the jawphone attachment on the subject's receiving system are unknown. Calibration measurements for this study were performed at 15 cm distance (beyond the minimum source distance as specified in ANSI S1.20-1998, "Procedures for calibration of underwater electro-acoustic transducers") and the measurements themselves exhibited substantial variance (see Fig. 2). AEP thresholds measured with jawphones were more variable than the direct field behavioral thresholds; however, variability in AEP thresholds seems similar to that observed in direct field measurements of EFR thresholds (Yuen *et al.*, 2005). The degree to which the jawphone calibration measurements match the effective stimulus to the subject is unknown and is likely frequency dependent.

The limitations in the jawphone calibration mean that direct comparisons between sensitivities assessed via jawphone and direct/free field are required to validate the calibrations for each specific jawphone. Inherent calibration problems may limit the utility of absolute threshold values obtained with jawphones. For example, in a previous assess-

ment of hearing sensitivity using jawphones (Brill *et al.*, 2001), the tonal stimulus was calibrated with a hydrophone 40 cm away from the sound source. This distance was 25 cm greater than that used in this study and exceeds the pathway distance from the pan (point of jawphone attachment) to the auditory bulla. However, within the same subject, differences between underwater behavioral thresholds measured with the jawphone and free transducer (Brill *et al.*, 2001) were within the range of differences between direct field behavioral and AEP thresholds observed in this study. Most important, direct field behavioral and jawphone AEP thresholds have agreed closely as to the shape of the audiogram and the upper cutoff frequency. This suggests that jawphone testing may be an acceptable substitute for underwater direct field measurements in many applications (e.g., hearing screening of a rehabilitating animal prior to release) where audiogram shape and cutoff frequency are of primary interest. In many environments, such as in-air testing of stranded cetaceans, jawphones may be the only practical method for testing higher frequencies and obtaining results comparable to those obtained in underwater direct field conditions.

The use of evoked potentials for odontocete audiometry has been practiced for several decades. The use of tone pips for audiometry predates the use of EFR (Popov and Supin, 1987, 1990; Szymanski *et al.*, 1999). The latter technique has been gaining favor in recent years because it produces a stimulus with narrower spectral spreading than does a tone pip. AEP audiograms obtained with tone pips as stimuli have been compared to behavioral audiograms in the same animal in killer whales (*Orcinus orca*; Szymanski *et al.*, 1999). This work demonstrated that electrophysiological estimates of hearing sensitivity obtained with tone pips range from 3 to 41 dB of the behavioral threshold, with average differences of ±8 dB. The procedure was useful in identifying the regions of best sensitivity and the upper limits of hearing. Qualitatively, both AEP audiograms obtained with tone pips and with EFR appear to show similar relationships to behavioral audiograms. Whether the frequency specificity provided by the EFR method provides noticeable gains given the variability inherent in evoked potential measures remains to be determined.

Estimates of hearing sensitivity in delphinids from recordings of the EFR show a similar degree of accuracy and precision relative to behavioral thresholds, regardless of the exact methodology employed for stimulus delivery (this study; Yuen *et al.*, 2005; Finneran and Houser, 2006). More promising to the future application of EFR methods to other marine mammal species is that the variation in accuracy and precision relative to behavioral thresholds is similar to that observed in human clinical studies (Lins *et al.*, 1995; Rance *et al.*, 1995; Aoyagi *et al.*, 1999; Dimitrijevic *et al.*, 2002; Vander Werff and Brown, 2005). The EFR estimation of delphinid hearing seems robust given its results under various modifications of the testing procedures. This characteristic of the EFR estimation technique should make it a useful tool in future efforts of meeting the NRC recommendation (NRC, 1994; 2000) to increase our knowledge base on marine mammal hearing through the testing of stranded or rehabilitating marine mammals.

V. CONCLUSIONS

(1) Underwater AEP measurements with a jawphone transducer produced comparable data to in-air AEP measurements with jawphones.
(2) Underwater or in-air AEP measurements using a jawphone may be a suitable proxy for direct field behavioral/AEP measurements in many circumstances.

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Comparison of in-air evoked potential and underwater behavioral hearing thresholds in four bottlenose dolphins (*Tursiops truncatus*)

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Traditional behavioral techniques for hearing assessment in marine mammals are limited by the time and access required to train subjects. Electrophysiological methods, where passive electrodes are used to measure auditory evoked potentials (AEPs), are attractive alternatives to behavioral techniques; however, there have been few attempts to compare AEP and behavioral results for the same subject. In this study, behavioral and AEP hearing thresholds were compared in four bottlenose dolphins. AEP thresholds were measured in-air using a piezoelectric sound projector embedded in a suction cup to deliver amplitude modulated tones to the dolphin through the lower jaw. Evoked potentials were recorded noninvasively using surface electrodes. Adaptive procedures allowed AEP hearing thresholds to be estimated from 10 to 150 kHz in a single ear in about 45 min. Behavioral thresholds were measured in a quiet pool and in San Diego Bay. AEP and behavioral threshold estimates agreed closely as to the upper cutoff frequency beyond which thresholds increased sharply. AEP thresholds were strongly correlated with pool behavioral thresholds across the range of hearing; differences between AEP and pool behavioral thresholds increased with threshold magnitude and ranged from 0 to +18 dB.
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I. INTRODUCTION

Marine mammal hearing capabilities have traditionally been assessed using behavioral response paradigms (e.g., Johnson, 1967; Schusterman and Moore, 1978; Moore and Schusterman, 1987; Awbrey *et al.*, 1988; Kastak and Schusterman, 1998; Finneran *et al.*, 2002; Kastelein *et al.*, 2002). In these methods, operant conditioning techniques are used to train animals to perform specific actions when they hear sounds. Features of the sound (e.g., amplitude, frequency) are varied and the subject's responses recorded and used to estimate thresholds or other desired features of the auditory system. These techniques provide the most direct assessment of the performance of the auditory system. For some smaller cetaceans, behavioral techniques have been used to define auditory system features such as temporal integration (Johnson, 1968), auditory filter widths (Au and Moore, 1990; Finneran *et al.*, 2002), auditory fatigue (Schlundt *et al.*, 2000; Nachtigall *et al.*, 2004), and receiving directivity (Au and Moore, 1984; Kastelein *et al.*, 2005).

Behavioral techniques require considerable access to subjects for training before reliable data can be obtained, thus preventing these methods from being used on larger marine mammals not routinely kept under human care. Behavioral approaches are also time consuming, both for initial training and data collection. The limited availability of subjects and the required time and costs have limited the number of individuals and species of marine mammals tested with behavioral techniques. At present, behavioral hearing thresholds have been obtained in only about 21 of the more than 100 extant marine mammal species. The National Research Council (National Research Council (NRC), 2000) stated that "population-level audiograms of many individuals are necessary for establishing baseline hearing capabilities and normal hearing loss in marine mammals" and has repeatedly recommended that studies be conducted to obtain such data (NRC, 1994; 2000). The NRC Ocean Studies Board further singled out the need to individually train behavioral hearing test subjects as the "major barrier to large-scale testing of the hearing of many individuals of the same species" (NRC, 2000). The need to specifically train individuals for hearing tests has prevented widespread testing, even at facilities with large numbers of trained marine mammals.

Electrophysiological hearing tests use passive electrodes to detect small voltages generated by the brain in response to acoustic stimuli. The voltages, called auditory evoked potentials (AEPs), are routinely measured in infants, children, and patients who are unwilling or unable to respond behaviorally. AEP methods are attractive alternatives to behavioral methods because they are fast and require minimal cooperation from the subject. AEPs have been measured in several marine mammal species, including bottlenose dolphins (Ridgway *et al.*, 1981; Popov and Supin, 1990b), harbor porpoises (*Phocoena phocoena*; Popov *et al.*, 1986), belugas (*Delphinapterus leucas*; Popov and Supin, 1987), killer whales (*Orca orcinus*; Szymanski *et al.*, 1999), tucuxi dolphins (*So-*

talia fluviatilis; Popov and Supin, 1990a), Amazon river dolphins (*Inia geoffrensis*; Popov and Supin, 1990c), the false killer whale (*Pseudorca crassidens*; Supin *et al.*, 2003; Yuen *et al.*, 2005), a striped dolphin (*Stenella coeruleoalba*; Andre *et al.*, 2003), Risso's dolphin (Grampus griseus; Dolphin, 1997) the finless porpoise (*Neophocaena phocaenoides asiaeorientalis*; Popov *et al.*, 2005), sea lions (*Zalophus californianus*; Bullock *et al.*, 1971) and harbor seals (*Phoca vitulina*; Wolski *et al.*, 2003).

The broader application of AEPs to the study of marine mammal hearing would permit more rapid collection of information on marine mammal auditory systems and address issues acknowledged by the NRC as limiting our understanding of marine mammal hearing at the population level. Unfortunately, most AEP measurements have occurred in laboratory settings using a single individual and there are few direct comparisons between AEP and behavioral hearing thresholds in the same individual.

This paper describes an AEP system developed to address a need for periodic hearing assessment of dolphins at the U.S. Navy Marine Mammal Program at the Space and Naval Warfare Systems Center, San Diego (SSC San Diego). Navy dolphins are trained to find and mark the location of underwater objects and waterborne intruders. To accomplish these tasks, they rely upon their biological sonar (echolocation) system. Since echolocation requires both generation of outgoing pulses and reception (hearing) of returning echoes, loss of hearing sensitivity would eventually decrease a dolphin's ability to detect and identify targets. Periodic hearing assessments of Navy dolphins are therefore needed to identify those animals with degraded hearing. This paper presents the operating principles and performance characteristics of the AEP system. AEP hearing thresholds obtained in air are compared to underwater behavioral thresholds in the same subjects to determine their suitability as proxies for underwater behavioral data. System effectiveness and the time required for hearing estimates in delphinids are discussed relative to the goals of routine screening of Navy dolphins and expanding our knowledge of population level audiometry within marine mammal species.

II. METHODS

A. Subjects

The subjects were four bottlenose dolphins: BLU (female, 39-y, 200 kg), BUG (male, 23-y, 190 kg), BUS (male, 24-y, 180 kg), and WEN (male, 21-y, 210 kg). All subjects had previously participated in cooperative psychophysical tasks, including auditory detection tasks. Subjects were housed in floating netted enclosures, 9×9 to 12×24 m, located in San Diego Bay (SD Bay), California. The study followed a protocol approved by the Institutional Animal Care and Use Committee of the Biosciences Division, SSC San Diego, and followed all applicable U.S. Department of Defense guidelines for the care of laboratory animals.

B. AEP measurements

AEP measurements were conducted in air. In-air measurements were preferable to underwater measurements because AEP amplitudes are generally larger in air compared to those collected underwater (seawater provides an alternative low resistance path for the electrical potentials), and the AEP measurements could be coupled to clinical examinations of dolphins, which are performed with the dolphin removed from water.

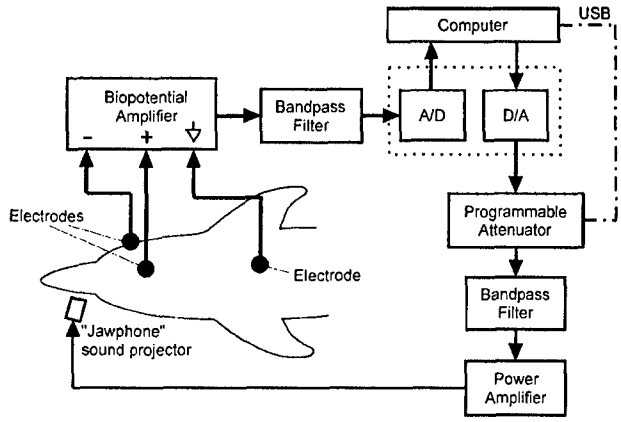

FIG. 1. AEP measurement system configuration.

1. Hardware

Figure 1 is a block diagram of the AEP measurement system. A personal computer (PC) with a multifunction data acquisition card (National Instruments PCI-MIO-16E-1) was used to generate sound stimuli and digitize the evoked responses. Sound stimuli were attenuated (Tucker-Davis Technologies PA-5), bandpass filtered (Krohn-Hite 3C module, 1–150 kHz), and amplified (Hafler P1000) before being presented to the subject. The attenuator featured a USB interface that allowed the PC to programmatically change the attenuation.

Sounds were presented to the subjects using piezoelectric sound projectors embedded in silicon rubber (Rhodia V-1065) suction cups. The suction cup-embedded projectors have been labeled "jawphones" in prior literature (Moore *et al.*, 1995; Brill *et al.*, 2001). The jawphone was placed on the subject's lower jaw, in the pan region, a site which has been previously demonstrated to be an important pathway for high frequency sound reception in dolphins (Brill *et al.*, 1988; Møhl *et al.*, 1999a). Jawphones were calibrated by measuring underwater rms sound pressure levels (SPLs) at a distance of 15 cm from the jawphone [Fig. 2(a)]. The 15-cm distance between jawphone and calibration hydrophone was based on the estimated distance between the jawphone attachment point and the auditory bullae. This distance was measured from a computed tomography (CT) scan of WEN (Houser *et al.*, 2004). SPLs were measured using a calibrated hydrophone (B&K 8105) and charge amplifier (B&K 2692 or 2635). Three individual jawphones, designated JP1, JP2, and JP4, were used. Table I details the size and operating range of the various jawphones. JP1 was used above 100 kHz; JP2 and JP4 were used at and below 100 kHz. Figure 2(b) compares the transmitting voltage response (the SPL at 15 cm in response to a 1-V jawphone input) mea-

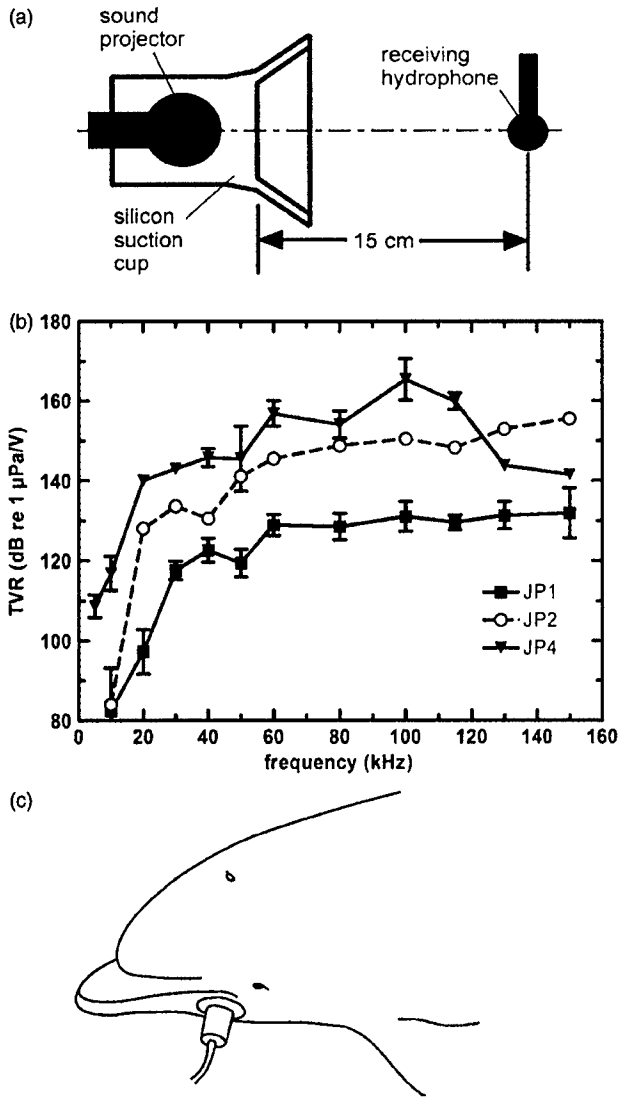

FIG. 2. (a) Jawphone construction and calibration arrangement. (b) Transmitting voltage response (TVR) from jawphones measured at 15 cm. (c) Example of jawphone position on subject.

sured from the various jawphones. Figure 2(c) shows the attachment site of the jawphone for testing the left ear.

Two types of surface electrodes were used to detect evoked potentials: disposable electrodes consisting of a flexible, conductive, self-adhesive Ag/Ag–Cl pad (Ambu Neuroline 710 series) and reusable 6 and 10 mm diameter gold cup electrodes (e.g., Grass FH-E6G series) embedded in 25 mm diameter silicon suction cups. Electrode placement sites were dried using gauze pads and/or alcohol swabs prior to placement. Disposable electrodes were subsequently covered with waterproof bandages (Nexcare Absolute Waterproof) to prevent contact with water periodically sprayed over the dolphin. Reusable electrodes were protected from contact with water by the suction cup itself. A three-electrode configuration was used: the active (+) electrode was placed near the vertex, approximately 10 cm posterior of the blowhole and offset approximately 2 cm contralateral of the ear being tested; the reference (−) electrode was placed contralateral of the ear being tested, just posterior to the external auditory meatus; and the ground (common) electrode was placed on the subject's back near the dorsal fin. When using reusable suction cup electrodes, the exact position of the active electrode varied depending on the shape of the subject's head, i.e., in some cases, curvature of the head prevented attachment of the suction cup electrodes at the desired site behind the blowhole. The electrode signals were amplified and filtered using a biopotential amplifier (Grass IP-511). The biopotential amplifier output was proportional to the voltage difference between the active and reference electrodes. The biopotential amplifier gain was fixed at 100 000. High and low-pass filters varied from 100 to 300 Hz and 3 to 10 kHz, respectively, depending on the stimulus modulation frequency and measurement sampling rate. Additional filtering (Krohn-Hite 3C, bandpass from 0.1 to 5 kHz) was sometimes used, depending on signal quality. The resulting signal was digitized by the PCI-MIO-16E-1 at either 15 or 20 kHz.

TABLE I. Jawphone properties.

Jawphone	Projector	Suction cup base diameter (cm)	Suction cup length (cm)	Frequency range (kHz)
JP1	EDO 1066	2.5	4.0	115–150
JP2	EDO 1066	4.0	6.0	10–100
JP4	ITC 1042	4.0	6.0	10–100

2. Stimuli

Sound stimuli consisted of sinusoidal amplitude modulated (SAM) tones, resulting in steady-state, periodic AEPs with a fundamental at the modulation frequency. An evoked response such as this that is phase-locked to the amplitude modulation rate is sometimes called the envelope following response (EFR) or auditory steady-state response (ASSR) (Dolphin and Mountain, 1993; Rance *et al.*, 1993; Dolphin *et al.*, 1995). Eleven carrier frequencies, from 10 to 150 kHz were tested. These frequencies were chosen to cover the effective range of bottlenose dolphin echolocation (Au, 1993). The modulation rate was 1 kHz. Modulation rate transfer function (MRTF) measurements have shown this rate to produce a strong evoked response in *T. truncatus* (Dolphin *et al.*, 1995; Supin and Popov, 2000; Finneran and Houser, 2004). This was verified by measuring MRTFs on BLU and WEN prior to threshold measurements. The amplitude modulation function was chosen to produce a stimulus with a three-component frequency spectrum (carrier plus sidebands).

BLU and WEN were tested using both intermittent and continuous SAM tones while BUG and BUS were tested only with continuous SAM tones. For intermittent presentations, tone durations varied from 12 to 15 ms (the majority were 13 ms), with a 1 ms cosine envelope rise and fall. Intermittent stimuli were presented at a rate of approximately 50/s. Continuous stimuli were presented for approximately 10 s.

Stimulus generation and evoked response recording were controlled using custom software. Artifact rejection was implemented in software; signals greater than 20 μV

were normally rejected. Frequency analysis was performed on 19 ms epochs for continuous stimuli and 10–13 ms epochs for intermittent stimuli. For continuous stimuli, the first epoch containing the response latency and initial transients was not included in the frequency analysis; for intermittent stimuli, the portions of the evoked response corresponding to the rise/fall of the stimulus were not included. Time durations for frequency analysis were constrained to integral multiples of 1 ms (the harmonic period of the modulation) to avoid spectral leakage in the frequency domain. Coherent averaging (typically 500–1000 averages) was performed in the frequency domain; this allowed the mean amplitude and phase at the modulation frequency, as well as confidence intervals, to be obtained.

Figure 3(a) shows examples of ASSR waveforms measured from BLU in response to an intermittent 30 kHz SAM tone. At high SPLs, responses are easily detectable from the waveforms; however, at lower SPLs the responses become indistinguishable from noise in the time domain. Figure 3(b) shows the frequency spectra of the waveforms from Fig. 3(a). The primary feature of interest for detection of the evoked response is the spectral peak at the 1 kHz modulation frequency.

3. Response detection

The magnitude-squared coherence (MSC) was calculated during each AEP measurement and used to objectively determine if the measured AEP component at the modulation frequency was statistically different from noise (Dobie and Wilson, 1989; Dobie, 1993; Dobie and Wilson, 1996). MSC is a ratio of the power (at a single frequency) contained in the "grand" coherent average to the average of the powers within individual "segments" or "subaverages" of the total data stream. The MSC provides a ratio of the signal power to signal-plus-noise power and varies from zero (all noise) to one (all signal). The MSC calculation used 20 subaverages. Critical values for MSC, using α=0.01, were obtained from Amos and Koopmans (1963) and Brillinger (1978). If the calculated MSC was greater than the critical value, the AEP at the modulation frequency was considered to be detected. This process of objective response detection provided a yes/no result for each AEP measurement and permitted adaptive procedures for adjusting stimulus levels (e.g., modified staircase technique). MSC calculations were done on-line during test procedures to permit the use of adaptive search techniques during data collection.

4. Data collection

Data collection began with a stimulus SPL of 80 dB re 1 μPa. An automated staircase technique was used to adjust stimulus levels to quickly reach threshold. Stimulus levels were decreased after each detected response (a "hit") and increased after each measurement without a detected response (a "miss"). The amount the stimulus was raised or lowered (the step size) started at 30 dB and was reduced after each "reversal"—a transition from a hit to a miss or a miss to a hit. The step size was multiplied by 0.4 after each miss-hit reversal and 0.6 after a hit-miss reversal. Unequal multipliers were used to avoid repeated testing at the same levels, provide a more continuous distribution of stimulus

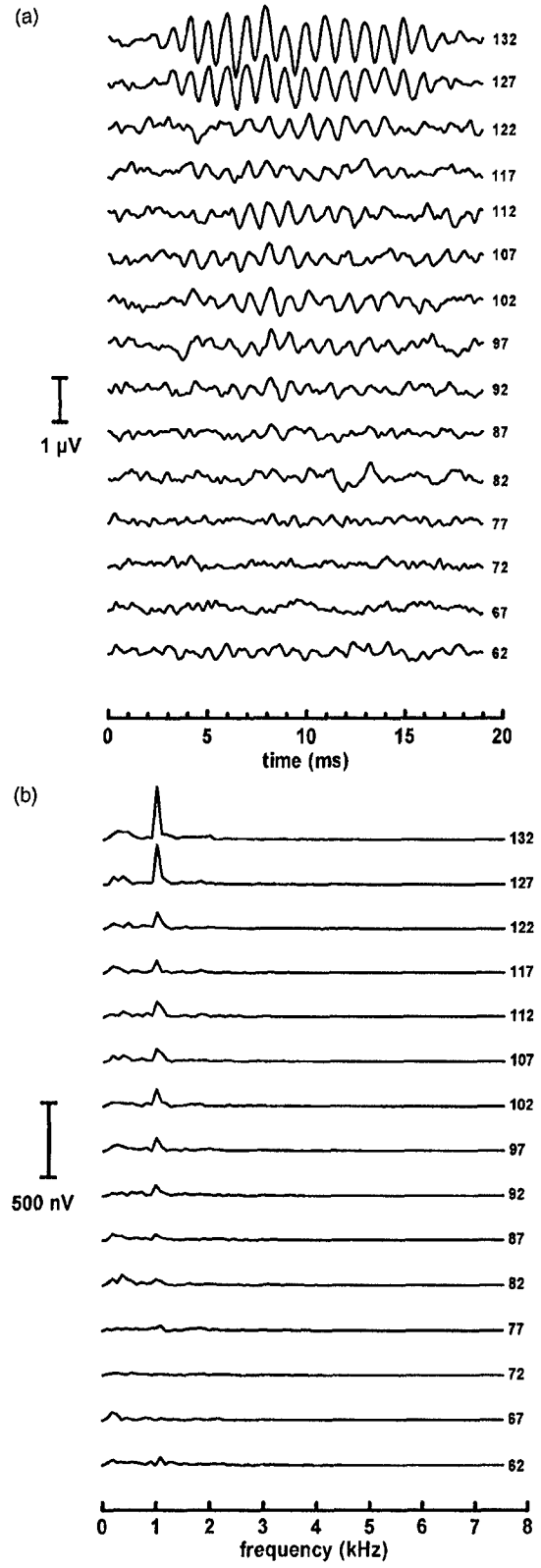

FIG. 3. (a) Steady-state AEP (a) waveforms and (b) frequency spectra measured at different stimulus SPLs for subject BLU, 30 kHz carrier frequency, 1 kHz modulation frequency. The frequency spectra were calculated from a 10 ms analysis window. The numbers next to each series indicate the stimulus SPL (dB re 1 μPa).

levels tested, and to increase the chance of obtaining a hit on the next trial. Multiple samples were not taken at the individual stimulus levels to avoid an uneven sampling of stimuli and ensure that time was available for sampling at all of the frequencies of interest. The staircase was terminated when the step size was reduced below 4 dB.

Considering only responses that were detected, a linear regression was then performed on the AEP amplitude versus stimulus SPL data. Detected responses with amplitudes greater than 400 nV were excluded from the regression analysis. Testing was concluded if the regression r^2 value from a minimum of four detected responses reached 0.9. If the minimum r^2 value was not obtained, additional measurements were conducted at SPLs selected to fill the gaps within the AEP amplitude versus stimulus SPL data. With each additional detected response, another regression was performed on the data series. This process was repeated until the criterion r^2 was met or until a maximum of eight detections at different SPLs was made. When necessary, additional stimulus levels were tested to ensure sufficient data for post-hoc threshold estimates. (This was most often required when a plateau in the AEP amplitude versus SPL data confounded the automated logic.) Using these techniques, a single ear could be tested (at eleven frequencies) in approximately 45 min.

5. Threshold estimation

A post-hoc linear regression technique was used to estimate hearing thresholds for each of the frequencies tested. Linear regression was first performed on the four detected responses with the lowest amplitudes. If the regression r^2 was less than 0.9, additional points obtained at consecutively higher stimulus levels were added to the regression analysis until the r^2 criterion of 0.9 was met or an obvious plateau was reached in the AEP amplitude data. The AEP threshold was defined as the stimulus SPL at which the extrapolated regression line reached 0 V. Almost all regressions were based on more than four points; however, in a few cases, only three points could be included in the regression. If a three point regression could not be performed using detected responses, the data at that frequency were excluded.

Figure 4 shows the amplitude (upper panel) and phase angle (lower panel) at the modulation frequency for the data shown in Fig. 3 and illustrates the threshold estimation procedure. The filled symbols represent detected responses according to the MSC calculation; open symbols indicate measurements that were not statistically different than noise. The solid line shows the linear regression applied according to rules outlined above. In this case, the four lowest amplitude responses resulted in an r^2=0.92, so no additional points were included. The dashed line shows the extrapolation of the regression to estimate the threshold (72 dB re 1 μPa).

Each ear of WEN and BLU was tested at least three times and the mean of the threshold estimates are reported here. BUG and BUS were only tested once (except BUG's right ear which was tested twice on the same day at 40 kHz).

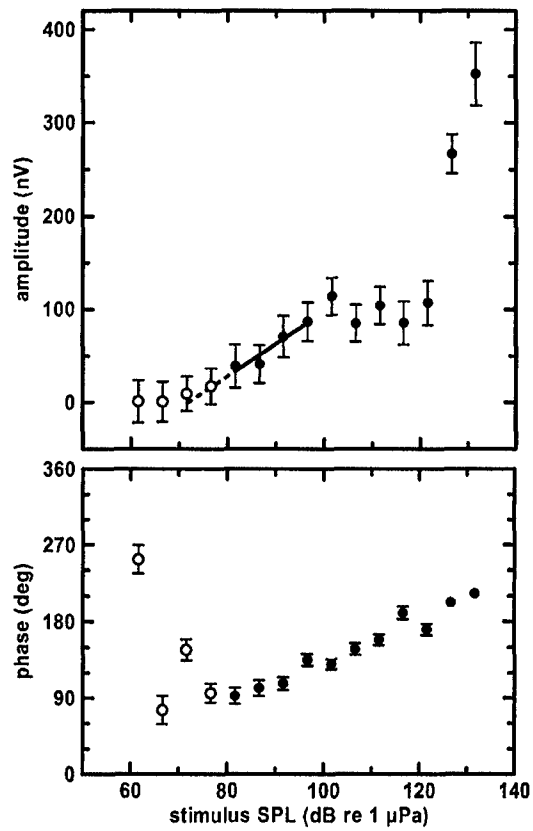

FIG. 4. Steady-state AEP (rms) amplitude and phase as functions of stimulus SPL for the data shown in Fig. 3. Symbols represent mean values; error bars show the 95% confidence intervals. The filled symbols represent detected responses (MSC values were greater than the critical value, MSC_{crit}); the open symbols represent measurements without statistically detected responses ($MSC < MSC_{crit}$). The solid line shows the linear regression performed on the lowest four detected responses (r^2=0.92). The dashed line is an extrapolation of the regression line to 0 V. The stimulus SPL at which the dashed line intersects the x-axis is the estimated threshold (72 dB re 1 μPa).

C. Behavioral measurements

Behavioral thresholds were measured underwater in SD Bay (all subjects) and an above-ground pool (BLU only). Ambient noise spectral density levels in the pool ranged from approximately 50 dB re 1 μPa^2/Hz (at 10 kHz) to 40 dB re 1 μPa^2/Hz (50 kHz); noise levels in San Diego Bay were approximately 20 dB higher (Finneran *et al.*, 2005).

Behavioral thresholds for BLU and WEN were obtained before or concurrently with AEP measurements (data were collected over a period of several months). BUG and BUS were tested under "blind" conditions; AEP measurements were conducted first, then behavioral measurements were performed by independent investigators. AEP thresholds were not disclosed until the behavioral testing was complete.

Table II compares test parameters for each subject. All subjects were behaviorally tested using 500 ms duration test tones with linear rise/fall times of 20 ms or larger. BLU, BUS, and WEN were trained to whistle in response to hearing test tones and to remain quiet otherwise. BUG was trained to press a response paddle in the presence of a tone. Fifty-percent of the trials were no-tone ("catch") trials, except for a few sessions with BUS where the tone/no-tone

TABLE II. Behavioral hearing test parameters.

	Subject			
	BLU	BUG	BUS	WEN
Location	Pool. SD Bay	SD Bay	SD Bay	SD Bay
Method	(Descending) Staircase	(Descending) Staircase	(Descending) Staircase	(Descending) Staircase
Trial presentation	MFR	Single interval	Single interval	Single interval
Response	Whistle	Paddle press	Whistle	Whistle
Tone duration	500 ms	500 ms	500 ms	500 ms
Step size	2 dB	2 dB	2 dB	2 dB
Threshold estimate	10 rev. (Pool) 6 rev. (SD Bay)	10 rev.	10 rev.	6 rev.
Reinforcement	Variable	Fixed	Fixed	Fixed

ratio was adjusted to 65/35 to maintain a false alarm rate of around 10%–15%. Stimulus SPLs were adjusted using a staircase procedure (Cornsweet, 1962) with a 2-dB step size for threshold estimates. Threshold estimates were based on 6 (WEN and BLU in SD Bay) or 10 (BUG, BUS, and BLU in the pool) consecutive hit-miss or miss-hit reversals. BUG, BUS, and WEN were tested in the context of a single interval experiment where the subject was rewarded (correct response) or recalled (incorrect response) after each trial. The behavioral threshold procedure for BLU was based on the Method of Free Response (MFR) (Egan *et al.*, 1961) and described in detail by (Finneran *et al.*, 2005). For all subjects, thresholds from independent sessions were averaged to yield a mean threshold estimate based on minimum of two (BUG, BUS) or three (BLU, WEN) independent measurements. At most frequencies, WEN and BLU were tested 4–6 times.

D. Statistical analysis

Left and right ear AEP thresholds for BLU obtained with intermittent and continuous stimuli were compared using a one-way ANOVA (GraphPad Software, 2003). A Tukey-Kramer post-test was used to compare all four data sets: Left ear, continuous; left ear, intermittent; right ear, continuous; right ear, intermittent.

III. RESULTS

Figure 5 presents the behavioral thresholds measured for BLU, BUG, BUS, and WEN. Three of the four subjects showed varying degrees of high-frequency hearing loss, with upper cutoff frequencies ranging from approximately 80 kHz (BUG) to 30 kHz (BUS). WEN possessed full range hearing with an upper frequency cutoff of approximately 140 kHz. Low-frequency thresholds in SD Bay were elevated, presumably from masking caused by the relatively high ambient noise levels.

Figure 6 compares AEP thresholds for BLU measured using both continuous and intermittent stimuli. For this subject, responses could not be detected above 100 kHz at the highest SPLs the jawphones could generate. No significant differences between threshold estimates using intermittent or continuous tones were observed for BLU ($p > 0.05$); for the remaining analyses, intermittent and continuous data were pooled to produce a single mean threshold at each frequency for each ear.

Figure 7 compares the left and right ear AEP thresholds estimates for BLU to behavioral thresholds measured in SD Bay and the pool. All threshold estimates reveal poor high frequency hearing, with AEP and pool behavioral threshold estimates rising at about 47 dB/octave above 30 kHz. Below 40 kHz, a clear separation exists between the behavioral thresholds measured in the pool and SD Bay, most likely from the ambient noise differences between the two environments.

Figure 8 compares BLU's AEP thresholds (ordinate) to the behavioral thresholds measured in the pool (abscissa). The dashed line represents the ideal case in which behavioral and AEP thresholds are identical. AEP threshold estimates tended to be higher than the behavioral estimates. Differences between thresholds (AEP minus behavioral) increased with threshold magnitude and ranged from 0 to +18 dB, with a mean of 8 dB. AEP thresholds were strongly corre-

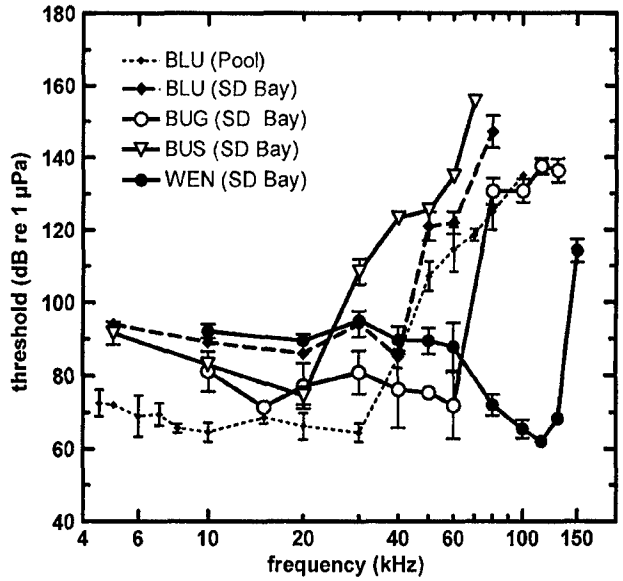

FIG. 5. Behavioral thresholds for BLU, BUG, BUS, and WEN. Symbols represent mean values; error bars show the mean ± one standard deviation.

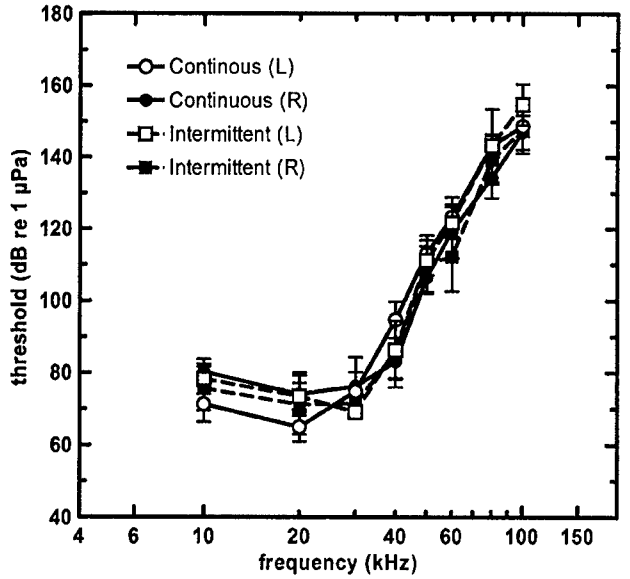

FIG. 6. Comparison of in-air steady-state AEP hearing thresholds for subject BLU obtained from left (L) and right (R) ears using continuous and intermittent stimuli. Symbols represent mean values; error bars show the mean ± one standard deviation. There were no statistically significant differences between the thresholds at a particular frequency.

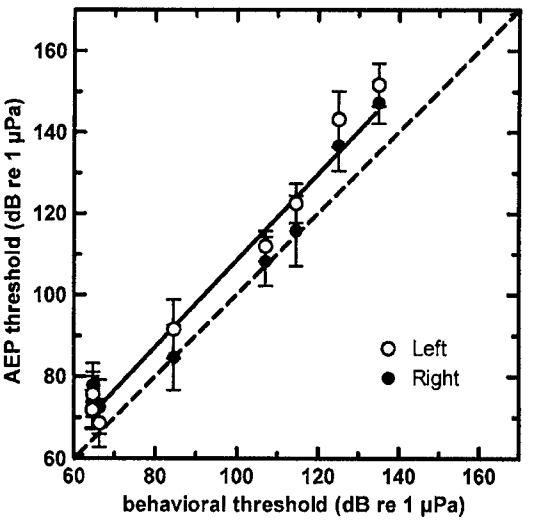

FIG. 8. In-air AEP threshold estimates vs behavioral thresholds measured in the pool for subject BLU. Symbols are mean values. Error bars represent ± one standard deviation. The solid line is a linear regression performed on the experimental data.

lated with behavioral thresholds across the range of hearing (r=0.98) with a regression equation slope of 1.1. The mean AEP thresholds from both ears ranged from 3 to 15 dB above the pool behavioral thresholds.

Figures 9–11 compare AEP and behavioral thresholds for WEN, BUG, and BUS, respectively. Both BUG and BUS exhibited poor high-frequency hearing, with thresholds increasing above 60 and 20 kHz, respectively. In contrast, WEN's thresholds remained relatively low across the expected functional range for dolphins and did not increase until the frequency exceeded 130 kHz. For all three subjects, AEP threshold estimates and behavioral estimates agreed closely as to the upper cutoff frequency beyond which thresholds increased sharply.

Figure 12 compares the AEP thresholds to the SD Bay behavioral thresholds for all four subjects. AEP threshold estimates below 40 kHz were generally lower than behavioral estimates, probably because the behavioral thresholds were masked by ambient noise at these frequencies. At high frequencies, AEP estimates tended to be higher than behavioral estimates, especially in WEN, the only subject with relatively low thresholds at high frequencies. Mean AEP thresholds (average of both ears) were within −26 to +20 dB of the SD Bay behavioral thresholds for all four subjects. AEP threshold estimates for individual ears ranged

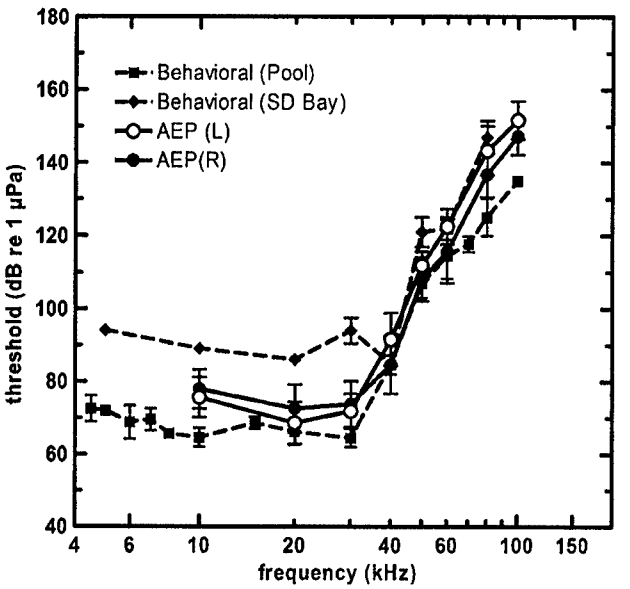

FIG. 7. In-air AEP and underwater behavioral hearing thresholds measured for BLU. Symbols are mean values. Error bars represent ± one standard deviation.

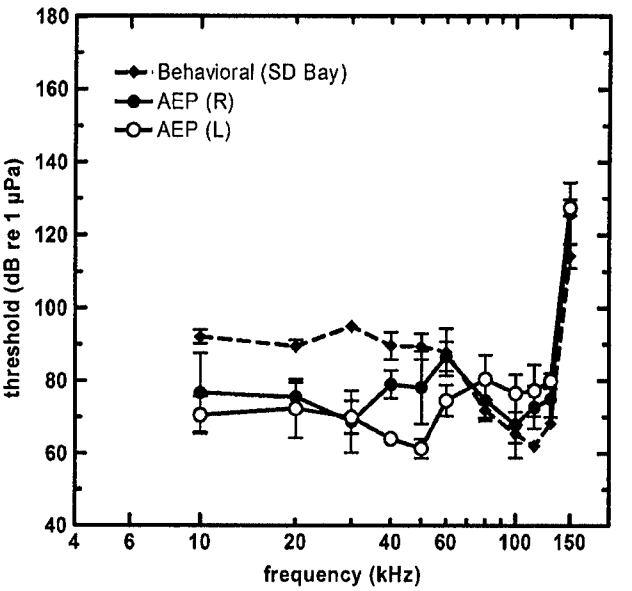

FIG. 9. In-air AEP and underwater behavioral hearing thresholds measured for WEN. Symbols are mean values. Error bars represent ± one standard deviation.

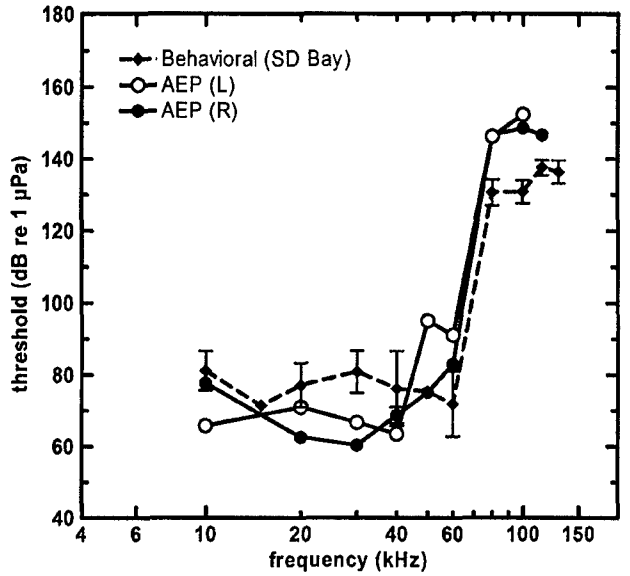

FIG. 10. In-air AEP and underwater behavioral hearing thresholds measured for BUG. Symbols are mean values. Error bars represent ± one standard deviation.

from −28 to +22 dB. Excluding comparisons at 40 kHz and below, where masking likely affected threshold measurements, mean AEP thresholds (average of both ears) were within −20 to +20 dB of the SD Bay behavioral thresholds for all four subjects, with a mean difference of +6 dB.

IV. DISCUSSION

A. Behavioral thresholds

Comparisons of behavioral thresholds between the BLU and WEN for SD Bay and the pool suggest that thresholds measured in SD Bay were masked by ambient noise at frequencies up to at least 40 kHz, and possibly higher. BUS and

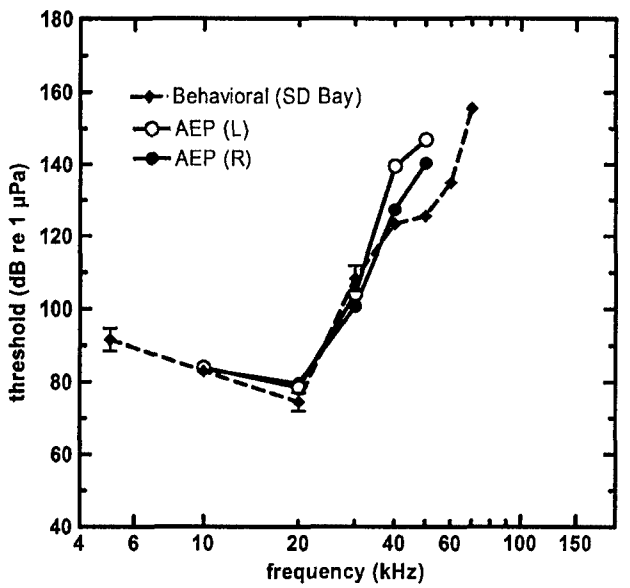

FIG. 11. In-air AEP and underwater behavioral hearing thresholds measured for BUS. Symbols are mean values. Error bars represent ± one standard deviation.

BUG had similar thresholds at low frequencies; however, their thresholds were consistently lower than thresholds for BLU and WEN. The similar shapes, but roughly 10 dB shift in amplitude, of the threshold curves for thresholds for WEN and BUG suggest that motivational, methodological, or calibration differences may have affected threshold estimates. An implicit assumption is often made that behavioral thresholds are correct and that any differences between AEP and behavior threshold estimates are attributed to errors in the AEP estimate. It must be remembered that the classical behavioral threshold is a statistical concept depending on experimental and motivational factors as well as the subject's sensitivity. Changes in a subject's response bias or differences in threshold estimation methods will affect the numeric thresholds obtained. Until standard methods for marine mammal behavioral threshold estimates are developed, comparisons between AEP and behaviorally measured data should consider the details of how the data were collected.

B. AEP thresholds

1. AEP methodology

The relationship between the AEP amplitude and the stimulus SPL, called the input/output function or AEP growth curve, is nonlinear (Fig. 4). The plateau and subsequent increase in the input/output function have been proposed to result from auditory adaptation during stimulation, followed by increased activation from spectral spreading at higher SPLs (Supin *et al.*, 2001). The shape may also reflect basilar membrane displacement, which exhibits a similar nonlinear response believed to arise from a combination of active and passive processes (Pickles, 1988). Identification of the linear regions and plateaus within the nonlinear input/output function requires sufficient measurements and under sampling the curve can result in unsatisfactory threshold estimates (Lins *et al.*, 1995; Vander Werff and Brown, 2005). The existence of a plateau can confound automatic threshold testing logic and, if occurring sufficiently close to the noise floor, prevent the application of regression techniques.

As the stimulus SPL is lowered, the AEP amplitude will, at some point, become indistinguishable from noise. Discriminating AEP responses from noise based only on visual observation cannot avoid some degree of subjectivity and a dependence on observer skill. Furthermore, AEP threshold estimates based on a regression technique can be markedly skewed by inclusion/rejection of individual points within the regression (Vander Werff and Brown, 2005).

This study employed an objective technique (the MSC) to distinguish responses from noise and avoid problems associated with observer subjectivity. This allowed objective decisions to be made regarding the presence or absence of a response. Data collection and stimulus adjustment could thus be automated to a great extent, since the presence or absence of a response could be used as a basis for adjusting the stimulus SPL for the next trial. Figure 4 illustrates the utility of statistical approaches; without objective response detection, one may be tempted to include the three highest amplitude undetected responses in the regression, producing a potentially erroneous threshold estimate.

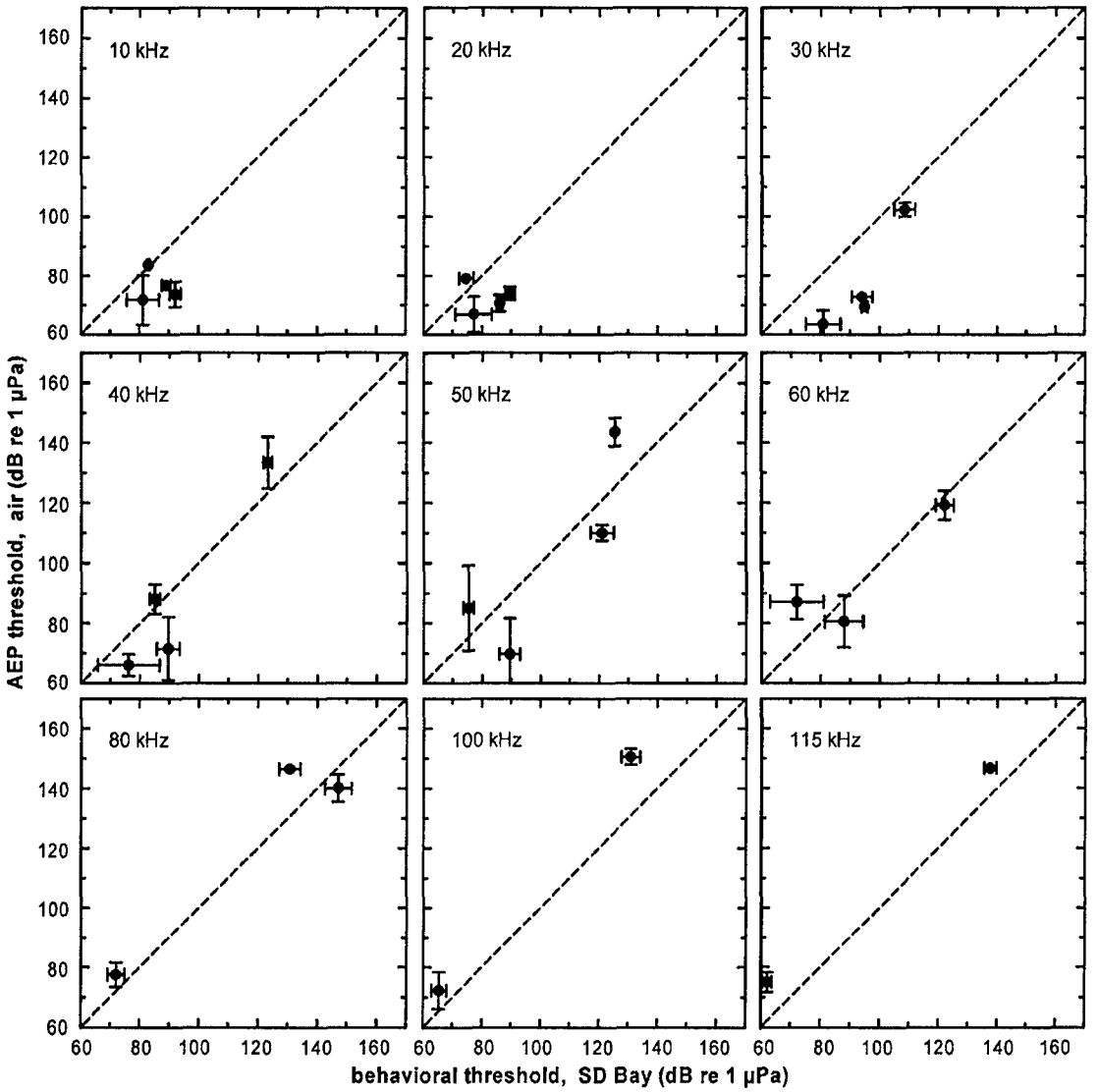

FIG. 12. In-air AEP thresholds plotted versus the San Diego Bay behavioral thresholds. Each panel shows data from all four subjects (if possible) for a single frequency. Error bars represent ± one standard deviation. The dotted line indicates where perfect agreement (equivalence) between the threshold predictions would occur. Note that frequencies below 40 kHz are likely to be masked, augmenting differences between behavioral and AEP estimates of hearing sensitivity at these frequencies.

2. Relationships between AEP and behavioral data

Because they were not affected by masking noise in SD Bay, behavioral thresholds in the pool provide more appropriate comparisons to the AEP thresholds than behavioral data obtained in SD Bay. The largest differences between the two threshold estimates occurred at the lowest (10 kHz) and highest frequencies (80 and 100 kHz). The magnitude and direction of the differences between AEP and behavioral thresholds agree with previous comparisons in humans with normal hearing and sensorineural hearing loss (Lins *et al.*, 1995; Rance *et al.*, 1995; Aoyagi *et al.*, 1999; Dimitrijevic *et al.*, 2002; Vander Werff and Brown, 2005) and show similarly strong correlations in predicted thresholds (Rance *et al.*, 1995; Vander Werff and Brown, 2005). The results reveal that in-air AEP thresholds measured using the techniques described here are useful proxies for underwater behavioral data, especially with respect to the audiogram shape and upper cutoff frequencies.

3. Left/right ear differences

Differences between BLU's left and right thresholds were small (relative to the standard deviation), ranging from 2 to 7 dB, whereas WEN's left ear had 12–17 dB lower thresholds between 40 and 60 kHz, but 6–10 dB higher thresholds between 80 and 130 kHz. BUG and BUS had similar thresholds for their left and right ears at most frequencies (most frequencies were only tested once, so there are no variance measures for these data). The ability to differentiate the relative hearing sensitivity between two ears would be beneficial in clinical diagnoses of auditory system dysfunction as well as the characterization of auditory system health in animals that might be used in psychoacoustic research. At present it is not clear how best to compare behavioral thresholds obtained binaurally with monaural AEP data. The mean of the left and right ear thresholds seems a reasonable approach, especially when the thresholds are

similar; however, one could imagine an animal with large sensitivity differences between ears relying on the more sensitive ear during binaural tests.

4. Continuous and intermittent stimuli

No difference between threshold estimates for BLU was found when using either continuous or intermittent stimuli. Continuous stimuli allow data to be acquired more rapidly because the same amount of time domain response data will take less time to acquire using continuous stimuli than with intermittent stimuli. The duration of the time segment available for spectral analysis dictates the resulting frequency resolution (frequency "bin" width). Assuming that the noise spectral density is relatively flat, smaller frequency bins will result in lower noise levels, making detection of responses near threshold easier. So, for identical data collection time periods, continuous stimuli would allow longer time segments for analysis and lower noise levels. The primary advantage of intermittent stimuli is that the response latency may be observed. This allows one to clearly separate physiological response from stimulus artifacts, which may have zero latency. In many cases, this advantage can offset the additional time that is required for data collection.

5. Approach limitations

The AEP threshold estimation in delphinids using the combined techniques described here is promising given that estimates demonstrate a similar degree of variation between behavioral and physiological estimates that are observed with other species. However, electrophysiological techniques have limitations when applied as surrogates for behavioral tests. Some limitations may be controlled from a methodological standpoint (e.g., the two methods typically do not use equivalent stimuli), while others are inherent to physiological measures (e.g., animals are "noisy" because of spontaneous brain activity and myogenic potentials). Differences between AEP and behavioral thresholds quantified for the methods used in this study should not be broadly applied because they are likely to vary as a function of methodology. For example, underwater, direct field stimulation of a subject may result in sound transmission to the ear via acoustic pathways that are not used when the sound projector is coupled to a point on the lower jaw of an animal that is out of the water.

The delivery of acoustic stimuli through a jawphone has been previously applied in studies of dolphin audition (Moore *et al.*, 1995; Mohl *et al.*, 1999; Brill *et al.*, 2001), but no standard approach exists for jawphone calibration. In this study, the jawphone was calibrated underwater as would be a free transducer with the receiving hydrophone 15 cm away. This approach does not account for potential distortion of the stimulus signal resulting from coupling the jawphone to the animal. The impact of the coupling is unknown. Nor can it presently be verified whether the direct path distance from the coupling point to the auditory bulla is representative of the true acoustic path to the ear, thus validating that distance for use in future calibrations. However, jawphones constructed for this project have effectively been calibrated against the animals on which they were used, i.e., by comparison of AEP and behavioral threshold estimates. The agreement between threshold estimates, within the variation expected between AEP and behavioral methods, suggests that the calibration used for this project is suitable for the types of animals and circumstances under which the jawphone is used.

6. Approach benefits

The AEP approach used here to estimate hearing thresholds in dolphins has characteristics that make it attractive for use on captive, stranded or wild caught marine mammals. The statistical approach to signal detection and defined rules for inclusion of data points used in the regression analysis diminish the potential bias introduced by the subjective selection of data for threshold estimation. Increasing the application of such objective approaches to AEP threshold estimates can help reduce interstudy variability and facilitate comparisons of results from different studies. Because it employs an automated search algorithm, automated statistical detection of signals and threshold estimation, and automatic transition between test frequencies, the approach permits rapid testing across a broad range of frequencies. The testing of 11 frequencies from 10 to 150 kHz was achieved in approximately 45 min, and the sensitivity of both ears was obtained in 1.5 h. It is important to note that the total of 1.5 h is a single test session in which the full range of hearing of both ears was tested. This relatively short testing time substantially exceeds the rate at which behavioral hearing tests can be performed (e.g., weeks to months) and permits multiple animals to be rapidly tested in sequence. Due to the potential impact of handling stress, the ability to rapidly test animals is a major consideration when dealing with stranded and rehabilitated animals and efforts to collect AEP threshold estimates from these animals will benefit from the implementation of rapid data acquisition systems.

V. CONCLUSIONS

The automated methodology described here offers advantages to assessing hearing sensitivity in delphinids by increasing the rapidity of data collection and the objectivity of the data analysis. Threshold estimates derived from this approach in four bottlenose dolphins demonstrated agreement with behavioral thresholds: (1) differences were consistent with the magnitude and variation of those observed in similar clinical studies; (2) the upper cutoff frequency of hearing sensitivity determined by both methods were close. AEP thresholds measured using the techniques described here are useful proxies for underwater behavioral data.

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Variation in the hearing sensitivity of a dolphin population determined through the use of evoked potential audiometry

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A portable electrophysiological data collection system was used to assess hearing in a captive population of bottlenose dolphins by recording auditory evoked potentials (AEPs). The AEP system used a transducer embedded in a suction cup to deliver amplitude modulated tones to the dolphin through the lower jaw. Evoked potentials were recorded noninvasively using surface electrodes. Adaptive procedures allowed hearing thresholds to be estimated from 10 to 150 kHz in a single ear in about 45 min. Hearing thresholds were measured in 42 bottlenose dolphins (28 male, 14 female), ranging in age from 4 to 47 years. Variations in hearing sensitivity with age and sex followed patterns seen in humans and terrestrial mammals: generally, within the population there was a progressive loss of high frequency hearing with age and an earlier onset of hearing loss in males than in females. Hearing loss generally occurred between the ages of 20 and 30, and all animals over the age of 27 had some degree of hearing loss. Two dolphins with profound hearing loss were found within the population. Aberrant hearing patterns were observed in related dolphins suggesting genetic links to hearing ability may exist. © *2006 Acoustical Society of America.*
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I. INTRODUCTION

Increased frequency of hearing impairment with age (presbycusis) and sex differences in the onset of hearing impairment have long been known for human populations. The 2003 survey by the National Center for Health Statistics (United States Department of Health and Human Services, 2005) on the health status of individuals within the United States indicated that incidence of profound hearing loss increased from 1% for those under the age of 44, to 15% for those over the age of 74. The incidence of hearing loss among males was nearly twice as high as that of females until the age of 75, supporting the pattern of age-related hearing loss and male bias toward hearing loss at a younger age in human populations. Within the United States, the causes of hearing loss are primarily attributed to noise exposure (33.7%), presbycusis (28%), and ear infections (12.2%) (United States Department of Health and Human Services, 1994).

It seems reasonable to expect that the auditory systems of marine mammals are similarly subject to impairment. Prior work on some odontocete species has demonstrated hearing loss in individuals (Ridgway and Carder, 1993, 1997; Brill *et al.*, 2001; Finneran *et al.*, 2005b; Yuen *et al.*, 2005), although the etiology of the deficit is not certain in most cases. That hearing loss occurs in marine mammals is an important consideration because the ability to produce, receive, and interpret sound permits most marine mammals to thrive in the ocean. Understanding how a marine mammal with hearing loss functions in natural contexts is important to understanding how sensory impairment affects the ability of marine mammals to survive and exploit their environment. Similarly, determining the impact that anthropogenic sound has on marine mammal populations is important to mitigating the environmental consequences of human activity in the world's oceans (National Research Council (NRC), 1994, 2000, 2003). To address either of these issues at the population level, the variation in hearing sensitivity within a population of marine mammals must be determined.

Behavioral audiometry has been the standard approach for assessing hearing sensitivity in marine mammals for many decades (see Nachtigall *et al.*, 2000, for review). Because behavioral approaches to audiometry require subjects to be conditioned to respond to acoustic stimuli, and because such conditioning requires long-term access to the subject, behavioral approaches have been cited as an impediment to large scale testing of hearing in any marine mammal species (NRC, 2000). Electrophysiological approaches to assessing hearing sensitivity increase the rapidity with which tests can be performed. Because they are relatively fast, and the auditory nerve of odontocetes is large (thus providing for a robust neural response to acoustic stimuli), such tests have become increasingly popular in odontocete audiometry (e.g., Popov and Supin, 1990a, 1990b; Szymanski *et al.*, 1999; Andre *et al.*, 2003; Nachtigall *et al.*, 2004; Yuen *et al.*, 2005; Houser and Finneran, 2006).

The presentation of a sinusoidal amplitude modulated (SAM) tone elicits a rhythmic evoked response from the au-

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ditory system, termed the envelope following response (EFR). Since the fundamental frequency of an EFR to a SAM tone matches the stimulus modulation rate (Campbell *et al.*, 1977; Hall, 1979; Stapells, 1984; Picton *et al.*, 1987), the magnitude of the evoked response can be assessed in the frequency domain. By tracking the magnitude of the evoked response at a range of stimulus levels, sensitivity at the tonal frequency can be obtained (e.g., Campbell *et al.*, 1977). This approach to electrophysiological estimates of hearing sensitivity provides substantial improvement in frequency specificity relative to other approaches (e.g., clicks or tone pips as stimuli). However, direct comparisons between behavioral and EFR thresholds have only recently been described in bottlenose dolphins (*Tursiops truncatus*) (Nachtigall *et al.*, 2004; Finneran and Houser, 2006; Houser and Finneran, 2006) and a false killer whale (*Pseudorca crassidens*) (Yuen *et al.*, 2005). In two of the studies, the transducer used for stimulus presentation was coupled to the lower jaw of a dolphin via a suction cup (Finneran and Houser, 2006; Houser and Finneran, 2006). The EFR thresholds were obtained on dolphins while they were submerged and while they were resting in air. In both instances, EFR thresholds were compared to behavioral thresholds obtained underwater with the dolphin in the direct field. Differences in EFR thresholds obtained on submerged animals and underwater behavioral thresholds (−20–21 dB) (Houser and Finneran, 2006) compared similarly to those observed between in-air EFR thresholds and underwater behavioral thresholds (−26–20 dB) (Finneran and Houser, 2006). Average differences and standard deviations of the differences were also similar (3±13 dB vs −2±13 dB, respectively). Most importantly, EFR and behavioral thresholds were shown to agree closely as to the shape of the audiogram and the upper cutoff frequency of hearing.

This paper presents population level estimates of auditory sensitivity for a cetacean, the bottlenose dolphin. Utilizing the EFR approach, the hearing sensitivity of 42 dolphins maintained by the United States Navy Marine Mammal Program was surveyed. The purpose of the study was to address hearing sensitivity of animals in the population with the expectation that hearing deficits would correlate with reduced echolocation performance. Variation in hearing sensitivity is discussed as a function of subject age and sex, providing the first insight into how hearing losses might develop in a population of marine mammals.

II. METHODS

A. Subjects

All subjects were Atlantic bottlenose dolphins (*Tursiops truncatus truncatus*) maintained by the United States Navy Marine Mammal Program at the Space and Naval Warfare Systems Center, San Diego, California (SSC San Diego). Subjects ranged from 4 to 47 years of age and consisted of 28 males and 14 females. Nine of the 42 animals were captive born. The distribution of subjects by age and sex is provided in Fig. 1. The study followed a protocol approved by the Institutional Animal Care and Use Committee of the Biosciences Division, SSC San Diego, and followed all applicable U.S. Department of Defense guidelines for the care of laboratory animals.

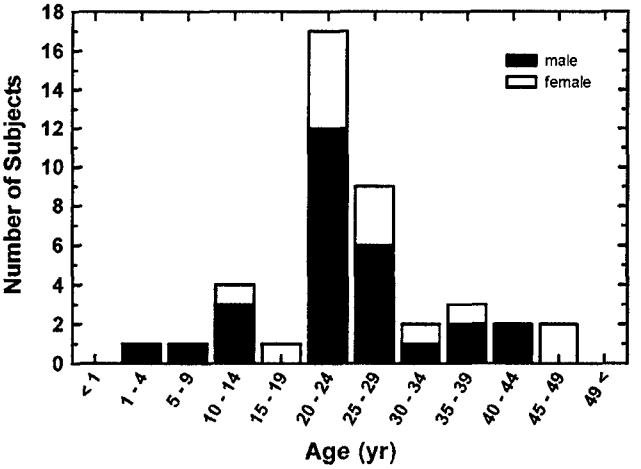

FIG. 1. Age and sex distribution of bottlenose dolphin subjects for which AEP audiograms were collected.

B. EFR Measurements

1. Stimuli, evoked responses, and response detection

The equipment and techniques for stimulus generation and EFR recording have been previously detailed (Finneran and Houser, 2006); only the salient features are described here. SAM tones were used as stimuli to generate the EFR. Eleven carrier frequencies, from 10 to 150 kHz, were tested. All carrier frequencies were 100% amplitude modulated at a rate of 1 kHz, which has been shown to produce a strong EFR in *T. truncatus* (Dolphin *et al.*, 1995; Supin and Popov, 2000). SAM tones were generated with a 1 ms rise/fall time, tone durations of 13–23 ms, and presentation rates of ~40–50/s. In order to improve the signal-to-noise ratio, tone durations were in some instances extended to 62 ms for a dolphin that was tested in the water (see below).

Stimuli were presented to the subjects via a jawphone (a piezoelectric sound projector embedded in a Rhodia V-1065 silicon rubber suction cup) coupled to the dolphin's lower jaw over the pan region (Moore *et al.*, 1995; Brill *et al.*, 2001). The calibration procedures and resulting transmitting voltage responses for the jawphones used in this study (JP1 and JP4), as well as the calibration of EFR-estimated thresholds against behavioral thresholds using these jawphones, are described in Finneran and Houser (2006).

All but one of the subjects was tested while resting out of the water on a padded beaching mat. This subject was tested while stationed on a biteplate and partially submerged (dorsal surface above the waterline) in San Diego Bay (SD Bay). This subject is identified by the superscript "c" in Table I. A different approach was used for this animal because age and poor eyesight prohibited him from voluntarily "beaching" himself out of the water. Since the jawphone permits coupling of the stimulus generator to the lower jaw, similar stimulus conditions can be created for animals that are underwater or in air. Prior comparisons of underwater and in-air estimates of hearing sensitivity derived from EFR measurements with jawphones have been shown to be com-

TABLE I. Subject ID number, age at time of testing, sex and upper cutoff frequency of hearing for each individual in the hearing survey. Unless otherwise noted, frequency-specific thresholds are presented for each individual and each ear. Thresholds are given as dB re: 1 μPa.

					Frequency (kHz)										
ID	Sex	Age	Ear	F_L(kHz)[f]	10	20	30	40	50	60	80	100	115	130	150
1	M	4	L	149	94	86	74	67	64	74	73	92	94	71	127
			R			84	77	71	62	79	75	92	94	74	120
2	M	7	L	147	99	75	83	71	63	77	66	71	73	72	
			R			88	85	76	73	79	78	73	64	73	129
3	M	13	L	73	88	105		71	64	73	154				
			R		75	83	95		66	67	140	163			
4	M	13	L	76	79	78	74	74	70	81	132	151			
			R		75	76	55	54	72	84	126	144			
5	M	13	L	142	80	73	72		70	75	90	79	79	84	145
			R		83	85	78		86		88	65	80	76	147
6[a]	F	12	L	149	88	83	67	74	69	74	77	89	90	78	120
			R		92	86	76	69	65	78	75	95	86	73	127
7[b,e]	M	41	L	53	87	121	115	83	94	155	137	167			
			R		92	137	133	111	116	153	158	173			
8	F	39	L	61	76	69	72	92	112	123	143	152			
			R		78	73	74	85	108	116	137	147			
9	F	28	L	100	75	75	80	79	74	81		116	147		
			R			73	69	72	76	77	75	123	142		
10	M	26	L	49	81	77	71	63	130						
			R		87	67	62	63	124	166					
11	M	23	L	71	66	71	67	64	95	91	146	152			
			R		78	63	60	69	75	83	147	149	147		
12	M	24	L	36	84	79	104	140	147						
			R		84	80	101	127	140						
13[a,d]	F	21	L	126											
			R		74	73	70	69	69		75	93	103	126	132
14	F	26	L	98	78	82	72	81	82	74	75	141	140	106	126
			R		81	82	71	79	99	75	78	116	141		
15	M	24	L	96	101	60	70	75	71	75	76	137			
			R		71	67	73	59	71	76	77	127			
16	F	20	L	142	80		68	63	72	74	74	77	81	100	134
			R				69	69	71						144
17	M	28	L	49	84	69	75	80	122	150					
			R		78	74	87	71	113	151					
18[c,d]	M	25	L												
			R				134		153	148	154	167	164	168	168
19[d]	M	40	L	50											
			R			78		97		146	155	151		139	
20	M	22	L	55	76	76	63	72	105	142	144				
			R		80	70		61	86	155					
21	M	24	L	73		89		86	84	93	142				
			R				91	80	56	83	133	150			
22	M	35	L	93	103	113	119	93	62	75	89	139	146		
			R		87	111	102	117	95	89	91	131			
23	F	27	L	149		89	90	70	64	74	77	88	98	80	121
			R		111	67	73	73	65	76	78	84	93	74	122
24[d]	F	20	L	54	85	74	68	69	115	129	135				
			R												
25	F	30	L	54	80	80	91	103	114	131	134	151			
			R		80	77	84	111	115	136	146	151			
26[c]	F	47	L												
			R												
27	M	25	L	55	69	84	85	62		136	124	153	153		
			R		91	75	79	105	107	133	141	150	159		
28	M	34	L	53	81	68	71	76	109	129	135	153			
			R		102	80	87	67	120	146					
29	M	39	L	125		72	73	67	73	72	72	90	100	131	136

TABLE I. (*Continued.*)

					Frequency (kHz)										
ID	Sex	Age	Ear	F_L(kHz)[f]	10	20	30	40	50	60	80	100	115	130	150
			R				78	71	71	81	86	93	96	128	
30[b]	M	26	L	64	104	109	135	70	71	107	140	148			
			R		100	121	86	84	75	122	153				
31[a]	M	22	L	43		100	82	116	131						
			R			87	86	113	117						
32	M	20	L	80	87	60	73	63	67	80	126	126	111	142	
			R		82	70	65	69	62	76	115	156			
33	M	26	L	39		70	68	131	135	139	164	167			
			R		118	63	61	122	128	113	154	150			
34	M	20	L	44	111	63	68	93	138	143	147	159			
			R		115	69	80	120	123	134	152	151			
35	F	23	L	146	81	75	70	80	65	76	79		88	84	144
			R		83	88		69	72	76	77	93	87	68	123
36	M	24	L	74		90	88	81	66	79	138	148			
			R		86		87	62	58	82	137	152			
37[d]	M	24	L	143											
			R		124	61	74	76	79	80	82	94	95	80	144
38	F	21	L	137	105	64	104	108	61	75	94	97	102		128
			R		105	64	79	108	61	75	85	102	102		128
39	F	46	L	126		66	67	60		77	110	86	81	137	150
			R		74		66	72	83	75	86	84	86	134	130
40	M	13	L	121	92	78	75	84	70	80	82	90	105	137	129
			R		81	79	66	67	54	84	70	98	115	129	
41	M	21	L	147	71	72	70	64	64	75	77	84	83	83	136
			R		77	79	73	80	78	84	71	73	81	78	131
42	F	17	L	108	85	84	72	72	58	81	79	103	136	129	125
			R		83	74	82	61	67	77	78	104	133	123	119

[a]Subjects received an intramuscular injection of midazolam, prior to testing, to reduce anxiety.
[b]Subjects with aberrant audiograms. Subject No. 7 is the father of subject No. 30.
[c]Subjects that are considered to have profound hearing loss based upon high thresholds or lack of evoked responses at the highest levels tested. No F_L was determined for these animals.
[d]Subjects for which thresholds were only obtained in one ear.
[e]This single subject was tested underwater because he could not voluntarily beach himself onto a padded mat.
[f]F_L is calculated from the averaged thresholds of the two ears, when possible.

parable (Houser and Finneran, 2006), so the data from this individual were pooled with the data from the other subjects. For all of the animals tested while resting out of the water on a padded mat, the total time out of the water was typically on the order of 90 min or less. During the testing period animals were periodically sprayed with water to keep the skin moist.

EFRs were measured using electrodes consisting of flexible, conductive, self-adhesive Ag/Ag–Cl pads (Ambu Neuroline 710 series) or 6 and 10 mm diameter gold cup electrodes (e.g., Grass FH-E6G series) embedded in 25 mm diameter silicon suction cups. Attachment sites were dried with gauze pads and alcohol swabs prior to placement of the electrodes. Since the dolphins were periodically sprayed with water during the testing period to keep the skin moist, disposable electrodes were covered with waterproof bandages (Nexcare Absolute Waterproof). Evoked responses were measured between a noninverting (+) electrode placed ~10 cm posterior of the rear edge of the blowhole and offset ~2 cm contralateral of the ear being tested and an inverting (–) electrode placed contralateral of the ear being tested, just posterior to the external auditory meatus. A common reference (ground) electrode was placed on the subject's back near the dorsal fin. Electrode signals were differentially amplified (100 000 gain), filtered (300–3000 Hz), and digitized at 15 or 20 kHz. Signals exceeding 20 μV were rejected from analysis. For each frequency and stimulus pairing, 500 evoked response epochs were recorded.

A small number of the test subjects (N=4) demonstrated an aversion to the attachment of the test equipment (see below). Movement by these individuals created myogenic artifacts sufficient to prevent the recording of evoked potentials. For three of these subjects (see Table I—subjects identified by the superscript "a"), an intramuscular injection of midazolam HCl (Versed; Hoffman-LaRoche, Inc.) at a dose of 0.075–0.1 mg/kg was administered to provide sedation throughout the procedure. Midazolam is a member of the family of sedatives known as the benzodiazepines (e.g., Valium and Xanax), of which at least Valium has been shown to have a mild effect on the latencies of short latency auditory evoked responses, but not the amplitude (Adams *et al.*, 1985). The sedatives are unlikely to have affected threshold estimates in these animals since only the amplitude of the evoked response is used for threshold estimation (see below).

Frequency analysis was performed on 11–21 ms ep-

ochs, excluding the first and last millisecond of the evoked response. Frequency analyses on data collected when tone durations were extended to 62 ms (for the male dolphin tested in water) were performed on 60 ms epochs. Leading and trailing portions of the evoked response were not included so as to avoid transients resulting from signal onset or termination. Durations for frequency analysis were constrained to integral multiples of 1 ms. Magnitude-squared coherence (MSC) was used to determine if the amplitude of the evoked response at the frequency of the modulation rate was significantly greater than measurement noise (Dobie and Wilson, 1989; Dobie, 1993; Dobie and Wilson, 1996). The MSC was calculated by dividing the total number of epochs obtained for each frequency/stimulus pairing into 20 subaverages. Using α=0.01, critical values for MSC, MSC_{crit}, were obtained from Amos and Koopmans (1963) and Brillinger (1978). EFRs with $MSC > MSC_{crit}$ were therefore statistically different from noise and were considered to be detected responses.

2. Data collection and threshold estimation

An automated modified staircase technique, similar to that described in Finneran and Houser (2006), was used to adjust stimulus levels and acquire data necessary for threshold estimation. Data collection began with a stimulus SPL of 80–100 dB re 1 μPa. The step size began at either 20 or 30 dB and was reduced after each reversal (i.e., change from a detection to no detection, or vice versa). The staircase procedure ended when the step size was $\leq$4 dB. A linear regression utilizing EFR amplitude as the response variable and stimulus SPL as the independent variable was subsequently performed. All detected responses were included in the regression except those exceeding 400 nV. The test was concluded if the regression r^2 value from a minimum of four detected responses reached 0.9; otherwise, additional measurements and regression analyses were performed until the criterion r^2 was met or a maximum of eight detections was made. This procedure for determining the termination of data collection for a specific test frequency ensured that sufficient data existed for the estimation of auditory thresholds at that frequency.

Following data collection, a rules-based modification of a linear regression technique was used to estimate the hearing threshold for each frequency tested (Supin *et al.*, 2001; Yuen *et al.*, 2005; Finneran and Houser, 2006). As previously, the analysis utilized detected responses recorded at each of the frequencies and their associated stimulus levels as the dependent and independent variables, respectively. Details of the rules-based approach are found in Finneran and Houser (2006). Threshold testing was discontinued prior to testing at 150 kHz if a threshold in excess of 140 dB re 1 μPa was obtained for a lower frequency.

3. Analysis

Thresholds were obtained for both ears in all but five individuals; these individuals are indicated by the superscript "d" in Table I. In these individuals, time limitations in the beaching mat resulting from noise issues, equipment malfunctions, or veterinary restrictions prevented testing of both ears. Thresholds were averaged for the left and right ears to produce a mean audiogram for each animal. When only one ear was tested, it was used as the representative audiogram. The upper frequency limit of hearing, F_L, arbitrarily defined as the frequency at which the threshold equaled 120 dB re 1 μPa, was calculated for each audiogram by linear interpolation. Audiograms were considered part of the baseline group if F_L exceeded 140 kHz and there were no notches in the audiogram that exceeded the 120 dB re 1 μPa threshold. Audiograms that qualified as base line were averaged to produce a mean base line audiogram to which all other audiograms could be compared. An analysis of covariance (ANCOVA) was applied to the distribution of F_L within the population using sex as the fixed factor and age as the covariate. Statistical analysis was conducted with STATISTICA® v.7.1 with a significance level of 0.05.

Patterns of variation in hearing sensitivity related to age were investigated by grouping subjects according to age and calculating mean thresholds across the range of hearing for each group. Means were determined by summing the thresholds of individual ears and dividing by the total number of ears tested for a given frequency in an age group. Age groups were divided accordingly: 0–9, 10–19, 20–24, 25–29, 30–39, and 40–47 yr. Since thresholds in excess of 140 dB re 1 μPa became more frequent at higher frequencies, many individuals with compromised hearing were not tested above 100 kHz.

III. RESULTS

Table I provides the age, sex, F_L, and audiometric information for each dolphin tested. The average age of subjects was 23.8 yr for males and 25.4 yr for females. The youngest and oldest males tested were 4 and 41 yr, respectively, and the youngest and oldest females were 12 and 47 yr, respectively. Bilateral testing of the ears was obtained for all but five of the subjects (N=38 for the left ear, N=41 for the right ear; these subjects are denoted by the subscript "d" in Table I). Comparisons between the records of animals sedated with midazolam HCl and those that were not sedated were similar and no differences in the range and mean amplitudes and latencies were noted.

The base line mean audiogram is shown in Fig. 2(a). Nine of the 42 animals qualified as having base line hearing. These animals ranged in age from 4 to 27 yr. All dolphins over the age of 27 had some degree of hearing loss when compared to the baseline audiogram. Examples of audiograms of the animals that did not qualify as baseline hearing are provided in Figs. 2(b)–2(d). Six of the animals tested had F_L between 100 and 140 kHz and 16 of them had F_L between 50 and 100 kHz [Figs. 2(b) and 2(c), respectively]. Of the remaining animals, seven had F_L below 50 kHz [Fig. 2(d)], two demonstrated aberrant audiograms, and two were considered to have profound hearing loss across the range of frequencies tested. The high frequency roll-off in sensitivity generally occurred across less than one octave.

In general, younger dolphins had a better range of hear-

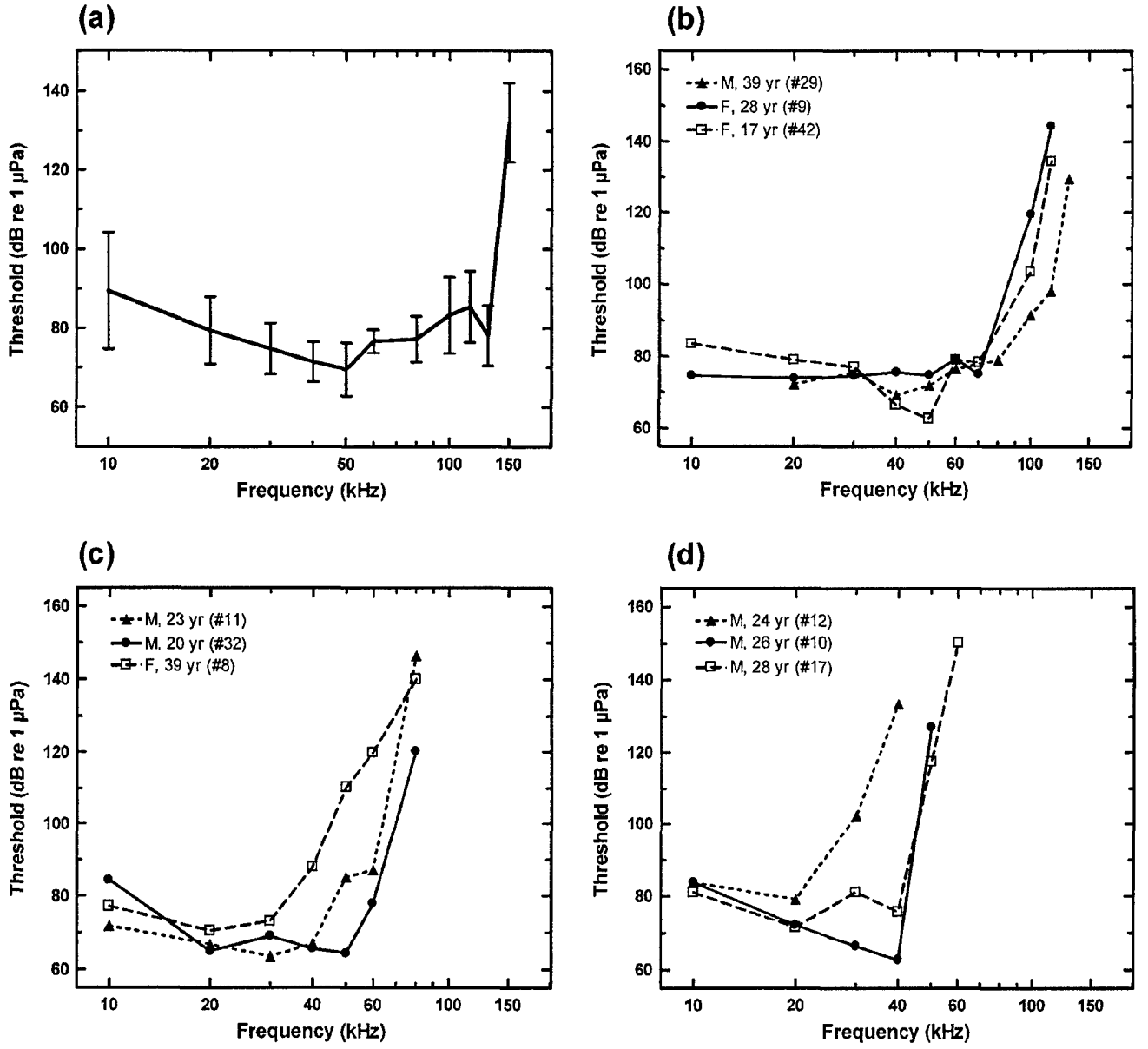

FIG. 2. (a) Mean base line audiogram (n=9). Symbols represent mean thresholds. Error bars indicate ±1 standard deviation. The other panels are representative audiograms for dolphins with upper cutoff frequencies between (b) 100 and 140, (c) 50 and 100, and (d) below 50 kHz. The values presented in the legend correspond to the sex of the animal (M or F), the age of the animal, and the ID number of the animal in Table I (the last value in parentheses).

ing than older dolphins and less variability in mean thresholds than older animals (Fig. 3 and Table II). As animals increased in age there was an overall trend for a reduction in sensitivity at progressively higher frequencies. The F_L generally appear to decline between the ages of 20 and 30 yr, although some animals older than 30 yr had a frequency range of hearing in excess of 120 kHz, and some animals younger than 20 yr showed hearing loss (Fig. 4). ANCOVA utilizing sex as a fixed factor and age as the covariate showed a significant impact of sex on the relationship between age and F_L (Table III). For a covariate mean of 24.3 yr, significant differences existed between the mean F_L of females (113 kHz) and males (81 kHz).

The aberrant audiograms of two male dolphins were characterized by notches below F_L (subjects are identified in Table I with the superscript "b"). The notches are reductions in sensitivity that occur between higher and lower frequencies to which the animal is more sensitive. Figure 5 depicts the aberrant audiogram of a 41 yr male (circles) characterized by a notch in hearing sensitivity at 20 and 30 kHz. The audiogram of this male shows a similar pattern of hearing sensitivity across the range of hearing to that of his male offspring (filled triangles), 15 years younger. The upper cutoff frequencies for the two animals differ by ~10 kHz.

Animals with profound hearing loss produced no detectable EFR across a broad range of frequencies (Fig. 6). For both of these animals, a 41 yr female and a 26 yr male, all detected evoked responses were in excess of 130 dB re 1 μPa. The inability to detect evoked responses always occurred at test frequencies below 50 kHz. These subjects are identified in Table I by the superscript "c".

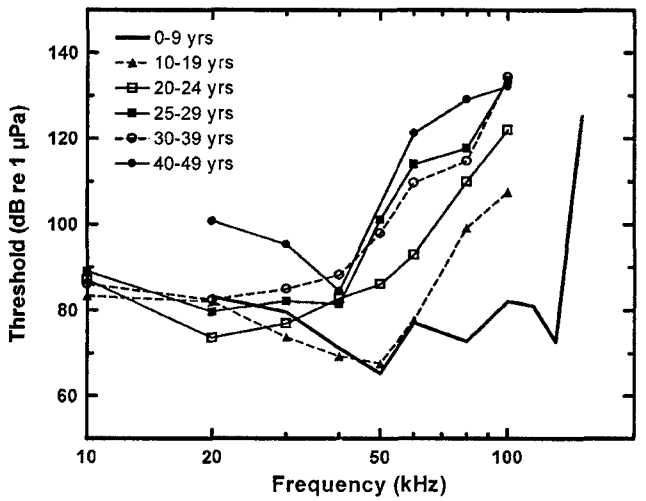

FIG. 3. Mean audiograms for bottlenose dolphins grouped by age. Mean threshold for a given frequency was not plotted if there were fewer than four thresholds obtained at that frequency. Except for the 0–9 yr age group, plots are terminated at 100 kHz; there were a number of individuals for which data above 100 kHz was not obtained because $F_L < 100$ kHz (see Table II). The plot for the 0–9 yr age group is extended so that the reduction in high frequency hearing for animals with a full range of hearing can be viewed for comparison.

IV. DISCUSSION

The variability in hearing sensitivity of a population of bottlenose dolphins observed in the present study follows patterns that are similar to those observed in human populations (Corso, 1959; Northern and Downs, 1971; United States Department of Health and Human Services, 2005) and animal models (Hunter and Willott, 1987; Boettcher, 2002); primarily, both sex and progression in age collectively contribute to reductions in the hearing range of bottlenose dolphins and the loss of hearing generally spreads from the higher frequencies to the lower frequencies. Most of the dolphins in the studied population showed reductions in the range of hearing between the ages of 20 and 30 yr, with the magnitude of the reduction being generally larger in males. Presbycusis in human and animal populations has long been documented (e.g., Corso, 1959), and this process is augmented by the deterioration of the hearing apparatus culminating from noise exposure throughout one's life (Macrae, 1971, 1991a, 1991b; Mills *et al.*, 1997). It seems reasonable that dolphins, having an inherently mammalian inner ear, would suffer from similar phenomena. Additionally, that

TABLE II. Mean threshold, standard deviation, and number of samples (thresholds for individual ears) for bottlenose dolphins grouped by age categories.

		Age (yr)						
Frequency (kHz)		0–9	10–19	20–24	25–29	30–39	40–49	All ages
10	Mean	96.4	83.3	87.0	89.0	86.0	84.3	86.7
	SD	3.6	5.8	15.3	14.9	10.7	9.1	12.6
	n	2	12	23	13	8	3	61
20	Mean	83.1	82.0	73.6	79.7	82.4	100.7	79.7
	SD	5.4	8.3	10.7	15.6	17.1	34.0	15.2
	n	4	12	25	16	9	4	70
30	Mean	79.6	73.7	76.9	82.1	84.9	95.3	79.9
	SD	5.2	10.2	12.5	21.3	15.5	34.1	16.7
	n	4	11	26	17	10	4	72
40	Mean	71.2	69.3	82.8	81.5	88.2	84.5	81.1
	SD	3.8	8.4	22.6	20.4	18.1	20.3	19.7
	n	4	9	29	16	10	5	73
50	Mean	65.3	67.6	86.1	101.1	97.9	97.5	87.3
	SD	5.4	7.7	27.9	28.9	21.4	16.5	26.4
	n	4	12	29	16	10	3	74
60	Mean	77.1	77.7	92.8	113.9	109.6	121.2	98.9
	SD	2.3	5.1	26.5	33.2	27.6	41.1	29.9
	n	4	11	23	16	10	5	69
80	Mean	72.8	99.1	109.9	117.7	114.7	129.1	109.2
	SD	5.0	29.8	31.6	37.5	29.5	30.8	32.5
	n	4	12	23	12	9	5	65
100	Mean	82.1	107.4	122.0	133.7	134.3	132.1	121.7
	SD	11.3	31.3	30.8	28.3	25.3	44.1	32.2
	n	4	11	19	12	9	5	60
115	Mean	81.0	103.0	98.2	137.3	114.1	83.7	107.4
	SD	15.1	23.0	19.0	25.2	27.8	3.5	27.8
	n	4	8	11	9	3	2	37
130	Mean	72.7	103.6	95.1	107.1	129.5	136.8	102.7
	SD	1.3	28.0	26.1	43.0	2.1	2.5	29.8
	n	4	8	8	4	2	3	29
150	Mean	125.3	130.1	134.2	134.1	135.7	140.4	132.6
	SD	4.8	11.4	7.6	22.4	···	14.1	11.5
	n	3	7	10	4	1	2	27

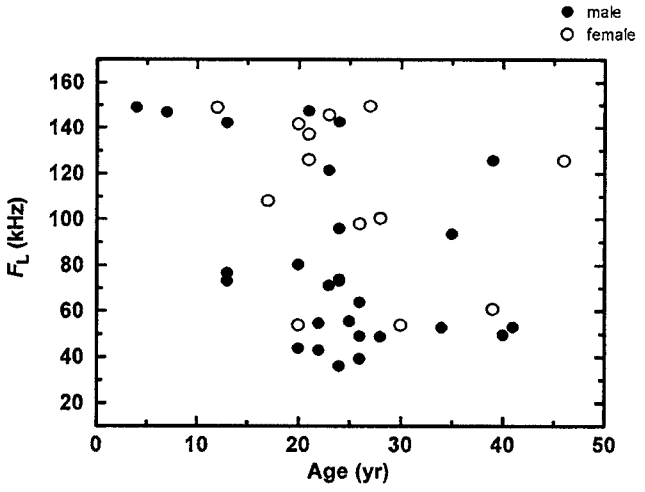

FIG. 4. Upper frequency limit of hearing (F_L), defined as the frequency at which the threshold equaled 120 dB re 1 μPa, as a function of animal age and sex.

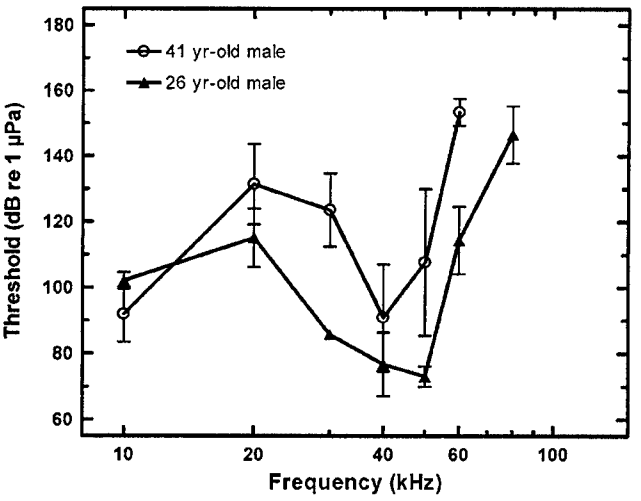

FIG. 5. Audiograms of a 41 yr-old male dolphin (No. 7 in Table I) and his 26 yr-old male offspring (No. 30 in Table I) showing unusual patterns of hearing loss. Threshold values are means of the left and right ears ±1 standard deviation (denoted by the error bars).

males tend to lose their higher frequency hearing at an earlier age than females suggests that similar physiological or behavioral factors contributing to earlier hearing loss in human males may also exist in dolphin populations.

The impact of aging on the ability of dolphins to utilize echolocation remains relatively unexplored. Dolphins have been noted to preferentially emit echolocation clicks in which the spectral content is dominated by frequencies to which they are sensitive (Houser *et al.*, 1999, 2005). This suggests active compensation for hearing deficits and the practice may be common in dolphins with age-related hearing loss. However, the interpretation of echoes received from ensonified targets may additionally be impacted by reductions in temporal sensitivity. Age-related temporal and spectral pattern processing deficits in humans are believed to interact with sensorineural hearing loss in the reduction of speech intelligibility (Fitzgibbons and Gordon-Salant, 2001, 2004; Divenyi *et al.*, 2005). This process is confounded by reverberation (Duquesnoy and Plomp, 1980), a common trait of the environments that bottlenose dolphins typically inhabit. The temporal processing capability of the dolphin has been studied by both psychophysical and physiological means (e.g., Johnson *et al.*, 1988; Dolphin *et al.*, 1995; Supin and Popov, 1995; Popov and Supin, 1997; Helweg *et al.*, 2003). Future studies, similarly designed but utilizing differently aged dolphins could be conducted to determine whether age impacts temporal or spectral pattern processing in the dolphin.

Humans, even under low noise exposure conditions, demonstrate a sex bias in the rate of hearing loss (Pearson *et al.*, 1994). The loss of high frequency hearing in this dolphin population follows a similar trend; however, noise exposure histories do not exist for the animals in this study so differences in noise exposure histories cannot be excluded from contributing to the variability in male-female comparisons. Reasons for the sex bias in human populations remain to be definitively determined, but onset of the phenomenon in males has been identified at ages as young as the late 20s and early 30s (Corso, 1959; Pearson *et al.*, 1994). This age range is similar to that observed for the appearance of reduced F_L in male dolphins and it seems reasonable to conclude that the onset of hearing loss in male dolphins tends to occur after sexually maturity.

Many of the audiograms for the tested animals had best sensitivities that were 10–20 dB less sensitive than those previously recorded by Johnson (1966). Differences in test-

TABLE III. Results of the ANCOVA performed on F_L utilizing sex as a fixed factor and age as a covariate.

	Df	MS	F	p
Intercept	1	82796.29	67.13	<0.001
Age	1	7940.03	6.43	0.016
Sex	1	9629.84	7.51	0.009
Error	37	1233.20		

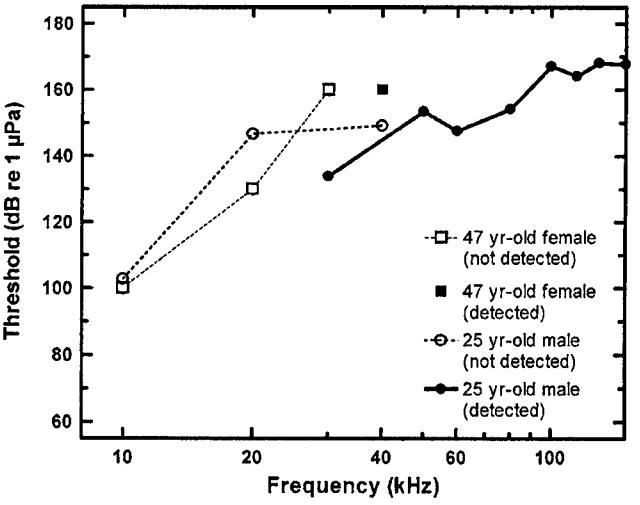

FIG. 6. Thresholds for two dolphins [a 47 yr-old female (No. 26 in Table I) and 26 yr-old male (No. 18 in Table I)] having significant high-frequency hearing loss. Filled symbols represent measured thresholds; open symbols indicate maximum SPLs for which no response was detected (the actual threshold would be higher). Note that thresholds are high for all frequencies at which EFRs were detected.

ing methodologies could explain the differences in threshold, since Johnson used a behavioral paradigm as opposed to the physiological measurements used here. The in-air method of estimating underwater hearing thresholds, using auditory evoked potentials (AEP) recordings obtained when a jawphone is used as the sound source, has been benchmarked against behavioral audiometry conducted within the same subjects, underwater, and in direct sound fields (Finneran and Houser, 2006). Considering behavioral audiometry conducted by Finneran and Houser (2006) on an animal in a pool, which is a closer approximation to the Johnson (1966) setup than the masked behavioral thresholds obtained by Finneran and Houser (2006) from animals housed in SD Bay, AEP threshold estimates are expected to be 0–18 dB higher than behavioral thresholds (with a mean of +8 dB). Thus, differences between the AEP threshold estimates presented here and the behavioral thresholds obtained by Johnson (1966) are near the expected range of differences for the test methodology.

The dolphin population studied here is maintained in netted enclosures within SD Bay. These animals are exposed not only to noise generated by the natural environment (dominated by snapping shrimp and conspecifics), but also to varying amounts of shipping, boating, and construction noise. Mean ambient noise spectral density levels within San Diego Bay range from 67 to 87 dB re: 1 $\mu Pa^2/Hz$ across the range of 0.1–50 kHz (Finneran *et al.*, 2005a). In future measurements of dolphin hearing sensitivity, it would be worthwhile to assess the ambient noise levels of the environment in which the animals lived. By this means, potential relationships between chronic noise exposure and variation in hearing sensitivity might be determined.

Some drug therapies may pose risks of ototoxicity, with the risk being generally related to the total dose administered and the rate of administration. Of particular concern are the aminoglycoside antibiotics which have the potential to produce irreversible hearing loss in mammals. Indeed, the aminoglycoside amikacin has not only been implicated in the loss of hearing in humans, but in odontocetes as well (Finneran *et al.*, 2005b). Some of the dolphins tested in this study, and that had notable hearing loss, had prior amikacin treatments for ailments in which gram-negative bacterial infections were suspected. Gentamicin is also of concern as the 25-yr old male dolphin determined to have profound hearing loss in this study (Fig. 6; No. 18 in Table I) received 1.0 g of gentamicin via combined intravenous and intramuscular injections several years prior to the determination of his hearing loss. Whether gentamicin or amikacin were contributing factors in the loss of hearing for any of these dolphins remains uncertain. The dosages administered were conservative in that exposure to the antibiotics was acute. Prolonged exposures are more likely to result in hair cell damage (Begg and Barclay, 1995; Aran *et al.*, 1999), as was observed in the case of a beluga treated for *Nocardia* spp. infection (Finneran *et al.*, 2005b). However, because of the documented deleterious effects of gentamicin and amikacin on human and lab animal hearing (Kahlmeter and Dahlager, 1984), and because there has never been a controlled study of the effects of any aminoglycoside on sensorineural hearing loss in a cetacean, the possibility that exposure to aminoglycoside antibiotics might contribute to hearing loss in delphinids deserves consideration. Additional data mining of the veterinary records of dolphins for which hearing sensitivity is known (such as might be obtained from marine parks and aquaria or during the rehabilitation of stranded marine mammals) will be useful in determining the impact of aminoglycoside antibiotics on the hearing sensitivity of odontocetes.

The similarity in the audiograms of the father and son pairing, and the atypical shape of the audiograms relative to the rest of the population, is suggestive of a familial link in progressive hearing loss. Genetic predispositions for hearing loss and auditory system abnormalities are documented in humans and laboratory animals (Brown, 1973; Erway *et al.*, 1993) and it seems reasonable that genetic causes of hearing loss would also exist in marine mammals. Whether the animals that were found with profound hearing loss had a genetic predisposition for the condition is unknown.

V. CONCLUSIONS

(1) Evoked potential audiometry was used to assess the hearing sensitivity of a population of bottlenose dolphins.

(2) Bottlenose dolphins experience progressive hearing loss with age (presbycusis), with the higher frequencies being affected first and males losing their hearing before females, as in humans.

(3) There may be genetic factors in an individual dolphin's susceptibility to hearing loss.

(4) The potential for certain antibiotics to contribute to cetacean hearing loss deserves continued investigation.

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