

RESEARCH ARTICLE

Hearing pathways in the Yangtze finless porpoise, *Neophocaena asiaeorientalis asiaeorientalis*

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ABSTRACT

How an animal receives sound may influence its use of sound. While ‘jaw hearing’ is well supported for odontocetes, work examining how sound is received across the head has been limited to a few representative species. The substantial variation in jaw and head morphology among odontocetes suggests variation in sound reception. Here, we address how a divergent subspecies, the Yangtze finless porpoise (*Neophocaena asiaeorientalis asiaeorientalis*) hears low-, mid- and high-frequency tones, as well as broadband clicks, comparing sounds presented at different locations across the head. Hearing was measured using auditory evoked potentials (AEPs). Click and tone stimuli (8, 54 and 120 kHz) were presented at nine locations on the head and body using a suction-cup transducer. Threshold differences were compared between frequencies and locations, and referenced to the underlying anatomy using computed tomography (CT) imaging of deceased animals of the same subspecies. The best hearing locations with minimum thresholds were found adjacent to a mandibular fat pad and overlaying the auditory bulla. Mean thresholds were not substantially different at locations from the rostrum tip to the ear (11.6 dB). This contrasts with tests with bottlenose dolphins and beluga whales, in which 30–40 dB threshold differences were found across the animals’ heads. Response latencies increased with decreasing response amplitudes, which suggests that latency and sensitivity are interrelated when considering sound reception across the odontocete head. The results suggest that there are differences among odontocetes in the anatomy related to receiving sound, and porpoises may have relatively less acoustic ‘shadowing’.

KEY WORDS: Sensory, Auditory brainstem response, Noise, Marine mammal, Odontocete, Communication

INTRODUCTION

In terrestrial mammals, structures such as the pinnae and ear canal serve to conduct sound to the middle and inner ear (Angell and Fite, 1901; Roffler and Butler, 1968). The shape of the outer ear influences

the spectra, amplitude and arrival time of sounds (Müller, 2004). Variations in these cues aid animals in sound source localization and consequently important biological activities, including locating predators and prey, navigating and communicating with conspecifics. Animals may also use directionality cues to limit the adverse impacts of noise exposure by turning away from sound sources to decrease received levels of noisome sounds (Heffner and Heffner, 1992).

Odontocetes (toothed whales, dolphins and porpoises) lack external pinnae. Most evidence indicates they receive sound using specialized adaptations around the lower jaw. For example, early anatomical studies proposed that sound preferentially travels through an acoustic window of the pan bone to the lower jaw (Norris, 1968). Fats within the odontocete mandible are similar to seawater in acoustic impedance, suggesting these fat bodies preferentially conduct sound to the inner ear (Varanasi and Malins, 1972). Biochemical analyses of the lipids within odontocete heads show that the ‘acoustic fats’ have a specific topographical arrangement within the lower jaw, perhaps affecting sound speed to channel incoming sound to the ears (Koopman et al., 2006; Koopman and Zahorodny, 2008). The jaw hearing hypothesis is supported by intracranial-recorded evoked potential and cochlear action potential studies, which demonstrated good hearing sensitivity from the dolphin lower jaw (Bullock et al., 1968; McCormick et al., 1970). Sound transmission measurements through head tissue samples indicated a concentration of sound through the throat and jaws (Norris and Harvey, 1974). Odontocete jaw hearing is also supported by psychophysical echolocation research and detailed, non-invasive physiological studies of dolphin received sensitivity (Brill et al., 1989; Möhl et al., 1999). Anatomical studies have shown discrete fat lobes associated with the lower jaw that vary in size and shape in each species (Ketten, 1994). The mandibular fat hearing hypothesis is supported by physiological data, particularly for the concept of an additional pathway near the bulla, which preferentially receives lower frequency sounds (Popov et al., 2008).

While the propensity of evidence clearly supports the foundation of ‘jaw hearing’, previous research has tended to focus on a few species, particularly on bottlenose dolphins (*Tursiops truncatus*). Yet, there are clear variations in the jaw and head morphologies of odontocete species suggesting at least subtle variation in how sound is received. Little attention has been paid to how divergent cetacean species, with varied jaw morphologies, receive sounds despite indications that they receive sound differently. For example, the harbor porpoise (*Phocoena phocoena*) has a significantly wider receiving beam than the bottlenose dolphin (Kastelein et al., 2005). The beluga (*Delphinapterus leucas*) demonstrates lowest hearing thresholds not from the lower jaw as in dolphins but rather at the tip of the lower rostrum (Mooney et al., 2008). Despite these physiological differences, no study has yet to comprehensively examine head/jaw received sensitivity while providing a comparison to auditory anatomy and the location of ‘acoustic’ fats.

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List of abbreviations

AEP	auditory evoked potential
CT	computed tomography
EFR	envelope following response
FFT	fast-Fourier transform
peRMS	peak-equivalent root mean square
SAM	sinusoidally amplitude modulated
SPL	sound pressure level
V_{p-p}	peak-to-peak voltage

Differences in how each species of odontocete receives sound may be significant as the shape of sound receivers can greatly influence directionality, localization and frequency sensitivity (Au and Hastings, 2009). Regulatory agencies and research review panels often stress the need for data from a greater number of species (National Academy of Sciences, 2005). However, with little data on hearing diversity it is unclear how to identify representative groups or even determine the applicability of such actions.

Here, we address how a divergent species of odontocete, the Yangtze finless porpoise [*Neophocaena asiaeorientalis asiaeorientalis* (Pilleri and Gahr, 1972)] receives sound. The Yangtze finless porpoise is a subspecies of the finless porpoise and the only freshwater porpoise population. They are a particular conservation issue as they inhabit the same busy waters as the now likely extinct baiji (*Lipotes vexillifer*) (Turvey et al., 2007). Porpoise populations are also declining rapidly (Mei et al., 2012; Zhao et al., 2008). Noise impacts on this subspecies are of concern because these animals inhabit waters with substantial acoustic sources including shipping, dredging and underwater construction. While hearing ranges have been established for this subspecies (Popov et al., 2005), there are no data regarding how they receive sound. The goal of this work was to examine the relative sensitivity of finless porpoise hearing across its head and lower jaw. The results are then viewed in light of localization capabilities and the potential impacts of anthropogenic noise.

RESULTS**Relative thresholds**

This work examined the hearing of two finless porpoises (see Materials and methods). Both animals showed good hearing sensitivities. The mean hearing thresholds for all frequencies and both animals were lowest and nearly identical at the porpoise jaw tip and mandibular (cheek) fat pad (Table 1). Thresholds were similarly low overlaying the bulla, at the meatus and at the lower jaw. The mean thresholds from all these locations were not significantly different (Fig. 1). The throat location was slightly, but

significantly, elevated relative to these locations (one-way ANOVA, $F_{7,58}=12.34$, $P<0.001$ and subsequent Tukey's pairwise comparison). Thresholds acquired from lower melon stimulation were substantially higher (>20 dB) than the areas of best sensitivity (jaw tip, mandibular fat pad, meatus and overlaying the bulla). Mean thresholds were highest when the transducer was placed on the animals' backs (a control location). No responses could be detected when the transducer was placed on the animals' flippers. The male and female porpoise did not demonstrate substantial differences in their hearing abilities.

Responses from the individual locations broken down by stimulus frequency show lowest thresholds when sound entered the mandibular fat pad or jaw tip for all frequencies except 8 kHz (Fig. 2). Stimulation at the throat location was slightly elevated for most frequencies and substantially (by 20 dB) for 8 kHz. Lowest thresholds for 8 kHz were found at the side locations: the meatus, overlaying the bulla and at the mandibular fat pad. For all frequencies except 120 kHz, stimulation at the lower portion of the melon resulted in substantially higher hearing thresholds (15–25 dB).

Relative latencies

Mean response latencies were measured using auditory evoked potential (AEP) waves I–IV. The latencies were generally lowest when transducer stimulation was at the mandibular fat pad and the lower jaw (Fig. 3). When the transducer was overlaying the animals' bullae, values were often similarly low. Latencies increased slightly using sound presented at the meatus. Throat-presented stimuli tended to have response latencies longer than those of the most rapid response regions (lower jaw, cheek, over bulla), but on a par with, or faster than, meatal stimulation. Relative response latencies increased with stimuli presented at the jaw tip, lower melon and back.

Response latencies were dependent upon sound levels and tended to increase as sound levels decreased (Figs 4, 5). This was clearly visible with a line drawn between the respective peak values of waves I–IV. This line shows a clear trend in longer response latencies as sound levels decreased (Fig. 4). These latencies (or increasing delays) were then plotted relative to the stimulus level. The maximum sound level presented was deemed 0 dB attenuation and 0 ms time delay. The difference in time from those of the original peak values was plotted for each attenuation step (5, 10, 15 or 20 dB). This was done for waves II–IV. While the spread is relatively high, there was a significant positive relationship between increasing sound attenuation and increasing physiological AEP

Table 1. Thresholds for both finless porpoises (*Neophocaena asiaeorientalis asiaeorientalis*) provided by individual stimulus frequencies and the mean of all thresholds, relative to the location of the suction-cup transducer-presented stimuli

	Location	Threshold (dB)				
		Mean \pm s.d.	Click	120 kHz	54 kHz	8 kHz
Lower melon	1	76.8 \pm 12.2	82.3	61.9	86.3	No response
Jaw tip	2	55.4 \pm 8.9 [‡]	65.3	50.9	49.0	56.5
Throat	3	67.0 \pm 12.3	70.4	62.0	59.5	76.4
Lower jaw	4	61.1 \pm 6.6	68.5	56.7	60.3	58.8
Mandibular fat pad	5	55.7 \pm 9.8 [‡]	65.7	53.7	42.5	61.0
Meatus	6	59.3 \pm 8.1 [‡]	70.2	59.1	53.3	54.6
Overlaying bulla	7	57.7 \pm 8.9 [‡]	68.3	54.8	49.0	58.7
Back	8	97.0 \pm 11.2 ^{*‡}	99.1	85.4	107.6	No response
Flipper	9	No response	No response	No response	No response	No response

*Thresholds from stimuli presented at the back are significantly greater than all others; [‡]thresholds are significantly different from those for stimuli presented at the lower melon (one-way ANOVA, $F_{7,58}=12.34$, $P<0.001$ and subsequent Tukey's pairwise comparison).

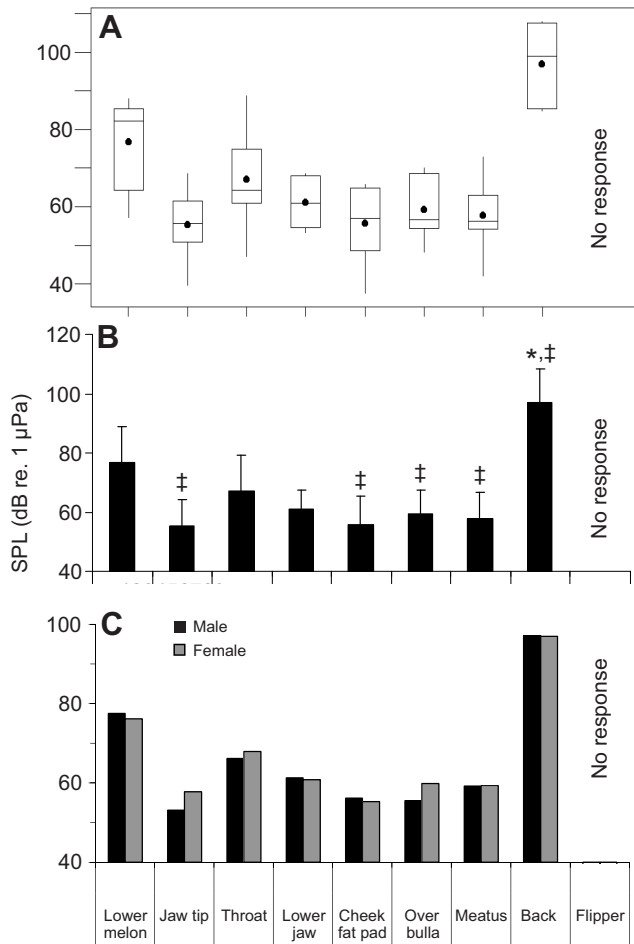


Fig. 1. Thresholds to all suction-cup transducer-presented stimuli from both finless porpoises (*Neophocaena asiaeorientalis asiaeorientalis*). (A) Boxplots of thresholds, showing mean value (dot), median and $\pm 25\%$ (box), and $\pm 50\%$ (whiskers). (B) Mean ± 1 s.d. of the thresholds. No response was detected from stimuli presented at the control flipper location. *The back location had significantly higher threshold values than all other locations (one-way ANOVA, $F_{7,58}=12.34$, $P<0.001$ and subsequent Tukey's pairwise comparison). †Thresholds from stimuli at the lower melon were significantly different from those at the jaw tip, mandibular fat pad, meatus, overlaying the bulla and back. (C) Threshold comparison of the male (black) and female (gray) porpoises. SPL, sound pressure level.

delays ($r^2=0.21$; $P<0.05$). In other words, as sound levels decreased, physiological delays increased. This was true across all stimulus locations and for both animals.

AEP comparisons to anatomy

The sensitivities from the transducer placements were plotted over the scans and three-dimensional reconstructions of the heads of the scanned stranded post-mortem specimens (Figs 6, 7). This provided an anatomical relationship for the physiological measurements. In part because of the condition of the stranded specimens, the blubber layers in some areas had similar attenuations to the mandibular fats, which led to some 'fogging' of the fat bodies in autosegmentations. The main jaw fat bodies were distinct but exact edges and dimensions were uncertain because of this overlap. The combined data from computed tomography (CT) assessments and dissections (Fig. 7A) revealed three fatty regions distinct from blubber: the melon and, bilaterally, an intramandibular and a lateral ovoid pad overlaying the thin pan bone region of each lower jaw.

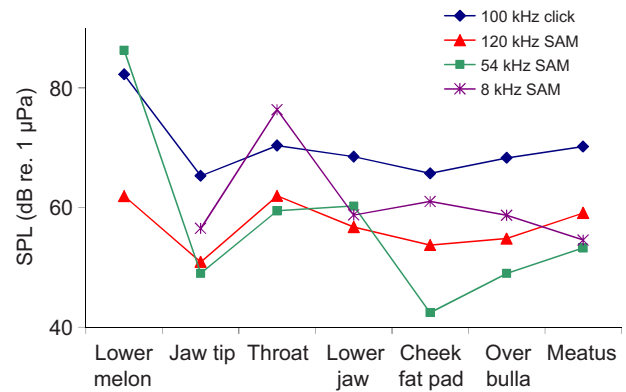


Fig. 2. Mean thresholds of both animals by stimulus frequency at respective stimulus locations. Data from the back were excluded. No response was detected from sounds presented at the flipper and those data were not plotted. SAM, sinusoidally amplitude modulated.

The mandibular placement, which provided lowest click thresholds (greatest sensitivity), overlaid the fat body at the pan bone area. The lower jaw location was just adjacent to this fat region. The location overlaying the bulla was also neighboring these tissues as shown in the VRT visualizations of the acoustic fats of the mandible. The jaw tip and meatus did not appear directly associated with visualized acoustic fat regions. However, the meatus location produced the lowest thresholds for the 8 kHz tone. The throat location was near, but not directly overlaying, the anterior portions of the mandibular fats, particularly the intramandibular fat bodies. The lower melon placement was near the fat of the melon body, but hearing thresholds were not particularly sensitive from this location.

DISCUSSION

Relative hearing

The best responses for the higher frequencies (54, 120 kHz tones, and click) were found with stimulation towards the front of the head and at the mandibular fat pad location. The lower frequency, 8 kHz, was detected best using lateral, meatal presented stimuli. While these data may not conclusively support a double acoustic window for the finless porpoise, they do indicate these porpoises hear low frequencies better from the side and higher frequencies better at anterior positions; and at least suggest the need for further investigations. Such a double-acoustic window has been proposed for the bottlenose dolphin (Popov et al., 2008). The porpoise's frequency-dependent general regions of low and high frequency sensitivity are less specific than in the bottlenose dolphin, which shows considerable (near 40 dB) differences across the head (Møhl et al., 1999). A double-acoustic window is intriguing for porpoises as they are considered to mainly produce high frequency echolocation sounds (Au et al., 1999; Li et al., 2005), which would be heard best from the front. Dolphins, in contrast, produce a range of sounds including lower frequency whistles and hear lower frequencies well from the side, presumably for evaluating whistle directionality (Lammers and Au, 2003; Popov et al., 2008). Yet, for porpoises, the ambient environment is filled with potentially important low frequency soundscape cues, and neonate finless porpoises also produce low frequency sounds (Li et al., 2008). Lateral low frequency hearing may facilitate localization of these sounds and orientation within the local soundscape.

Examining relative sensitivities based on frequency does reflect some data scatter, although the broadband clicks showed less threshold fluctuation across most of the head (except from the melon

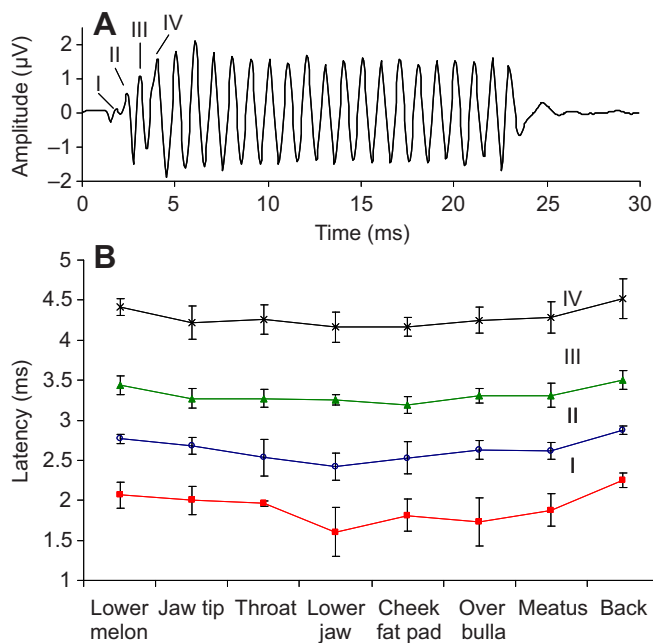


Fig. 3. Representative AEP waveform and respective latencies by location. (A) Auditory evoked potential (AEP) waveform to a click stimulus presented at the 'lower jaw' location at 135 dB peak to peak ($\text{dB}_{\text{p-p}}$). The first four 'positive' inflections of the first click are indicated (I–IV). (B) Latencies of the four waves from all stimulus locations with a detectable response. The respective waves (I, II, III, IV) are labeled to the right.

site, which did not conduct sounds well) (Figs 2, 6, 7). These clicks generated elevated thresholds compared with the tones, which is expected for brief signals and considering the difference between signals presented in dB peak to peak ($\text{dB}_{\text{p-p}}$; clicks) and those of the tones presented in dB root mean square (dB_{RMS}) (Au et al., 2002). If the tones were compared in $\text{dB}_{\text{p-p}}$, this difference in thresholds was negligible. The generally higher thresholds for melon stimulation probably reflect its acoustic isolation from the auditory system. Interestingly, the 120 kHz threshold at the melon was least elevated relative to the other frequencies. The 120 kHz tone was near the center frequency of porpoise echolocation signals (Li et al., 2005). This suggests a potential means to hear their own clicks or echoes, but in an attenuated manner. Thresholds from throat stimuli were also elevated, suggesting that, despite predictions in other odontocetes (Cranford et al., 2008), this is not a preferential auditory pathway for the finless porpoise.

Lowest thresholds were found at the mandibular fat pad and lower jaw, which reinforces previous hypotheses suggesting acoustic fats preferentially conduct sound to the ear (Bullock et al., 1968; Norris, 1968). The lower jaw of these porpoises has limited extramandibular fat, suggesting that, like in dolphins, sound is either conducted to the adjacent fat body or is transmitted through the pan bone to the intramandibular fat and then to the ear, as Norris also proposed investigating (Norris, 1968). How sound could be effectively transmitted through the impedance mismatch of bone is uncertain.

The responses and sensitivities from locations that are not associated with acoustic fats were particularly striking (Fig. 7). This includes the jaw tip, throat, the meatus and overlaying the bulla. Responses were even generated from the animal's back. Hearing from the back is quite unusual and simply reflects that sound is transmitted through the body's tissues. Similar empirical (Møhl et al., 1999; Mooney et al., 2008) and modeling (Cranford et al., 2008) studies have suggested good hearing from head pathways not

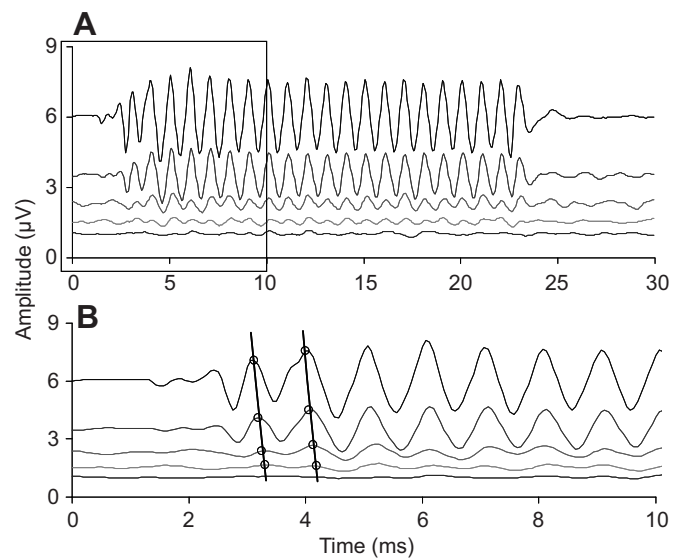


Fig. 4. Waveforms and latencies with respect to stimulus amplitude. (A) AEP responses to a 100 kHz centered click at 105, 95, 85, 80 and 75 $\text{dB}_{\text{p-p}}$. (B) Close-up of the AEP waveforms illustrated by the boxed region in A. Regressions of the peak positive waves III and IV are overlaid. Note that the regressions slope towards increasing delays for corresponding peak values.

necessarily associated with acoustic fat. One concern here is that the suction-cup transducer may have generated responses through means not considered primary odontocete auditory mechanisms (e.g. bone conduction). Yet, soft tissue pathways offer the most parsimonious entryway into the bulla (Cranford et al., 2010). Further, prior work has shown using a suction-cup transducer produces thresholds that are similar to free-field stimuli, suggesting a consistent hearing mechanism between the two methods (Finneran and Houser, 2006). This method also provides consistent stimuli at a fixed difference, which is vital in near-field experiments (Kalmijn, 1988). In water, sounds are more often received by porpoises in a free-field plane wave across the head and body. Sound reception and conduction to the ear may be influenced by the subtle differences in preferential acoustic pathways. These small differences likely play a large role in sound processing and properly utilizing acoustic signals.

While hearing thresholds were primarily lowest at the jaw tip, it is likely that sound presented at this location influenced responses by accessing both ears. The same is probably true for the throat

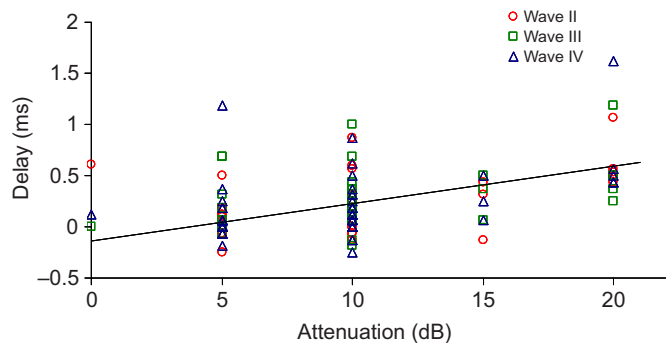


Fig. 5. Delay of waves II, III and IV relative to stimulus attenuation (dB). Response delays increase with decreasing sensation levels, indicated by the regression. All stimulus frequencies are combined.

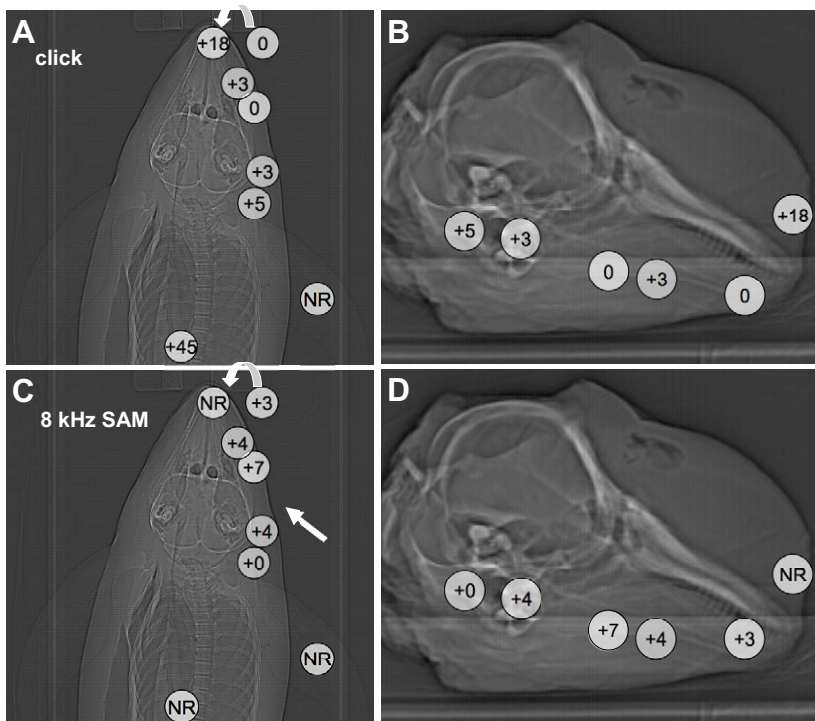


Fig. 6. Computed tomography topograms of both specimens showing the head, bone and body structure. Circles reflect the sound levels (in dB) of the relative thresholds, where zero was the lowest threshold. Higher numbers reflect the relatively higher thresholds; NR indicates no response was measured. A and B reflect high-frequency click thresholds. C and D are from the 8 kHz amplitude modulated tone. A, C and B, D correspond to specimens 1 and 2, respectively.

measurements. In both situations the transducer was placed on the midline. Similar low ‘midline’ thresholds have been shown in the beluga whale and the bottlenose dolphin (Møhl et al., 1999; Mooney et al., 2008). Sound reaching both ears likely increases evoked response amplitudes relative to ipsilateral stimulations, and thresholds were comparatively low. Sounds presented from locations on the side of the head (lower jaw to the ear) were likely received primarily by the ipsilateral ear. (They also may have been shaded on the contra-lateral side, although these differences were not measured.) Because minimum thresholds were from ipsilateral locations of the mandibular fat pad and overlaying the porpoise bulla, this suggests that these were particularly sensitive locations.

Yet, mean thresholds were not substantially different at locations from the jaw tip to the ear (~12 dB), suggesting that the porpoise

receives sound similarly from multiple locations and sensitivity cues may be quite subtle for the finless porpoise. The slight differences in thresholds across the finless porpoise head suggest a broad receiving beam and directivity index. If so, this would be similar to the harbor porpoise’s (*Phocoena phocoena*) auditory receiving angles (Kastelein et al., 2005), and may reflect conserved auditory similarities (e.g. Popov et al., 2006) despite the substantial niche separations. The relative similarity of sensitivities across the finless porpoise head is in contrast to findings in the bottlenose dolphin and beluga whale, for which 30–40 dB differences occur across the animals’ heads (Møhl et al., 1999; Mooney et al., 2008). This suggests that while there are consistencies in lower jaw hearing, substantial differences can be found in how divergent odontocetes receive sound.

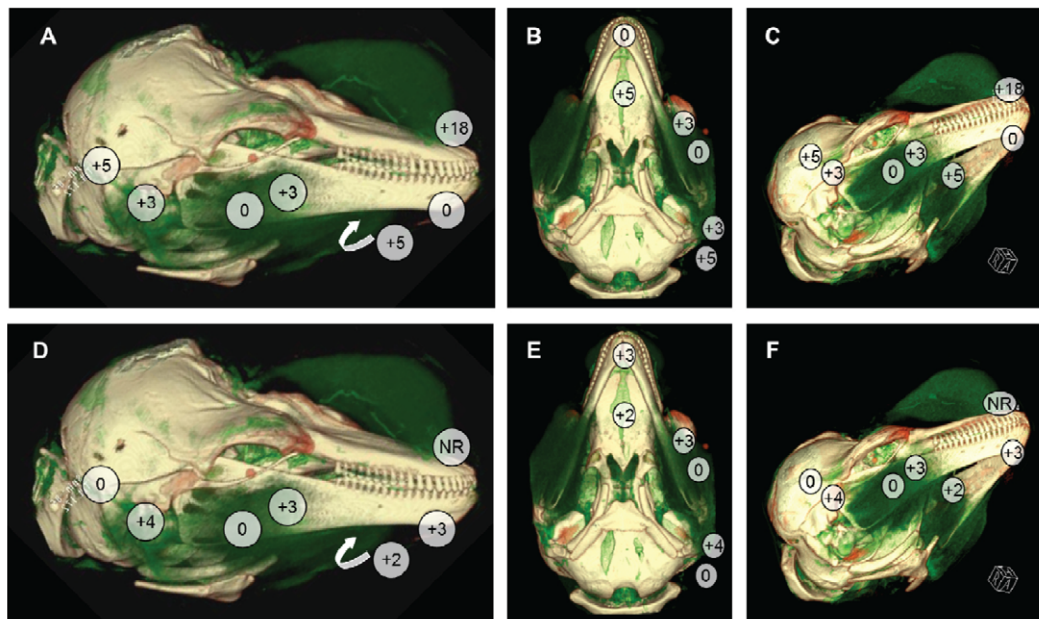


Fig. 7. Three-dimensional reconstruction of the computed tomography images. White, bone; green, fat bodies. Circles reflect the sound levels (in dB) of the relative thresholds, where zero was the lowest threshold. (A–C) Relative threshold values for the broadband clicks. (D–F) Values for the 8 kHz tone. Side, bottom and off-axis views are presented for these frequencies.

Differences may be due to niche separations. For one, bottlenose dolphins probably use long-range echolocation compared with a river porpoise (the river may restrict ranges). Coastal porpoises may not need a high directivity index if their sonar is range limited by the riverine environment. In general, these differences are not very surprising when considering that porpoises are quite phylogenetically divergent from other odontocetes, and finless porpoises themselves are even farther removed (5.60–11.60 million years) (McGowen et al., 2009). Determining whether these differences result from species morphology, simple size differences or other factors requires further investigation.

With over 70 species in varying aquatic niches (Perrin et al., 2009), finding auditory differences among divergent odontocete species is expected. In addition, factors other than hearing also influence jaw morphology. Yet, as the shape and size of a receiver contributes substantially to transducer performance (Au and Hastings, 2009), complex mandibular anatomy seemingly plays a major role in how sounds are gathered and utilized by the auditory system. This is reflected in variations in received sensitivity. These results seem to suggest that with species anatomical differences, there are correlates of divergent auditory capabilities.

Signal-to-noise ratios may be low for finless porpoises because they receive sound well at multiple locations. They may be sensitive to a greater proportion of both biologically important sounds and confounding signals. Sounds at off-the-midline axis angles may not be substantially shaded. This may aid in detecting low amplitude biologically important cues. In a high noise environment, such as the Yangtze River, detecting certain important signals from noise may be difficult for the finless porpoise. While high frequency echolocation signals may not be masked by most noise, detection of lower frequency communication signals, and the sounds of particular fish prey (Li et al., 2008; Luczkovich et al., 2000), may be affected by lower signal-to-noise ratios.

Relative latencies

Response latencies varied according to stimulus location and relative distance to the ear. The greatest latencies were found when stimuli were presented farther from the ear, at the animals' back and at the lower melon. Minimum response latencies were found at the animals' lower jaw and mandibular fat pad locations. These were not the shortest routes to the ear, suggesting sound pathways that may be lowest in impedance and preferential for sound conduction. These data agree with sound speed measurements collected from deceased odontocetes (Norris and Harvey, 1974).

Latencies might also reflect the orientation of sound entering the bulla. In this manner, a plane wave is not entirely dissipated by its reception and conduction from multiple points on the head. Rather, sound received at different locations could be summated in time. For example, low amplitude returning echoes (which are effectively a plane wave) would be difficult to detect if received as a point source. But a plane wave, which does not reach all parts of the head at the same time, might be effectively enhanced by anatomically induced latencies guiding the plane wave across the head, i.e. anatomy may help coordinate sound to the bulla, increasing the physiological responses to sound. This may be advantageous for detecting short duration, low amplitude sounds, such as echolocation echoes, but disadvantageous for detecting intense impulse sounds, such as those from some anthropogenic sources.

There was a slight increase in response latency with decreasing sound level (Figs 4, 5). Such findings have been established in terrestrial mammals as well as in other odontocetes (Don et al., 1984; Popov et al., 2008). We see from prior work that sound levels

are directly tied to response magnitude, and this magnitude is linked to response latency (Popov et al., 2008). Because sound levels will often be different at ipsilateral and contralateral ears, these results imply that sound levels can affect responses for sound source localization in two manners: response amplitude and latency. A sound from one side, for this example the right, would likely be higher in amplitude at one (in this case the right) ear. Thus, physiological response amplitudes in the right ear are higher, but also faster, compared with those to lower amplitude sounds on the left (contra-lateral) side of the head. These physiological mechanism may be in part adaptations to sound speed in water (~5× faster than in air), compensating for the minimal intra-aural time and loudness differences of an aquatic medium (Mooney et al., 2012; Moore et al., 1995). Thus, finless porpoises, and likely other odontocetes, have multiple means to direct and shade sound within the head.

These differences in latencies are small but their description in multiple animals and distinct odontocete species suggests that they are robust. Notably, odontocetes have rapid integration times, perhaps in part to help discriminate these acoustic differences (Au et al., 1988), as these differences in latencies may also provide clues to sound reception. The decreased latencies and increased response amplitudes of preferential sound pathways may play an important role in the use of sound. Conversely, sounds received at other locations may be somewhat limited by decreased response amplitudes and increased latencies. Comparative form and function investigations in other species will address the differences and consistencies that comprise odontocete auditory systems.

The differences in relative hearing sensitivities between the finless porpoise and the bottlenose dolphin and beluga whale hearing data indicate that additional auditory variations are likely to be found among taxonomic groups. This consideration underscores the need for care when applying hearing data from representative animals to divergent species. These differences also suggest that species may differ not only based upon audiograms but also in more subtle auditory adaptations. Thus, multiple auditory characteristics (e.g. audiograms, temporal resolution, received sensitivity parameters, filter shapes and form–function studies) will better inform comparisons between species and potential applications of hearing studies.

MATERIALS AND METHODS

Hearing measurements were made in April 2010 on two Yangtze finless porpoises using AEP methodology. The AEP technique is increasingly utilized in marine mammals as a means to rapidly, passively and non-invasively investigate hearing (reviewed in Mooney et al., 2012; Nachtigall et al., 2007; Supin et al., 2001). The animal subjects, one male and one female, were originally from the wild, but have resided at the Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, China, for the past 6 and 14 years, respectively. At the time of the experiments, the adult male (Abao) was estimated to be 15 years old, weighed 47.8 kg and was 145 cm in length. The female (Ying Ying) weighed 41.8 kg, was 142 cm in length, and was estimated to be of a similar age. The two were housed in cement pools with four other conspecifics. Pools were filled with filtered freshwater from the nearby Yangtze River. Hearing data were first collected from the male over three consecutive days. There was then a 3 day break before data were collected from the female.

The basic audiograms of these animals were measured in water several years prior to this research (Popov et al., 2005) and probe hearing tests related to this study reflected normal odontocete hearing capabilities (Mooney et al., 2011). All received sensitivity hearing examinations were obtained out of the water. The test subject porpoises rested on two foam mats on the top ledge of a tiered pool. Water was drained just prior to the experiments and the porpoise within the pool was gently lifted and placed on the mats.

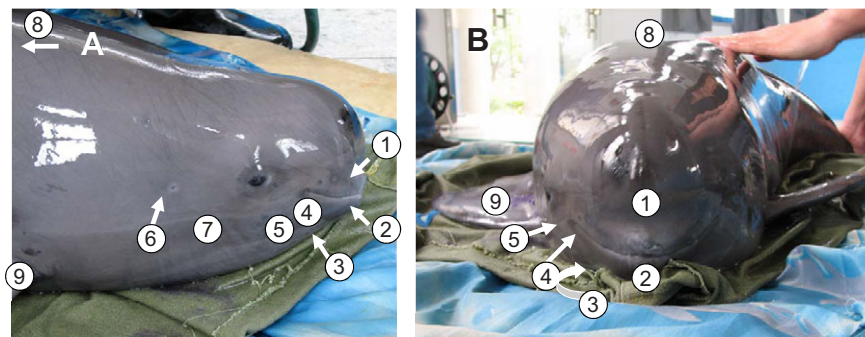


Fig. 8. Finless porpoise (*Neophocaena asiaeorientalis asiaeorientalis*) from side (A) and front (B) viewpoints. Stimulus locations are indicated by the numbered circles. 1, lower melon; 2, jaw tip; 3, throat; 4, lower jaw; 5, mandibular fat pad; 6, overlaying the auditory bulla; 7, meatus; 8, back; 9, flipper.

This study was conducted with the approval of the Institute of Hydrobiology of the Chinese Academy of Sciences and the Woods Hole Oceanographic Institution's Animal Care and Utilization Committee (protocol number DRK no. 3).

Stimulus presentation and evoked potential recording

Once the animal was properly stationed, three custom-built silicone suction cups (KE1300T, Shin-Etsu, Tokyo, Japan) with embedded gold electrodes (Grass Technologies, Warwick, RI, USA) were attached using conductive electrode gel (Signagel, Parker Laboratories, Fairfield, NJ, USA). An active (non-inverting) electrode was placed along the midline of the animal 3–4 cm behind the blowhole. The reference electrode was placed on the dorsal peduncle, and a ground electrode was placed on the animal's tail fin. The electrodes were connected to a biological amplifier (CP511, Grass Technologies), which amplified all responses 10,000-fold and bandpass filtered them from 300 to 3000 Hz. This bioamplifier was connected to a BNC breakout box (2110, National Instruments Corporation, Austin, TX, USA) and a PCMCIA-6062E data acquisition card implemented in a laptop computer. Using a custom-written LabView program (National Instruments), the data acquisition card converted the analog signal to a digital record at a 16 kHz sampling rate. The data were stored on the laptop.

Acoustic stimuli were created using the same custom-written LabView program, laptop, and data acquisition card. Outgoing signals were produced at a 512 kHz update rate. Signal amplitudes were controlled using a HP 350D attenuator and projected to the animal through a custom-built 'jawphone' or suction-cup transducer. This consisted of a Reson 4013 transducer (Slingerup, Denmark) implanted in a custom-built silicone suction cup. The transducer was attached to the animal using the electrogel to eliminate reflective air gaps between the cup and the animals' skin.

To measure location-based receiving sensitivity, the suction-cup transducer was attached at nine specific locations on the animal's head and body. These were the lower melon, jaw tip, throat, lower jaw, mandibular fat pad, overlaying (outward from) the auditory bulla, meatus, back and flipper (Fig. 8). The first seven locations were used to map the relative thresholds of the animal across its head. The back and flipper positions were used as controls. Thresholds were measured using four different stimuli: broadband clicks centered at 100 kHz (see Mooney et al., 2011), and sinusoidally amplitude modulated (SAM) tones at carrier frequencies of 8, 54 and 120 kHz. All stimuli were presented in 20 ms bouts at a 20 s^{-1} rate. Carrier signals within the bout were modulated (tones) or presented (clicks) at a rate of 1 kHz. The individual click stimuli were $20\text{ }\mu\text{s}$ in duration. Evoked response recordings were of 30 ms duration and began coincident with stimulus presentation. Stimuli were presented 1000 times for each sound level and a corresponding response was collected for each sound presentation. These 1000 responses were averaged using the custom-written software and stored for later data analyses. This total number of stimuli and sweeps is relatively standard for odontocetes because it establishes a robust response while averaging down background noise to a relatively low level (Nachtigall et al., 2007). A record for a particular frequency and sound level is then acquired in 50 s and thresholds are obtained in just a few minutes.

Calibrations and data analysis

Suction-cup transducer stimuli were calibrated in the water at the research facility before the experiment using the same sounds as in the hearing tests.

Received measurements were made using a Reson 4040 transducer. The projector and receiver were placed 1 m apart at 1 m depth. The use of the short-duration SAM tone sweeps (i.e. several ms) and relatively low sound levels (i.e. reflections were significantly attenuated relative to the direct 1 m path) reduced the interference of overlapping standing waves and facilitated the calibration. The received signals were viewed on an oscilloscope (Tektronix TPS 2014, Beaverton, OR, USA) and the peak-to-peak voltages (V_{p-p}) were measured. For the SAM tones, this V_{p-p} was converted to peak-equivalent root mean square voltage (peRMS) by subtracting 15 dB. The peRMS was taken as the RMS voltage and used to calculate the sound pressure level (SPL) for that frequency (dB re. $1\text{ }\mu\text{Pa}$). This method is often used for calculating the modulating amplitude of these stimuli, which are most easily initially quantified by their peak-to-peak values (e.g. Mooney et al., 2010; Nachtigall et al., 2008; Nachtigall et al., 2005). SPLs of the clicks were measured using V_{p-p} as is standard for measuring odontocete click intensities due to the inherent brevity of the signals (Au, 1993). Hereafter, dB levels of click SPLs are presented in dB_{p-p} re. $1\text{ }\mu\text{Pa}$ and SAM tone dB are presented in dB_{peRMS} re. $1\text{ }\mu\text{Pa}$. While calibration measurements were in the free- and far-fields, it should be acknowledged that stimuli presented in this manner are inherently near-field stimuli. Thus, this method provides comparisons to hearing in the free-field, but with the caveat that there may be differences between free-field and contact transducer measurements (Cook et al., 2006). Actual hearing differences between methods appear minimal (Finneran and Houser, 2006).

In order to determine the regions of 'best' response, two primary variables were analyzed. The first was the relative threshold of response at the varying positions. The second was the latency of the AEP responses. For thresholds, SPLs started at an estimated 20–30 dB above previous audiogram thresholds of the finless porpoise (Popov et al., 2005). With a detectable stimulus, an envelope following response (EFR) was visible as part of the custom-written AEP program. A 16 ms portion of the response was fast-Fourier transformed (FFT; 256 points) and viewed in the frequency spectrum. The magnitude of the EFR was reflected by a peak in the FFT at the 1 kHz modulation rate (Supin and Popov, 1995). Sound levels were then decreased in steps of 5–10 dB until responses (EFRs and FFT peaks) were no longer visually detectable for two to three trials. Each threshold took 5–10 min to collect and 5–10 thresholds were collected per session. Two sessions were collected per day.

Actual threshold estimates were calculated offline. For each of the frequencies and projecting transducer placements, the FFT peak at each stimulus intensity was a function of the SPL of the stimulus (Nachtigall et al., 2005). A linear regression addressing the data points obtained was extrapolated to zero, the hypothetical point where there would be no response to the stimulus. This zero point had to be extrapolated because the low level of biological electrical noise always present in the records masks the actual zero point. By estimating the zero response level it was possible to predict the threshold for each frequency and transducer placement presented to the animal. For AEPs, multiple methods can be used to calculate thresholds (Finneran et al., 2009; Hall, 2007). This FFT-based method is well established, rapid and, importantly, produces thresholds similar to behavioral techniques (Supin et al., 2001; Szymanski et al., 1999; Yuen et al., 2005), which are considered a standard for sound detection.

Response latencies were measured by establishing the time (ms) between stimulus onset and the point of maximal change in neuronal firing; thus,

latencies were measured as the time value of positive peak of the first four AEP waves, which were designated as wave I, II, III and IV, respectively. These waves were likely the response to the first click or SAM stimulus (Mooney et al., 2006; Supin et al., 2001). This was confirmed by counting back 20 individual waves (from 20 individual stimuli) from the last significant AEP wave in the EFR. Latencies relative to stimulus locations were pooled between animals and calculated using all frequencies.

Latencies relative to stimulus amplitude were compared separately. In this case, time values of wave II–IV peaks were compared with the attenuation from maximum stimulus level. The maximum stimulus level was considered 0 dB and 0 ms delay. The increase in response latency was then plotted for each decreasing stimulus level. Wave I (usually of smallest amplitude) data were not included in these analyses as they were not always detectable at lower stimulus levels. Data were pooled across frequencies and animals. All analyses were conducted using Excel, Matlab and Minitab software. Threshold differences between locations were evaluated with a repeated measures ANOVA and a Tukey's *post hoc* test. While the order of locations from which measurements were taken was random, the repeated measures was used because it assumes there was perhaps some relationship between the variables (for example, multiple thresholds from one animal and the tones and clicks were measured at a location during one session).

CT measures

CT scans were acquired from two finless porpoise specimens that stranded in the month prior to the hearing test experiments. The animals had been frozen and were gradually thawed for scanning. For one specimen, only the head was available for imaging. The specimens were scanned at the Zhongnan Hospital, Wuhan University, on a Siemens Somatom Sensation 16. Both scans were obtained in the transaxial plane using a spiral protocol of 120 kV and 118 effective mAS. Images were formatted at 1 mm slice widths and a 512 matrix in bone and soft tissue kernels.

Attenuation-based segmentations and three-dimensional reconstructions were obtained using Siemens proprietary VRT software. Reconstructions were completed for the skull, mandibles, tympanoperiotic complex, outer mandibular fat body and intra-mandibular fat body based on Hounsfield Unit readings consistent with the characteristic X-ray attenuation values for each of these tissues. This auto-segmentation procedure was followed by visual inspection of the segmented fields to correct any contour errors. Dissections of the specimens were performed to verify the position and dimensions of anatomical structures delineated in the reconstructions.

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Competing interests

The authors declare no competing financial interests.

Author contributions

T.A.M., S.L. and D.R.K. participated in the conception, design and execution of the experiment(s), interpretation of the findings being published, and drafting and revising the article. K.W. and D.W. assisted with execution of the experiment(s), interpretation of the findings being published, and drafting and revising the article.

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