RESEARCH ARTICLE

Aerial audiograms of several California sea lions (*Zalophus californianus*) and Steller sea lions (*Eumetopias jubatus*) measured using single and multiple simultaneous auditory steady-state response methods

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SUMMARY

Measurements of the electrophysiological auditory steady-state response (ASSR) have proven to be efficient for evaluating hearing sensitivity in odontocete cetaceans. In an effort to expand these methods to pinnipeds, ASSRs elicited by single and multiple simultaneous tones were used to measure aerial hearing thresholds in several California sea lions (*Zalophus californianus*) and Steller sea lions (*Eumetopias jubatus*). There were no significant differences between thresholds measured using the single and multiple ASSR methods, despite the more rapid nature of data collection using the multiple ASSR method. There was a high degree of variability in ASSR thresholds among subjects; thresholds covered a range of ~40 dB at each tested frequency. As expected, ASSR thresholds were elevated relative to previously reported psychophysical thresholds for California and Steller sea lions. The features of high-frequency hearing limit and relative sensitivity of most ASSR audiograms were, however, similar to those of psychophysical audiograms, suggesting that ASSR methods can be used to improve understanding of hearing demographics in sea lions, especially with respect to high-frequency hearing. Thresholds for one Steller sea lion were substantially elevated relative to all other subjects, demonstrating that ASSR methods can be used to detect hearing loss in sea lions.

Key words: pinniped, sea lion, hearing, audiogram, evoked potential, auditory steady-state response.

INTRODUCTION

Aerial hearing data for otariid pinnipeds (sea lions and fur seals) have demonstrated that these species are sensitive to aerial sound across a wide range of frequencies (Schusterman, 1974; Moore and Schusterman, 1987; Babushina et al., 1991; Kastak and Schusterman, 1998; Mulsow and Reichmuth, 2010), with absolute sensitivities similar to those of terrestrial carnivores (Fay, 1988). These findings are consistent with the importance of aerial vocalizations in otariid behaviors such as the maintenance of breeding territories (Peterson and Bartholomew, 1969; Fernandez-Juricic et al., 2001; Gwilliam et al., 2008) and the mutual recognition of mothers and pups (Trillmich, 1981; Gisiner and Schusterman, 1991; Insley et al., 2003). Although the psychophysical methods that have produced most of these aerial hearing data are considered the 'gold standard' of measuring auditory perception, they require extensive training of subjects in captive environments. This process is often time consuming, expensive and restricted in terms of the species that are suitable for housing in captive research environments. As a result, despite over 35 years of scientific interest in the amphibious hearing capabilities of otariids, there are little available data aside from those obtained from the few individuals that have acted as subjects for each study.

Recent reports concerning the effects of noise on marine mammals have highlighted the need for population-level hearing information in sea lions and other pinnipeds (National Research Council, 2003; National Research Council, 2005; Southall et al., 2007). To increase the number of species and individuals for which data are available, these reports have recommended the use of auditory evoked potential (AEP) methods, which measure the minute voltages generated by the auditory nervous system in response to sound. AEP methods do not require the active participation of a subject, and are therefore being increasingly used as a supplement to psychophysical methods (Supin et al., 2001; Mann et al., 2005; Nachtigall et al., 2007; Houser et al., 2008a; Mulsow and Reichmuth, 2010).

In contrast to AEP studies with odontocete cetaceans (dolphins and porpoises), relatively few studies have used AEPs to examine hearing in pinnipeds. The first AEP studies with pinnipeds used intracranial electrodes to record directly from structures in the auditory nervous system (Bullock et al., 1971; Ridgway and Joyce, 1975). More recent studies have used subdermal electrodes to record far-field AEPs in chemically immobilized or anesthetized subjects, and have demonstrated that non-invasive AEP measurements can provide a good estimate of aerial hearing sensitivity in harbor seals (*Phoca vitulina*) (Wolski et al., 2003) and Steller sea lions (*Eumetopias jubatus*) (Mulsow and Reichmuth, 2010). AEP methods therefore seem to be an efficient means of providing data that complement existing pinniped psychophysical hearing data.

Hearing sensitivity measurements obtained using the auditory steady-state response (ASSR), an AEP that is phase-locked to a modulation rate imposed on a sound, have proven to be particularly useful in acquiring data from odontocete cetaceans in wild and captive settings (Cook et al., 2004; Nachtigall et al., 2005; Nachtigall et al., 2008; Popov et al., 2007; Houser et al., 2008b). The phase-locked nature of the ASSR is advantageous in that it allows for objective statistical evaluation of the hearing threshold. As opposed to traditional visual methods of detecting the presence of an AEP in the time-domain electrophysiological waveform, an ASSR can be detected as a statistically significant peak in the spectrum of the electrophysiological record at a frequency that corresponds to the stimulus modulation rate (Kuwada et al., 1986; Lins et al., 1995; Dobie and Wilson, 1996). The level of a frequency-specific stimulus can be progressively attenuated until a level of no ASSR is reached, allowing for estimation of a subject's behavioral hearing threshold.

Although ASSR audiometry was originally conducted by presenting one test frequency at a time, more recent studies have shown that the simultaneous presentation of multiple sinusoidally amplitude-modulated (SAM) tones with unique center and modulation frequencies elicits ASSRs that are phase-locked to each unique modulation rate (Lins et al., 1995; John et al., 1998). The use of multiple simultaneous frequencies allows the hearing threshold at each frequency to be assessed simultaneously. The multiple ASSR method has been shown to produce hearing sensitivity measurements comparable to those of the single ASSR method in both humans (Lins and Picton, 1995; John et al., 1998; John et al., 2002b) and dolphins (Finneran and Houser, 2007; Finneran et al., 2008). This procedure leads to a reduced testing time relative to the single ASSR method, and makes it a desirable choice in situations where testing time is limited, such as pinniped anesthetic procedures (Haulena and Heath, 2001). Although close spacing of frequency components in multiple ASSR procedures can lead to interactions that confound threshold estimation, one-octave spacing of test frequencies appears to be sufficient for threshold measurements with humans and dolphins (Lins and Picton, 1995; John et al., 1998; Branstetter et al., 2008).

This study compared the use of single and multiple ASSR methods in measuring aerial hearing thresholds for several California sea lions, *Zalophus californianus* (Lesson 1828), and Steller sea lions, *Eumetopias jubatus* Schreber 1776. Thresholds measured using both methods were compared at matched frequencies with one-octave spacing. Prior to threshold testing, a function describing ASSR amplitude as a function of modulation rate, called the modulation rate transfer function (MRTF), was obtained. This function described the ability of the sea lion auditory system to temporally resolve amplitude fluctuations imposed on a pure-tone stimulus, and guided the choice of the stimulus modulation rates used for subsequent threshold testing. The results of this investigation augment existing psychophysical data regarding otariid aerial hearing sensitivity, and represent a step in the refinement of AEP methods for measuring pinniped hearing.

MATERIALS AND METHODS
Subjects and testing environments

The subjects of the study were seven California sea lions and five Steller sea lions. The California sea lions (Table 1) were housed at The Marine Mammal Center (TMMC) in Sausalito, CA, USA, following stranding, and were scheduled for necessary euthanasia because of domoic acid toxicosis. Although domoic acid has neurotoxic effects at the level of the limbic system, it does not appear to damage the brainstem (Silvagni et al., 2005), the region of the brain

that generates the ASSRs recorded in the present study. Testing was conducted between 5 August 2007 and 31 October 2008. The Steller sea lions (Table 2) were permanently housed at Vancouver Aquarium (VANAQ) in Vancouver, British Columbia, Canada. They were tested between 17 July 2007 and 21 November 2008 during scheduled research procedures, and were revived following testing. All of the sea lions were kept under gas anesthesia with isoflurane during evoked potential recordings. In some cases, the California sea lions were administered one (or a combination) of the following drugs prior to receiving isoflurane anesthesia: butorphanol, midazolam, medetomidine or atropine. As the ASSRs that were recorded in this study arise from activity in the brainstem, they are therefore generally resistant to the effects of these drugs (Hall, 2007; Reichmuth et al., 2007). While under anesthesia, the subjects were continuously monitored by an attending veterinarian or veterinary technician.

Testing of sea lions took place in a surgical room at TMMC and a dry workspace at VANAQ; thus, the environments were not specially constructed for sound attenuation. Ambient noise levels were measured in each environment with a sound level meter (Type 2250, Brüel & Kjær, Nærum, Denmark), using a 1 min unweighted recording and 1/3-octave band analysis, in a configuration that approximated the conditions during the study (Fig. 1). Intermittent sound generated by compression of the ventilators used to deliver gas anesthesia caused the noise level probability distributions at most frequencies to be positively skewed [i.e. the majority of actual noise levels during testing were generally less than the 1 min L_{eq} (the equivalent continuous sound pressure level)]. The ambient noise levels at the subjects' ears were in fact lower than those shown in Fig. 1, as the headphones used during testing (see below) attenuated ambient noise by 3–17 dB at test frequencies. Although noise levels
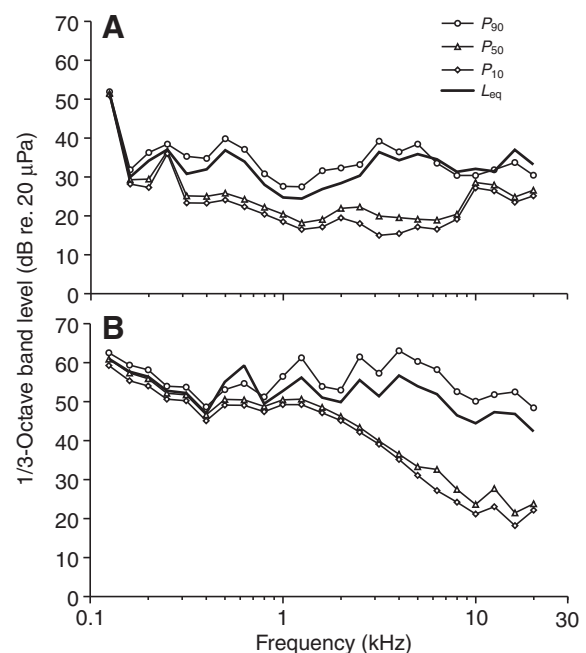

Fig. 1. Ambient noise in (A) the procedure room at The Marine Mammal Center and (B) the procedure room at Vancouver Aquarium. The 10th, 50th and 90th percentiles (P_{10}, P_{50} and P_{90}, respectively) of the noise distribution are shown in addition to the equivalent continuous sound pressure level (L_{eq}). Note the positive skew of the noise level distributions due to the intermittent activity of the anesthesia ventilators. Actual noise levels received by the subjects were lower because of attenuation by the earmuffs placed over subjects' headphones.

Table 1. Subject parameters for all tested California sea lions

ID	Sex	Mass (kg)	Length (cm)	Age	Procedure	Modulation rate (Hz)				
						2 kHz	4 kHz	8 kHz	16 kHz	32 kHz
SWA	M	80	188	S	MRTF	–	–	–	–	–
DON	F	40	130	J	MRTF	–	–	–	–	–
GUY	M	20	112	J	ASSR+MASSR	200 (200)	200 (224)	200 (176)	200 (152)	200 (128)
HHH	F	71	174	A	ASSR+MASSR	200 (200)	200 (224)	200 (176)	200 (152)	200 (128)
DAN	F	62	162	A	ASSR+MASSR	200 (200)	200 (224)	200 (176)	200 (152)	200
LAP	F	83	171	A	ASSR+MASSR	200 (200)	200 (224)	200 (176)	200 (152)	200
NIM	M	68	169	S	ASSR+MASSR	200 (200)	200 (224)	200 (176)	200 (152)	200
BEW	F	55	142	S	ASSR+MASSR	200 (200)	200 (224)	200 (176)	200 (152)	200
MBI	F	22	115	J	ASSR+MASSR	200 (200)	200 (224)	200 (176)	200 (152)	200
KIM	F	39	132	S	MASSR ×5	(200)	(224)	(176)	(152)	–
DAW	F	78	160	A	MASSR ×5	(200)	(224)	(176)	(152)	–
BUS	F	62	160	S	MASSR ×5	(200)	(224)	(176)	(152)	–

Subjects are listed in the order in which they were tested. Age classes of the subjects are given as: J, juvenile; S, sub-adult; A, adult. MRTF, all subjects in the modulation rate transfer function study; ASSR+MASSR, all subjects for which one single ASSR and one multiple ASSR audiogram were obtained; MASSR ×5, subjects for which five repeated multiple ASSR audiograms were acquired. Modulation rates for SAM tones are given where appropriate. The values without parentheses indicate the rate used for single ASSR measurements and the values in parentheses indicate the rates used for multiple ASSR measurements.

above 20 kHz were not measured because of limitations of the sound level meter, they were likely comparable to those near 20 kHz.

Signal calibration

Signals were presented diotically to headphones (TDH-39, Telephonics Corporation, Farmingdale, NY, USA) fitted with sound attenuating earmuffs that fit over a subject's ears without altering the natural orientation of the pinnae. A probe microphone (0.25–14 kHz ±3 dB, ER-7C, Etymotic Research, Elk Grove Village, IL, USA) was placed underneath a headphone near the meeting of the pinna and the scalp. Signals from the microphone were first low-pass filtered (–3 dB at 200 kHz, 3C series, Krohn-Hite Corporation, Brockton, MA, USA) to prevent aliasing, followed by analog-to-digital conversion at 500 kHz by a data acquisition (DAQ) card (USB-6251, National Instruments Corporation, Austin, TX, USA) and subsequent averaging using the Evoked Response Study Tool software (EVREST) (Finneran, 2008; Finneran, 2009). Calibration levels for the each of the frequencies were based on the mean r.m.s. acoustic pressures [sound pressure level (SPL)] measured during electrophysiological testing of California sea lion subjects conducted from 2007 to 2008, including the subjects of this experiment. The received levels were corrected based on the frequency response of the ER-7C probe microphone. The mean SPLs from these measurements were used as calibration values in order to reduce variability caused by small changes in the placement of the probe microphone under the headphones. The transmitting

voltage responses of the two headphones were within 3 dB at all test frequencies except 16 kHz, where there was a 15 dB difference. The large difference at 16 kHz likely had a small effect on the measured thresholds at this frequency (see Discussion). For all frequencies, the higher of the two SPLs coming from the two headphones was used as the calibration value.

Determination of optimal modulation rates

MRTF measurements were made with two California sea lions, identified as SWA and DON (Table 1), and a single Steller sea lion identified as ROG (Table 2). The center frequency and SPL of the SAM tones were held constant while the modulation rate was systematically varied. The tones had a center frequency of 10 kHz, a modulation depth of 100%, an SPL of 86 dB re. 20 µPa (dB SPL hereafter) and a duration of 60 ms. Modulation rates for the California sea lions varied from 133 to 233 Hz, in 33 Hz intervals, with additional testing at 150 and 250 Hz for SWA. This limited range of modulation rates was chosen to coincide with the range previously shown to produce high-amplitude ASSRs in California sea lions (Mulsow and Reichmuth, 2007a; Mulsow and Reichmuth, 2007b). Because MRTF measurements had not yet been reported for Steller sea lions, the range of modulation rates was more extensive, ranging from 100 to 400 Hz in 33 Hz intervals and 400 to 800 Hz in 50 Hz intervals, with additional testing at 50, 150, 250 and 350 Hz. The tones were presented at a rate of ~7 stimuli s^{-1}, with the polarities alternated on successive presentations to reduce

Table 2. Subject parameters for all tested Steller sea lions

ID	Sex	Mass (kg)	Length (cm)	Age (years)	Procedure	Modulation rate (Hz)								
						1 kHz	2 kHz	4 kHz	5 kHz	8 kHz	10 kHz	16 kHz	20 kHz	32 kHz
EDE	F	150	211	7	ASSR	153.9	153.9	–	153.9	153.9	153.9	–	153.9	–
ROG	F	140	195	5	MASSR, MRTF	(200)	(200)	(128)	(128)	(176)	(176)	(152)	(152)	152
IZZ	F	143	206	5	MASSR	(200)	(200)	(128)	(128)	(176)	(176)	(152)	(152)	152
ASH	F	147	204	5	MASSR	(216)	(216)	(144)	(144)	(192)	(192)	(168)	(168)	168
WILL	F	136	197	5	MASSR	(216)	(216)	(144)	(144)	(192)	(192)	(168)	(168)	152

Subjects are listed in the order in which they were tested. MRTF, the subject included in the modulation rate transfer function study; ASSR, the subject for which all thresholds were determined using single ASSR methods; MASSR, subjects for which multiple ASSR audiograms were used to obtain thresholds (except at 32 kHz, see Materials and methods). Modulation rates for SAM tones are given where appropriate. The values without parentheses indicate the rate used for single ASSR measurements and the values in parentheses indicate the rates used for multiple ASSR measurements.

stimulus artifacts in the electrophysiological records. The tones were generated at an update rate of 0.5 MHz using EVREST and underwent digital-to-analog conversion using the USB-6251 DAQ card. The tones were then passed through a Krohn-Hite 3C series low-pass filter (–3 dB at 150 kHz), a custom attenuator (0–70 dB) and the TDH-39 headphones.

Electrophysiological signals were recorded using a three-electrode montage comprising 12 mm×30 gauge stainless steel subdermal electrodes (F-E3M-72, Grass Technologies, West Warwick, RI, USA). An active (non-inverting) electrode was placed on the dorsal midline of the head, midway between the ears. The reference (inverting) and ground electrodes were placed on the animal's dorsal side, just posterior to the ribcage (SWA, DON) or just above the flipper (ROG). Signals were amplified (100 dB) and band-pass filtered (SWA, DON: 0.03 to 1 kHz; ROG: 0.03 to 3 kHz) using a biopotential amplifier (IP511, Grass Technologies), and then digitized with the USB-6251 DAQ card at a rate of 5.6 kHz. Signals were acquired in discrete 60 ms epochs, synchronized with the stimulus onset and averaged to reduce electrical background noise. Final ASSR waveforms were generated from an average of 2000 (SWA, DON) or 1000 (ROG) epochs. Epochs with peak amplitude greater than 50 μV (SWA, DON) or 35 μV (ROG) were considered to contain spurious electrical artifacts and were excluded from averaging. A fast Fourier transform (FFT) was conducted on the 60 ms ASSR, resulting in a 16.7 Hz frequency bin resolution. This frequency resolution ensured that each of the modulation rates was centered on a frequency bin in the spectra. The amplitude of the ASSR at the stimulus modulation rate was plotted as a function of modulation rate to visualize the MRTF.

ASSR audiometry
California sea lions

ASSR procedures were conducted with seven California sea lions. Thresholds were determined for SAM tones with center frequencies of 2, 4, 8, 16 and 32 kHz. The tones had 100% amplitude modulation depth and were generated by EVREST software at an update rate of 1 MHz and amplitude modulated at rates that corresponded to high-amplitude ASSRs in the previously determined MRTFs (Table 1; see Results). The tones were continuous in nature for each SPL tested; thus, no 'stimulus-on' or 'stimulus-off' neural potentials were present in the ASSR recordings. Electrophysiological signals were amplified by 100 dB and band-pass filtered (0.1–1 kHz, –6 dB) using the IP511 biopotential amplifier. The EVREST software sampled and recorded 125 ms epochs at a rate of 8 kHz. Epochs with a peak voltage greater than 25 μV were excluded from averaging. An FFT of the average of the 125 ms epoch waveforms was used to transform the time-domain electrophysiological response into the frequency domain with 8 Hz frequency resolution. This resolution resulted in a frequency bin that was centered on each of the modulation rates. The software, hardware and the electrode types were identical to those in the MRTF study. The active electrode was placed on the dorsal midline, midway between the two ears, the reference was placed on the dorsal midline, at the base of the neck, and the ground was placed on the dorsal side immediately posterior to the ribcage. This configuration was used as it was found to be superior for reducing extraneous cardiac signals relative to the configuration used in the MRTF study.

For single ASSR testing, thresholds were sequentially measured at each frequency. The rate of amplitude modulation for the SAM tones was 200 Hz. Following the collection of 250 epochs, a magnitude-squared-coherence (MSC) signal-to-noise statistical test was used to determine whether an ASSR was present in the spectrum at the 200 Hz modulation rate. The MSC test created 20 subaverages from the grand average waveform, and provided a ratio of the power at 200 Hz in the grand average to the average power at 200 Hz in the subaverages (Dobie and Wilson, 1989; Dobie and Wilson, 1996). An ASSR was considered present if the MSC statistic was larger than a critical value at the level of α=0.01 (Amos and Koopmans, 1963; Brillinger, 1978). If an ASSR was not detected using the MSC test after 1000 averaged epochs, it was considered that no ASSR was present. SAM tones at each frequency were set to an initial SPL estimated to be above a subject's threshold. When an ASSR was detected using the MSC test (a hit), the SPL for the subsequent trial was attenuated by 10 dB. This continued until a statistically significant ASSR could not be detected after 1000 epochs (a miss), after which the SPL in the subsequent trial was increased by 5 dB. Thresholds were defined as the SPL corresponding to the lowest hit. To confirm that the threshold had been reached, misses at 5 and 10 dB below the lowest hit were collected at each frequency.

During multiple ASSR testing, several frequencies were tested simultaneously, with each frequency independently amplitude modulated at a rate that produced high-amplitude ASSRs in California sea lions (Table 1; see Results). To adequately resolve the spectral peaks corresponding to ASSRs, the minimum separation between modulation rates was 24 Hz. The stimulus that was delivered to the headphones comprised the sum of all independently modulated test frequencies. Thresholds at 32 kHz were found to be markedly elevated relative to other tested frequencies for the first two sea lions tested (see Results); therefore, all further multiple ASSR testing excluded 32 kHz in order to reduce the possibility of a high-amplitude 32 kHz component confounding threshold measurements at other frequencies.

The 8 Hz frequency resolution of the spectrum resulted in two frequency bins between each of the bins containing the SAM tone modulation frequencies. An MSC test using 20 subaverages was used to determine whether a statistically significant (α=0.01) response was present at each of the stimulus modulation rates after 1000 epoch waveforms were collected. The procedure was used for adjustment of test frequency SPLs following a miss or hit, and the threshold criteria were the same as those used for single ASSR testing. After threshold criteria had been met for a test frequency, the SPL of that component was then kept constant at the level of the last hit. The SPL of frequencies for which threshold criteria had not yet been met continued to be adjusted in the manner described above, until thresholds had been determined for all frequencies. All other stimulus properties and ASSR recording settings were identical to those described above for the single ASSR study.

Following the collection of ASSR thresholds, two-tailed one-sample t-tests were used to determine whether the mean differences of the multiple and single ASSR thresholds at each frequency were significantly different from zero.

To determine the degree of variability in ASSR thresholds among and within subjects, repeated multiple ASSR threshold measurements were made with three California sea lions (Table 2) using methods comparable to those described above. Five replicate audiograms with thresholds at 2, 4, 8 and 16 kHz were acquired for each subject. To avoid pseudoreplication, the headphones and the recording electrodes were removed from subjects DAW and BUS after the collection of each audiogram and were replaced before replicates. A mixed-model variance component analysis, using subject and frequency (nested within subject) as effects, was used to quantify the variance in the repeated multiple ASSR audiograms.

Steller sea lions

Four female Steller sea lions participated in ASSR audiometry using only multiple ASSR methods (Table 2). Multiple ASSR testing consisted of two sets of measurements for each subject: one in which thresholds at 2, 4, 8 and 16 kHz were obtained simultaneously, and a second in which thresholds at 1, 5, 10 and 20 kHz were obtained simultaneously (Table 2). A minimum separation of one octave between the test frequencies was used in order to minimize potential interactions between frequencies. Modulation rates were chosen based on the MRTF obtained with ROG (see Results), with a minimum separation between modulation rates of 24 Hz. Thresholds at 32 kHz were determined using the single ASSR method to prevent a high-amplitude 32 kHz component confounding threshold measurements at other frequencies. SAM tones were updated at a rate of 1 MHz and amplitude modulated at rates corresponding to relatively high-amplitude ASSRs in the Steller sea lion MRTF (Table 2; see Results). All other stimulus properties, recording settings, electrode placements and threshold determination procedures were identical to those described above for ASSR audiometry with California sea lions.

Following the observation of aberrantly low signal-to-noise ratios in the AEPs of a female Steller sea lion (EDE, Table 2), threshold data were collected at six frequencies using the single ASSR method. The hardware and software were identical to that used during the other ASSR procedures. ASSRs were amplified by 100 dB and band-pass filtered using the IP511 biopotential amplifier (0.03–1 kHz, –6 dB), recorded in epochs of 64.97 ms and sampled at a rate of 5.6 kHz by EVREST. Objective determination of threshold was conducted in the same manner as that described above, except that a miss was defined by 2000 epochs without a detectable ASSR.

RESULTS
Determination of optimal modulation rates

The MRTFs for the two California sea lions are shown in Fig. 2A. Although the absolute magnitudes of the ASSRs were markedly different for the two subjects, the functions have a similar shape, with the highest amplitude between modulation rates of 150 and 200 Hz. The MRTF for the Steller sea lion is shown in Fig. 2B. The MRTF is similar to those of the California sea lions in that the highest ASSR amplitudes are found between 100 and 200 Hz. ASSR amplitude decreased with increasing modulation rate past 150 Hz, and the fundamental response amplitude declined to 10% of its maximum amplitude between 600 and 800 Hz. This decrease was not monotonic, however, as there were peaks and valleys in the MRTF.

ASSR audiometry

Single and multiple ASSR thresholds for the seven California sea lions are shown in Fig. 3, and the mean thresholds are shown in Fig. 4. Sensitivity typically improved with increasing frequency, up to a sharp decrease in sensitivity at 32 kHz relative to other frequencies. There was substantial variability among the subjects' thresholds; a range of ~40 dB existed between the lowest and the highest thresholds at each frequency. This resulted in large standard deviations at each frequency (between 5 and 17 dB). Multiple ASSR thresholds were similar to single ASSR thresholds: 64% of multiple ASSR thresholds were within 5 dB from single ASSR thresholds, and 82% were within 10 dB (Fig. 5). Mean differences between the thresholds obtained with the two methods were not significantly different from zero. Data collection for the single ASSR audiograms took an average of ~1 h to complete. It took an average of ~20 min
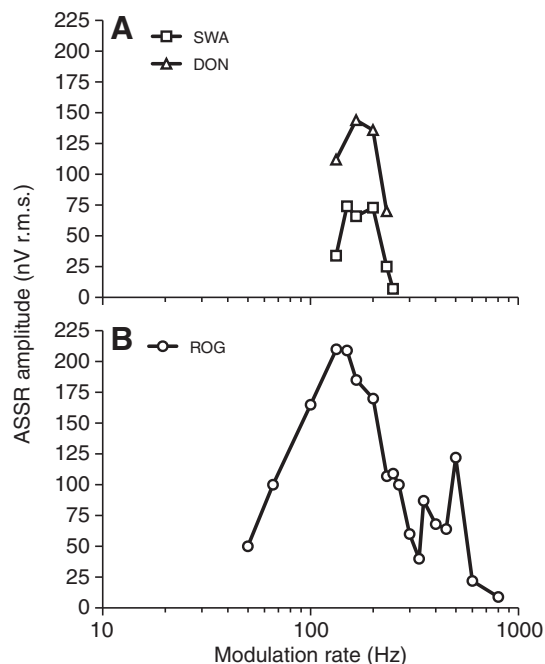

Fig. 2. Modulation rate transfer functions for (A) two California sea lions and (B) a Steller sea lion. Stimuli were 86 dB SPL sinusoidally amplitude-modulated tones with a center frequency of 10 kHz. The shape of the function is generally similar for all subjects between 100 and 200 Hz.

to measure thresholds at 2, 4, 8 and 16 kHz using the multiple ASSR method.

Despite the 15 dB difference in the SPLs of the left and right headphones at 16 kHz, thresholds at this frequency were not outliers relative to other frequencies. To further evaluate the influence of this difference, some preliminary threshold measurements in which the SPL delivered from each headphone was adjusted independently were made using a California sea lion. Thresholds at 16 kHz with the same SPL in each ear were within 5 dB of thresholds in which the SPL in each headphone differed by 15 dB (where thresholds were defined by the higher of the two SPLs). This is similar to the results of Conijn et al., who found that human auditory brainstem response thresholds for diotic stimuli were on average 5.5 dB lower than thresholds for monaural stimuli (Conijn et al., 1990).

The mean thresholds and standard deviations for the replicate audiograms collected with California sea lions KIM, DAW and BUS are shown in Fig. 6. There was relatively low intrasubject variability, with standard deviations of less than 10 dB at each frequency. Variance component analysis showed that 75% of the variability was due to the effect of subject and 14% was due to the effect of frequency.

Thresholds for Steller sea lions (ROG, IZZ, ASH and WIL) are shown in Fig. 7, and mean thresholds are shown in Fig. 8. The audiograms were qualitatively similar to one another: all displayed a region of best sensitivity near 10 kHz, with reduced sensitivity at the low frequencies and a steep rise at the high frequencies. A reduction in sensitivity at 5 kHz relative to nearby frequencies was observed in each subject. Standard deviations of thresholds were between 6 and 12 dB, similar to those of the California sea lions. It took ~40 min to obtain the hearing curve for each Steller sea lion. Thresholds at 1, 2, 5 and 8 kHz with EDE were elevated by over
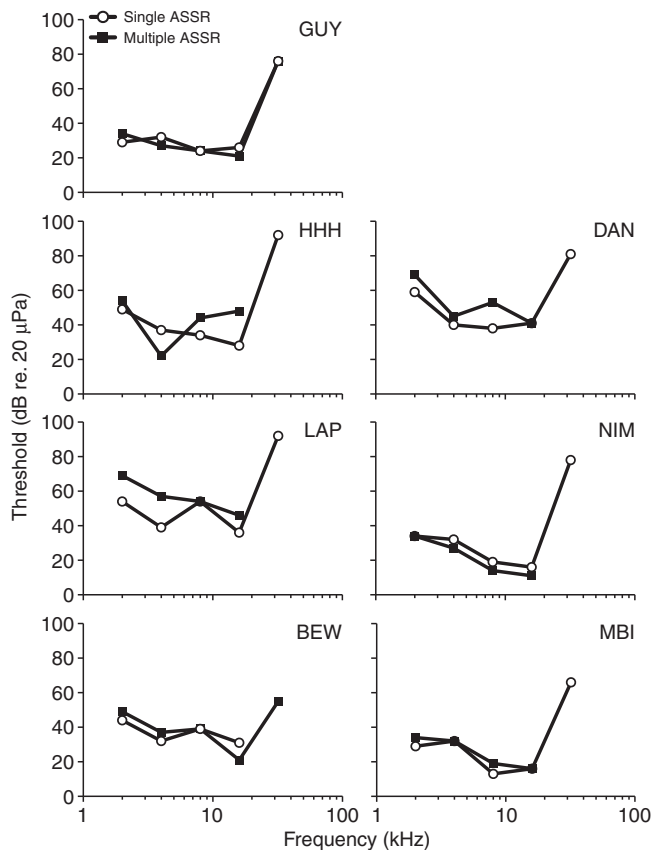
Fig. 3. Aerial hearing thresholds for seven California sea lions measured using single and multiple auditory steady-state response (ASSR) methods. The multiple ASSR method was used to obtain threshold data at 32 kHz only for GUY and HHH (see Materials and methods); however, no ASSR was detected at 32 kHz using multiple ASSR methods for HHH.

two standard deviations relative to the thresholds obtained for the four Steller sea lions tested in November 2008. A significant ASSR could not be detected at 10 or 20 kHz for EDE, even with extended and replicated testing sessions.

DISCUSSION
Determination of optimal modulation rates

The shapes of the MRTFs for both of the California sea lions tested are similar, and they are comparable within the examined frequency range to those previously acquired using rhythmic click and tone-burst trains (Mulsow and Reichmuth, 2007a; Mulsow and Reichmuth, 2007b). The shape of the Steller sea lion MRTF is generally similar to that of California sea lions (Mulsow and Reichmuth, 2007a; Mulsow and Reichmuth, 2007b) (this study) in terms of the range of modulation rates that elicit the highest-amplitude ASSRs, and the highest rates for which an ASSR can be recorded. Similar temporal resolution in the Steller sea lion relative to the California sea lion is not surprising, considering the close phylogenetic relationship (see Heyning and Lento, 2002) and comparable aerial hearing capabilities in these two otariids (Schusterman, 1974; Moore and Schusterman, 1987; Mulsow and Reichmuth, 2010). These findings suggest that the SAM tone modulation rates similar to those used in this study will be an appropriate starting point for ASSR studies with other otariid species.
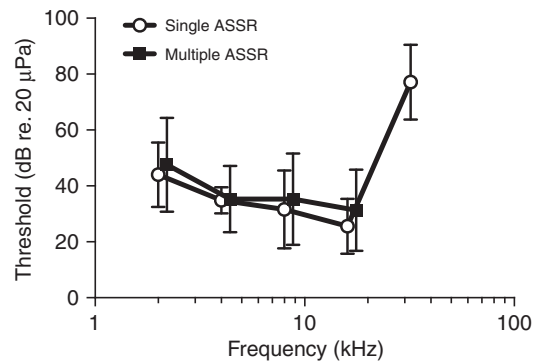

Fig. 4. Mean (±1 s.d.) single and multiple ASSR thresholds for the seven California sea lions shown in Fig. 3. Multiple ASSR thresholds are shifted slightly along the frequency axis for clarity. For 32 kHz, only the means of single ASSR measurements are shown, as multiple ASSR measurements were conducted for only two of the seven subjects (see Fig. 3 and Materials and methods).

Aerial hearing sensitivity

The ASSR audiograms for most of the California and Steller sea lions are qualitatively similar to previously reported psychophysical audiograms for these species in that sensitivity typically increases with increasing frequency up to 10 kHz, and then decreases towards a cutoff between 20 and 32 kHz (Schusterman, 1974; Moore and Schusterman, 1987; Mulsow and Reichmuth, 2010). The similarity between the high-frequency hearing limits of the California sea lions from a wild population, the Steller sea lions housed permanently at VANAQ and the otariids previously tested using psychophysical methods suggests that the high-frequency hearing limit is a robust feature of audition in both captive and free-ranging populations. However, the subjects that were available for testing during this study were limited to females and young males, and it is not yet possible to form any conclusions on how auditory sensitivity and frequency range may vary as functions of sex and age. Given the important role that hearing plays in otariid survival and reproduction (Schusterman et al., 2002), age-related hearing loss could have a significant effect on the individual fitness of these animals.

Although aerial hearing is of primary importance for detecting vocalizations related to otariid reproductive behavior, it is likely that underwater hearing is also important for detecting and localizing
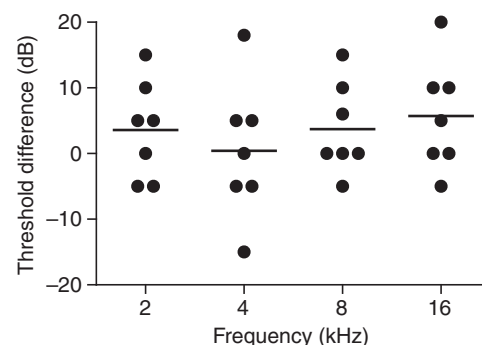

Fig. 5. Differences between multiple and single ASSR thresholds (defined as multiple ASSR threshold minus single ASSR threshold) for the seven California sea lions shown in Fig. 3. The mean difference for each frequency is indicated by a horizontal line. Mean differences were not significantly different from zero at any of the frequencies.
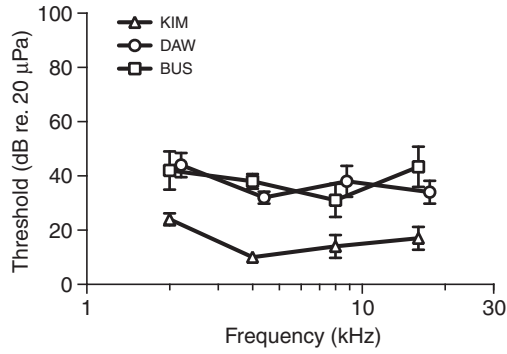
Fig. 6. Mean (±1 s.d.) multiple ASSR thresholds (N=5 threshold measurements per frequency, per subject) for three California sea lions. Data points for DAW are shifted slightly along the frequency axis for clarity.

prey and predators, and for orientating and navigating under water (Schusterman et al., 2000). Additionally, it is recent increases in underwater anthropogenic noise levels that have primarily raised concern regarding marine mammal hearing. In contrast to the phocid (true or earless) seals, whose high-frequency hearing limit is substantially higher underwater relative to in air, the high-frequency limits of aerial and underwater hearing in otariids are similar (Hemilä et al., 2006). This may allow for aerial ASSR audiometric methods to rapidly estimate the underwater frequency range of hearing in otariids.

Although the high-frequency ends of the ASSR audiograms agree well with psychophysical data from California and Steller sea lions, thresholds at the lowest frequencies are notably elevated (Moore and Schusterman, 1987; Mulsow and Reichmuth, 2010). Although the circumaural earmuffs on the headphones attenuated ambient noise, noise levels during this study were higher than those reported in psychophysical studies. Auditory masking is a potential explanation for the elevation of thresholds at the low frequencies (Southall et al., 2003); however, this effect is hard to quantify because of the spectrally and temporally complicated nature of the noise at TMMC and VANAQ. The relative elevation of the low-frequency thresholds is also potentially due to the inability of lower-frequency SAM tones to elicit synchronous in-phase firing of hair

cells in the inner ear (Picton et al., 2003). Studies with humans have demonstrated that modifications to the stimulus modulation envelope and the use of frequency-modulated stimuli can aid in overcoming this physiological limitation (Cohen et al., 1991; John et al., 2001; John et al., 2002a), and similar adjustments can be assessed in future pinniped studies.

The elevation of the Steller sea lion thresholds at 5 kHz was unexpected, and not present in the previously reported psychophysical or ASSR aerial audiograms of a young Steller sea lion (Mulsow and Reichmuth, 2010). The ASSR audiogram of that individual was obtained using the same headphones, stimulus presentation and recording hardware as those used in the present study, and very similar software setups. The audiogram did not show a similar elevation at 5 kHz, although the data were obtained in a different environment. Likewise, preliminary data obtained for IZZ in July 2007, obtained using the same headphones and a similar setup, did not display a peak in her audiogram at 5 kHz. It seems unlikely that all of the Steller sea lions tested at VANAQ possess a hearing deficit that is restricted to a narrow range in the middle of the audiogram. Additionally, the masking of 5 kHz thresholds by ambient noise does not seem probable, as noise measurements did not reveal excessive noise at this frequency. This peak is potentially an artifact of the methods employed in the study, although a plausible explanation has proven difficult to determine.

Audiogram variability

The aerial sensitivities in both species displayed a high degree of intersubject variability, although the high-frequency hearing limit was fairly consistent among subjects. The relatively low intrasubject variability in the thresholds for KIM, DAW and BUS suggest that large standard deviations observed with the other California and Steller sea lions are not a result of equipment related issues, such as differences in the placement of electrodes and headphones. Two alternative hypotheses regarding variability in the audiograms can be proposed. The first is that the subjects have hearing ranges (i.e. high-frequency hearing limits) that are nearly identical to those of previously tested otariids, but sensitivities that are relatively elevated and highly variable. A second hypothesis is that the true hearing capabilities are similar among the subjects in terms of both sensitivity and frequency range. In this case, the variability in thresholds is
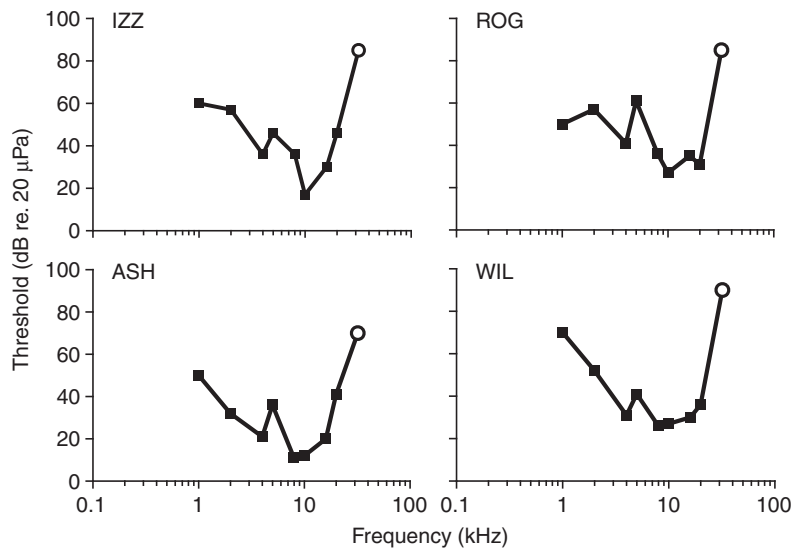

Fig. 7. Aerial hearing thresholds for four Steller sea lions measured using the multiple ASSR method (filled squares), except for data points at 32 kHz, where thresholds were measured using the single ASSR method (open circles).
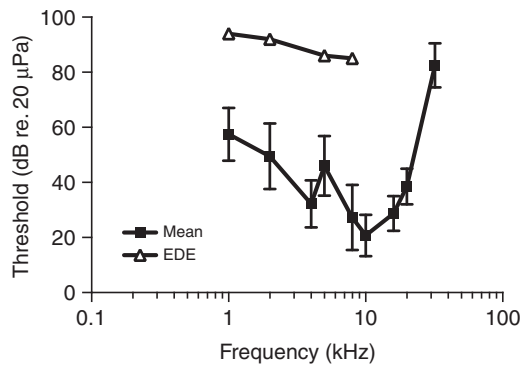
Fig. 8. A comparison of mean (±1 s.d.) aerial hearing thresholds for the four Steller sea lions shown in Fig. 7 and thresholds obtained using the single ASSR method for a fifth Steller sea lion (EDE). No significant ASSR was detected for EDE at 10 or 20 kHz using the highest possible level of 96 dB SPL.

likely due to the experimenters' ability to detect near-threshold responses using electrophysiological methods, rather than the occurrence of a hearing deficit in many subjects.

The latter hypothesis seems more plausible than the former. ASSR thresholds in normal-hearing human subjects are typically elevated relative to behavioral thresholds, and standard deviations of the difference between ASSR and behavioral thresholds are on the order of 10 to 15 dB (Picton et al., 2003). This level of variability is consistent with the results of the present study. Additionally, although there is a large degree of variability in thresholds, most of the audiograms in this study have similar shapes. Most of the variability was due to intrasubject threshold differences at each frequency, as opposed to differences in relative sensitivity across frequencies. All of the subjects appear to have a similar high-frequency hearing limit, and a uniform hearing loss across all frequencies is probably unlikely, especially considering that age-related hearing loss in otariids and other mammals is manifested at the high-frequency end of the audiogram (Schusterman et al., 2002).

In contrast to the high degree of variability observed in the ASSR thresholds, the aerial psychophysical data available for otariids show a remarkable degree of consistency among subjects and species in terms of absolute thresholds and range of hearing (Mulsow and Reichmuth, 2010). This demonstrates an important trade-off in measurements of hearing sensitivity. The reduced time frame and increased subject pool that is afforded by electrophysiological methods must be weighed against their reduced accuracy relative to psychophysical methods. With this trade-off in mind, electrophysiological and psychophysical methods should be viewed as complementary methods for obtaining hearing data in animal species, each with specific advantages and disadvantages.

Comparison of single and multiple ASSR thresholds

The lack of significant differences between single and multiple ASSR thresholds demonstrates that one-octave spacing of test frequencies is an appropriate choice for measuring hearing sensitivity in otariids. This finding is consistent with previous studies involving humans (Lins et al., 1995; John et al., 1998) and dolphins (Branstetter et al., 2008; Finneran et al., 2008) that have not found interactions using one-octave spacing.

The greatest advantage of the multiple ASSR method is the reduced time necessary required to measure thresholds at multiple

frequencies. This is of particular importance in testing the hearing of otariids, which are normally placed under anesthesia in order to provide conditions that optimize both subject comfort and high signal-to-noise ratios for ASSR recording (Reichmuth et al., 2007). The duration of otariid anesthetic procedures is generally minimized for safety reasons (Haulena and Heath, 2001), and the multiple ASSR method may provide a critical advantage in this context. This is perhaps best demonstrated by the audiograms obtained for the Steller sea lions. The anesthetic procedures allowed sufficient time to measure thresholds at 2, 4, 8 and 16 kHz, followed by testing at 1, 5, 10 and 20 kHz, and finally at 32 kHz. This procedure provided a high-resolution audiogram that was especially useful for assessing the high-frequency hearing limit.

Although recent interest in non-human ASSR procedures has primarily come from those who study marine mammal hearing, the utility of these rapid audiological methods need not be limited to this group. The most obvious extension of ASSR methods is to terrestrial carnivores, as these species possess auditory systems that are anatomically similar to those of otariids (Repenning, 1972). This application is supported by a previous electrophysiological study that found similar temporal resolution capabilities among three species of pinniped and the domestic dog (*Canis lupus familiaris*), and a recent methodological study that examined the use of ASSR methods in clinical veterinary settings with canines (Markessis et al., 2006; Mulsow and Reichmuth, 2007a). The current single and multiple ASSR methods can potentially be adapted as an efficient means of obtaining additional comparative and clinical data for terrestrial carnivores.

Assessment of hearing loss

Elevated thresholds between 1 and 8 kHz and the lack of a detectable response at the highest level of 96 dB SPL at 10 and 20 kHz strongly suggest that the hearing of EDE is compromised. As the stimuli were delivered diotically, the elevated thresholds imply that this hearing loss is binaural. Medical records indicated that EDE had not been treated with ototoxic antibiotics and had no history of excessive noise exposure. Also, EDE was similar in age (7 years old) to the other Steller sea lions that were tested (5 years old), making it unlikely that age-related hearing loss is the cause of the hearing deficit in this individual.

This finding demonstrates that ASSR measurements can be useful in detecting substantial hearing loss in otariids. ASSR testing of captive subjects can provide a rapid method of monitoring auditory health, and similar testing with free-ranging and stranded otariids can provide useful data regarding the prevalence of hearing loss in wild populations. However, further testing is necessary to determine the relationship between ASSR thresholds and the degree and nature of hearing loss in otariids, especially considering the relatively high variability in the thresholds measured during this study.

Conclusions

1. The ASSR audiograms of several California sea lions and Steller sea lions were similar to psychophysical audiograms for these species in terms of high-frequency hearing limit and relative sensitivity, although the ASSR thresholds were elevated and variable compared to psychophysical thresholds.

2. Multiple ASSR thresholds at four frequencies with one-octave spacing were not significantly different than single ASSR thresholds, despite a substantial reduction in the time required to obtain an audiogram.

3. Thresholds for one Steller sea lion were markedly elevated relative to those of other subjects. ASSR methods are likely to be a useful tool for rapidly detecting the presence of hearing loss in otariids.
4. ASSR methods can be used to improve understanding of hearing demographics in sea lions, especially with respect to high-frequency hearing.

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