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Toothed whale auditory brainstem responses measured with a non-invasive, on-animal tag

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Abstract: Empirical measurements of odontocete hearing are limited to captive individuals, constituting a fraction of species across the suborder. Data from more species could be available if such measurements were collected from unrestrained animals in the wild. This study investigated whether electrophysiological hearing data could be recorded from a trained harbor porpoise (Phocoena phocoena) using a non-invasive, animal-attached tag. The results demonstrate that auditory brainstem responses to external and self-generated stimuli can be measured from a stationary odontocete using an animal-attached recorder. With additional development, tag-based electrophysiological platforms may facilitate the collection of hearing data from freely swimming odontocetes in the wild. © 2021 Author(s). All article content, except where otherwise noted, is licensed under a Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

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1. Introduction

Hearing is a vital sensory modality in echolocating bats and toothed whales. Information gained from echolocation is derived from the reception and processing of the returning echo signal by the auditory system. Auditory research is thus fundamental to understand and interpret the biology and behavior of odontocetes and to assess potential impacts of anthropogenic noise. Empirical measurements of odontocete hearing have been collected with psychoacoustic and electrophysiological experiments. In psychoacoustic hearing tests, a subject is trained in a laboratory environment via operant conditioning to behaviorally indicate the detection of a sound. This method gives highly reliable results and is the gold-standard method in audiometry but is time consuming and hence expensive. Alternatively, electrophysiological hearing tests involve the measurement of time-averaged auditory brainstem responses (ABRs) elicited by the presentation of acoustic stimuli in the auditory nerve, brainstem, and large auditory centers of the odontocete brain that can be measured non-invasively and from the skin with electrodes attached with suction cups. While this method is less sensitive than behavioral tests, resulting in overestimation of auditory thresholds, it is also less time consuming and more economical.

A significant limitation to odontocete auditory research is the small number of species and individuals with which such research can be conducted. Both auditory study methods currently require the involvement of either subjects trained via operant conditioning, subjects that have stranded, or subjects from the wild that are temporarily restrained for opportunistic study. Thus, hearing data are published for only 20 of the approximately 76 extant odontocete species and often from only a few individuals. Most species tested are either delphinids or phocoenids, whereas few data exist on other odontocete families. More data from a larger diversity of species would be available if empirical measurements could be collected from unrestrained animals in the wild. Since psychophysical methods require extensive training in a laboratory setting, electrophysiological measurements seem the most plausible methodology to meet this goal.

In all electrophysiological hearing studies with odontocetes to date, ABR signals are conducted along wires from on-animal electrodes to data acquisition hardware located above-water, essentially tethering the subject to the equipment location and constraining its movement to the immediate surrounding area. A fundamental requirement for in situ ABR measurement from unrestrained wild odontocetes is thus untethering, or de-coupling, the subject from above-water hardware. Technological advancements have led to the use of miniaturized biologging or biotelemetry sensor tags that can be
attached to animals to collect in situ biological data from individuals in the wild. Such tags have been pivotal in recent decades to the study of cetaceans and other marine mammals since most cetacean behavior occurs underwater and out of view of human observers.\textsuperscript{13–15} Some tags have been outfitted with non-invasive, suction cup electrodes to record electrophysiological signals from wild individuals. Such studies are challenging due to increased movement-related and myogenic biopotential noise that comes from an actively moving test subject, which can mask the targeted biopotential signal, yet tag-based electrophysiological recorders have enabled collection of electrocardiograms (ECGs) and the study of diving bradycardia from freely moving captive and wild cetaceans.\textsuperscript{16–18} While ABRs are low amplitude signals compared to an ECG and thus myogenic and movement-related noise pose an increased challenge, it nonetheless seems plausible that an on-animal tag could also be used to record electrophysiological auditory data as well, which has yet to be explored. As a first step toward assessing the feasibility of making such measurements, here we investigated whether ABRs could be measured from a stationary harbor porpoise (*Phocoena phocoena*) with suction-cup-attached digital tags ("DTAGs," constructed by author M.J.) that recorded an electroencephalogram (EEG) data stream.

2. Methods

2.1 Test subject and experiment overview

Experiments were carried out on a trained captive porpoise at Fjord & Bælt in Kerteminde, Denmark. The porpoise enclosure consisted of three netted pens with a natural sandy substrate 2–4 m deep connected to a natural harbor. Experiments were conducted in the largest pen (11 × 20 m). The 25-year-old female porpoise, named Freja, has been kept at the facility since 1997 after being by-caught in a pond net. Freja has been used in previously published research studies involving both on-animal tags and electrophysiological measurements.\textsuperscript{18,19} For all experiments, Freja completed the same trained behavioral task in which she was cued to station horizontally underwater on a biteplate at a depth of 1 m for a duration of 40 s, after which she was bridged with a whistle and returned to the trainer to receive a fish reward. One experimental session consisted of seven or eight stationing trials.

When she was stationed on the biteplate, we recorded ABR data from Freja using one of three different data acquisition systems per experimental session. First, we used a DTAG3-ECG to measure ABRs that were elicited in response to external stimuli projected to the animal [Fig. 1(a)]. Second, as a comparison, we also measured ABRs using a typical tethered data acquisition system under identical experimental circumstances and using the same stimuli [Fig. 1(b)]. Third, we used a DTAG4-ECG to measure ABRs that were elicited by the porpoise’s own outgoing echolocation clicks while similarly stationing on the biteplate in the absence of externally generated stimuli [Fig. 1(c)]. The amplitude and wave pattern of ABR signals collected via the tag-based EEG data streams ("tag-ABRs") were then compared to similar data collected with the typical surface-based, or tethered, ABR measurement system ("tethered ABRs").

2.2 External stimulus design and presentation

External acoustic stimuli were used to elicit ABRs during sessions with the DTAG3-ECG and tethered system trials. The stimulus was an on-axis recording of Freja’s own echolocation click that was replicated into a train of 1915 clicks with an inter-click interval (ICI) of 15.6 ms (30 s total duration). A series of ten high-frequency, higher-intensity upsweeps (210–220 kHz with 1 ms duration per sweep) were added to the beginning of the stimulus click train to help locate trials in the tag acoustic recordings. The sweep train was above the frequency range of porpoise hearing.\textsuperscript{20} The combined signal was saved to a 16 bit WAV file. Stimulus projection was controlled from a laptop computer via a custom LabView

Fig. 1. Pictures (top panels) and diagrams (bottom panels) illustrating the on-animal placements and equipment setup of the ABR recording systems used in this study, including (a) the DTAG3-ECG, (b) the surface-based ("tethered") data acquisition system, and (c) the DTAG4-ECG. White arrows indicate the placements of the suction cup electrodes. ADC, analog-to-digital converter.
program. The digital stimuli were created with a sampling rate of 500 kHz, converted to analog signals on a National Instruments USB-6356 DAQ device, amplified with a PA1001 amplifier (ETEC, Frederiksværk, Denmark), and projected from a Reson 4034 transducer (Reson, Slangerup, Denmark) placed 1.5 m in front of the biteplate at a depth of 1 m. The transmitting efficiency of the transducer was measured multiple times over the course of data collection and was consistent from session to session. After projection from the transducer, the stimulus had a centroid frequency of 121 kHz and a –10 dB energy duration of 40 μs. The stimulus projection level was varied between trials to facilitate the possibility of estimating an auditory threshold for the stimulus from each session, and a given level was only projected once per session.

2.3 Tag-based recording and analysis of ABRs to external stimuli (DTAG3-ECG)

A DTAG3-ECG was attached to Freja along her dorsal midline via suction cups 4–6 cm behind her blowhole. Two electrodes, embedded in separate suction cups and wired to the tag, were attached along the dorsal midline. One cup was placed 3 cm behind the blowhole and one 6 cm offset from, and in front of, the dorsal fin [Fig. 1(a)], and the tag body was electrically grounded to the seawater. An electrical conducting gel was applied to facilitate electrical contact between the electrodes and the skin. In the tag, the differentially combined electrode signals were low-pass filtered at 1.7 kHz (~12 dB/octave roll-off) and amplified by 30 dB before being digitized at a rate of 5 kHz with 16-bit resolution. Acoustic data from the two tag hydrophones were simultaneously recorded at a rate of 500 kHz and saved on-board with 16-bit resolution. Tag recordings were initiated prior to the tag's application to Freja's back, with all data streams recorded continuously until the tag was removed from the porpoise after completion of the session.

The tag data were analyzed using custom MATLAB functions (Version 2020a, Mathworks, Natick, MA). The start time of each trial was determined by identification of the pre-stimulus frequency sweeps in spectrograms of the tag's acoustic recording. A 40 s section of the subsequent acoustic data was bandpass filtered from 120 to 140 kHz, and an amplitude-based click detector was applied to mark each stimulus received on the tag. Outgoing echolocation clicks produced by the porpoise during a trial were distinguished from the fixed-interval stimulus clicks based on ICI differences and removed from further analysis. Stimuli were difficult to detect in the tag recordings at received levels below approximately 112 dB re 1 μPa peak-to-peak (pp). In these trials, the expected times of arrival of the stimulus clicks on the tag were extrapolated from the more intense pre-stimulus frequency sweeps based on the fixed intervals of the stimulus.

The 40 s sections of electrode data were first compensated for the frequency characteristics of the on-board filter by applying a frequency-dependent amplitude correction between 100 and 2400 Hz to the two-sided fast Fourier transform (FFT) of the full EEG data waveform and then conversion back to the temporal domain with an inverse-FFT. Full EEG waveforms for each trial were then bandpass filtered from 0.3 to 2.4 kHz (seventh order Butterworth filter), encompassing the dominant energy in porpoise ABRs, and 20 ms sections of the EEG data were extracted starting at each stimulus reception on the tag. The extracted EEG segments were interpolated by a factor of 10 using an FFT interpolation function in MATLAB (“interpft”) and averaged over successive stimuli to enhance the ABR signal relative to the EEG noise.

2.4 Surface-based recording of ABRs to external stimuli (tethered system)

Additional sessions were done using the same behavioral paradigm and acoustic stimuli; however, ABRs were recorded using the typical tethered hardware setup, wherein suction cup electrodes were placed in similar locations on the porpoise but connected via wires to a recording system on land [Fig. 1(b)]. The tethered ABR data stream was amplified 10 000 times and filtered from 0.3 to 3 kHz using a custom differential amplifier box. The amplified and filtered data were digitized at a rate of 1 MHz with a National Instruments (Austin, TX) USB-6356 data acquisition box and then saved as a WAV-format file on the same computer that was used to project the stimulus. The outgoing stimulus signal was synchronized on the data acquisition board along with the electrophysiological data, and 15 ms sections of the electrode data following the each stimulus projection were separated and averaged to extract the ABR from noise, following the same procedure as for the tag-based data analysis. Due to timing and training constraints during this phase of the data collection, only 1000 stimulus clicks were presented to the porpoise per tethered trial.

2.5 Tag-based recordings of ABRs to self-generated echolocation clicks (DTAG4-ECG)

Outgoing echolocation clicks also elicit measurable ABRs in odontocetes. To measure self-generated click ABRs from Freja, a DTAG4-ECG with two wired suction cup electrodes was applied to the porpoise in the same manner and position as described previously for the DTAG3-ECG [Fig. 1(c)]. Data acquisition was initiated prior to the start of an experimental session, after which acoustic and EEG data streams were recorded continuously. Acoustic data were recorded on the single tag hydrophone at a sample rate of 576 kHz and saved on-board with 16-bit resolution. Concurrent electrophysiological data were low-pass filtered on-board at 2.5 kHz (~18 dB/octave filter roll-off) and amplified by 34 dB before being digitized at a rate of 9 kHz with 16-bit resolution and saved on-board.

With the tag attached, the porpoise stationed on the biteplate for multiple 40 s trials while no external stimulus was projected. The porpoise's spontaneous clicks were identified by filtering the acoustic data with a seventh order, 180–200 kHz Butterworth filter to ensure the detections were triggered by outgoing clicks and not echoes. Odontocete ABRs to individual stimuli are around 6 ms in duration, and stimuli presented at shorter intervals cause ABRs to
begin to overlap and fuse into a more complex evoked response. We therefore focused our analysis on outgoing clicks with ICIs of 10 ms or greater to facilitate comparison with the external stimulus trials. A total of 860 clicks were produced across eight trials with the DTAG4-ECG that had a separation of 10 ms or more from adjacent clicks. After identifying such clicks, the corresponding electrode data were processed in a similar manner to the DTAG3 trials. The EEG data stream was first compensated for the frequency roll-off characteristics of the on-board filter between 100 and 4000 Hz and then bandpass filtered from 500 to 3000 Hz to encompass the dominant ABR frequencies, and a 20 ms section of the electrode data surrounding each detected outgoing click was extracted for subsequent averaging and analysis.

2.6 Comparison between systems

Although the presented dataset is too small for a statistical comparison between the electrophysiological recording systems, we examined the ABR signal-to-noise ratios (SNRs) over successive epoch averaging. SNR values were calculated using a single point amplitude variance estimate of the electrophysiological noise at $t = 4$ ms re: stimulus reception. Signal amplitudes were calculated as the root mean square (rms) amplitude from $t = 1$ to 6 ms re: stimulus reception. Detailed descriptions of the SNR calculation can be found in the previously published literature.26,27

3. Results and discussion

3.1 ABRs to external stimuli

Averaged evoked potentials to external stimuli from the DTAG3-ECG and the tethered system exhibited wave deflections between 1 and 5 ms from the approximate time the sound reached the auditory bullae [Figs. 2(a) and 2(b)]. The main deflections of the tag-based and tethered EEG waveforms both peaked at 3.8 ms. The deflection patterns and timing show close correspondence to tethered ABRs collected from Freja previously28,29 as well as tethered ABRs reported from other porpoise individuals and odontocete species.19,24,25,28,30 This similarity leads us to conclude that the evoked responses measured with the DTAG3-ECG are ABRs to the presented acoustic stimuli.

With both setups, ABR magnitudes, calculated as the peak to peak between the P4 wave and its preceding trough, decreased with decreasing received levels to the point where an ABR was no longer visible [Figs. 2(a) and 2(b)]. Thresholds to the stimulus were estimated by plotting the ABR magnitudes from a single session as a function of the stimulus received level and fitting a linear regression to points with clearly visible response waves [Fig. 2(c)]. Three thresholds were estimated from three experimental sessions with the DTAG3-ECG (105, 107, and 109 dB re 1 $\mu$Pa pp), and two thresholds were estimated from two sessions with the tethered system (105 and 109 dB re 1 $\mu$Pa pp). Although a malfunction in the stimulus amplifier limited the maximum stimulus levels used to elicit tethered ABRs, the ABR magnitudes were similar between the two hardware setups across the range of stimulus levels that were tested [Fig. 2(c)]. The similarity in ABR amplitudes across acoustic received levels as well as the similar resulting threshold estimates show that the

![Fig. 2. Example ABRs collected with the DTAG3-ECG (a) and the tethered recording system (b). Red dots in the top panels illustrate peak-to-peak magnitude calculations. Bottom panels illustrate ABR amplitude variation according to increasing acoustic stimulus levels within a single experimental session. (c) Top panel: Example threshold calculations from the ABR responses in (a) and (b). Thresholds were estimated as the point where linear regressions fit over the detected ABR responses (solid lines) crossed the zero value of the y axis. (c) Bottom panel: Comparison of all ABR magnitudes recorded with the tag-based and surface-based data acquisition systems during threshold sessions. Solid markers denote visually clear responses used for linear regression thresholds.](image)
externally stimulated ABR data from the DTAG3-ECG are consistent with data collected with the tethered ABR setup that is widely used in odontocete research.

3.2 ABRs to self-generated echolocation clicks

The averaged EEG elicited by self-generated clicks and recorded with the DTAG4 revealed wave deflections from approximately 2 to 5 ms after the click recording time [Fig. 3(a)], which were similar to previously published click-ABR results obtained from another porpoise via a tethered measurement system. The maximum positive deflection occurred at 3.8 ms, corresponding to the peak wave delays of the externally stimulated ABRs. This indicates the observed biopotential represents an ABR to the porpoise’s self-generated echolocation signals. The click-evoked ABR was still observed when averaged from smaller numbers of clicks [Fig. 3(b)].

3.3 System noise comparison

All three biopotential recording systems exhibited a low noise floor [Fig. 3(c)], although there were differences between systems. The DTAG3-ECG circuit exhibited spectral peaks and an elevated noise floor below 1 kHz in comparison to the tethered system, while the DTAG4-ECG noise floor was 7–10 dB higher than the other two systems above 0.5 kHz. Despite these differences, the SNR of ABRs recorded with all systems improved with increased epoch averaging [Fig. 3(d)].

3.4 Potential application and future research

These results show that ABRs to external and self-generated stimuli can be measured from an animal-attached tag, which may benefit future studies of odontocete hearing and the sensory ecology of echolocation. All odontocete ABR measurements to date have been collected via tethered data acquisition systems in combination with stationary experimental protocols, where the electrodes attached to a submerged animal are connected to equipment above the water surface via wires, and the position of the test subject and any external sound source or echolocation target are fixed for the duration of an auditory measurement. Yet echolocation is acoustically and perceptually dynamic; both the production and reception of sounds are controlled and adjusted according to a variety of factors. Existing experimental paradigms are thus constrained in how well they can mimic natural echolocation circumstances. Auditory measurements during mobile echolocation tasks that involve the search, approach, and terminal phases of prey capture would be a valuable way to improve our understanding of auditory dynamics during natural acoustic behavior.

The tag-based ABR measurements in this study successfully uncoupled the test subject from recording equipment that must remain in air and are an important first step toward implementing such mobile experimental paradigms. However, ABR measurements from an actively locomoting animal have not been described in the previously published literature and involve significant additional challenges. Most animal ABR experiments involve anesthetized subjects to

![Fig. 3.](image-url)
reduce movement-related and myogenic noise that can otherwise mask the evoked biopotential signal. Anesthesia is not possible or required with odontocetes, from which large ABRs can still be measured while lightly engaging muscles to maintain a proper stationing position in a hoop or on a biteplate. Although this is a promising start, it remains to be determined whether the ABR is measurable in the face of increased biopotential noise resulting from active locomotion. Future research should investigate whether tag-based ABRs can be measured from individuals during active locomotion as well as from additional odontocete species. The use of captive individuals that are trained in public display or research facilities will enable systematic exploration and testing of this possibility.

The measurement of tag-based ABRs raises the possibility that empirical auditory data could be collected from individuals in the wild if the issues of myogenic and movement-related noise can be overcome. To our knowledge, there are no electrophysiological hearing measurements from any free-moving, non-human vertebrate in the wild due to layered methodological challenges. Beyond uncoupling a subject from stationary equipment, a free-moving measurement also requires accurately placing and maintaining electrode positions on a moving subject as well as accounting for variation in the orientation and distance of the subject to the source of externally generated stimuli. One solution to the latter challenge could involve placing a sound source on the tag itself.32 If the challenge of controlling and timing tag-based sound projections can be resolved, then noise or stimulus sounds produced by a tag may improve our ability to assess when increases in anthropogenic noise mask detection of biologically relevant signals in nature such as echolocation clicks or echoes. However, echolocating animals such as odontocetes are their own stimulus generators, making it plausible that auditory data could also be collected from individuals without the need for external stimuli. ABR responses have been recorded not only from outgoing clicks of an echolocating odontocete, but also by returning echoes. However, echolocating animals such as odontocetes are their own stimulus generators, making it plausible that auditory data could also be collected from individuals without the need for external stimuli. ABR responses have been recorded not only from outgoing clicks of an echolocating odontocete, but also by returning echoes.21,22 Tag recordings of click- and echo-ABRs could enable studies of auditory processing as animals solve naturalistic echolocation problems that incorporate movement as part of the echolocation strategy. Since sensory information is a fundamental regulator of behavior, future collection of in situ auditory data from free-moving odontocetes in-natura would be a novel and broadly valuable data collection paradigm for the study of auditory processing, sensory ecology, and animal behavior.

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References and links


