

High frequency echolocation, ear morphology, and the marine–freshwater transition: A comparative study of extant and extinct toothed whales



Carolina S. Gutstein^{a,b,c,*}, Constanza P. Figueroa-Bravo^b, Nicholas D. Pyenson^{c,d}, Roberto E. Yury-Yañez^e, Mario A. Cozzuol^f, Mauricio Canals^a

^a Laboratorio de Ecofisiología, Departamento de Ecología, Facultad de Ciencias, Universidad de Chile, Las Palmeras 3425, Ñuñoa, Santiago, Chile

^b Red Paleontológica U. Chile, Laboratorio de Ontogenia y Filogenia, Departamento de Biología, Facultad de Ciencias, Universidad de Chile, Las Palmeras 3425, Ñuñoa, Santiago, Chile

^c Department of Paleobiology, National Museum of Natural History, Smithsonian Institution, P.O. Box 37012, Washington DC 20013-7012, USA

^d Departments of Mammalogy and Paleontology, Burke Museum of Nature and Culture, Seattle, WA 98195-3010, USA

^e Laboratorio de Zoología de Vertebrados, Facultad de Ciencias, Universidad de Chile, Las Palmeras 3425, Ñuñoa, Santiago, Chile

^f Departamento de Zoologia, Instituto Ciências Biológicas, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627, Pampulha, 31270-910 Belo Horizonte, MG, Brazil

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ABSTRACT

This study compares the bony ear morphology of freshwater and marine odontocetes (toothed whales). Odontocetes are unique among marine mammals in two important respects: 1) they use echolocation; 2) at least three lineages have independently evolved obligate freshwater habits from marine ancestries. Freshwater odontocetes include the so-called “river dolphins,” a paraphyletic group that each evolved convergent external morphological characters that distinguish them from oceanic dolphins (Delphinoidea). In addition to their convergent external morphology, “river dolphins” all have echolocation that use one peak (narrow-band) frequency around 100 kHz, compared to oceanic delphinoids which use a two peak (bimodal) frequency ranging from 40 to 140 kHz. The differences in echolocation suggest that the sensory systems responsible for detecting these different sound frequencies should also differ, although quantitative assessments of the cetacean hearing system remain understudied and taxonomically undersampled. To test if ear bone morphology reflects underlying environmentally driven differences in echolocation ability, we assembled a dataset of odontocete periotics ($n = 114$) from extant and fossil species. We examined 18 external and three internal linear periotic measurements, the latter of which were examined using cone-beam scanning tomography. Results from multivariate canonical ordination analyses show that periotic height, periotic thickness and pars cochlearis width collectively explain the largest amount of interspecific variation in our dataset. Because these particular ear bone measurements correspond to acoustic hearing ranges, we propose that they are also proxies for environmental preference (i.e., marine, freshwater and intermediate habitats) and may be useful for deciphering environmental preferences of extinct odontocetes.

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1. Introduction

Echolocation is a sophisticated biosonar system that has evolved independently in distantly related mammals (tenrecs, shrews, toothed whales, microchiropteran bats and Egyptian fruit bats; Gould, 1965; Gould et al., 1964; Au, 1993; Au et al., 2000) and several bird species (Brinkløv et al., 2013). Toothed whales (Odontoceti) are the only

mammals that have evolved this system for use underwater (Lindberg and Pyenson, 2007), where they use sound to both navigate and locate prey (Au, 1993; Au et al., 2000). To navigate underwater, odontocetes generate and transmit high-frequency sound from the forehead using a complex system of muscles, air sacs, and fat bodies, including the large, conical melon (Cranford et al., 1996; McKenna et al., 2011). Sound emitted from the forehead is then transmitted into the underwater environment, and its returning echoes are received via mandibular fat bodies, which articulate directly with acoustically isolated outer ear bones (i.e., the tympanic bullae) of the skull (Nummela et al., 2004; Cranford and Krysl, 2008). This process has been elucidated through a combination of experimental work directly with live individuals in laboratory settings (e.g., Lawrence and Schevill, 1956; Norris, 1969) and anatomical investigations on postmortem specimens (e.g., Cranford

* Corresponding author at: Laboratorio de Ecofisiología, Departamento de Ecología, Facultad de Ciencias, Universidad de Chile, Las Palmeras 3425, Ñuñoa, Santiago, Chile. Tel./fax: +56 2 8958513.

E-mail addresses: sgcarolina@gmail.com (C.S. Gutstein), fbconstanza@gmail.com (C.P. Figueroa-Bravo), pyenson@si.edu (N.D. Pyenson), robbyury@ug.uchile.cl (R.E. Yury-Yañez), cozzuol@icb.ufmg.br (M.A. Cozzuol), mcanals@uchile.cl (M. Canals).

et al., 2008). Most in vivo experiments investigated marine dolphins (delphinoids), whereas fewer studies have looked at freshwater odontocetes (Herald et al., 1969; Pilleri, 1974).

Freshwater odontocetes are the so-called “river dolphins,” a paraphyletic grouping of four species in three lineages, one of which recently went extinct. “River dolphins” have external morphological characters that distinguish them from marine delphinoids. They include odontocete lineages that are represented by the living genera *Inia*, *Platanista*, and the recently extinct *Lipotes* (Simpson, 1945; Rice, 1998; Hamilton et al., 2001; Nikaido et al., 2001; Pyenson, 2009). *Pontoporia* is typically included among the “river dolphins,” and considered as such in this study, although it predominantly inhabits coastal to estuarine environments. On the other hand, the delphinoid species *Sotalia fluviatilis* inhabits freshwater river mouths of Amazonia, but is rarely included as a “river dolphin” because it lacks the external morphological specializations found in *Inia*, *Platanista*, *Lipotes* and *Pontoporia* (see below).

The first systematic considerations of “river dolphins” (Simpson, 1945; Kasuya, 1973) implied a single evolutionary origin for their freshwater distributions from globally distributed marine ancestors. More recent molecular (Hamilton et al., 2001; Nikaido et al., 2001; May-Collado and Agnarsson, 2006; Steeman et al., 2009) and morphological (Geisler et al., 2011) work confirms the paraphyly of this group. The revised phylogenetic arrangement, along with the biogeography of extant “river dolphins,” supports three independent invasions of freshwater habitats that are associated with a suite of convergent morphological specializations: 1) *Platanista* spp. endemic to the Indus and Ganges basins of South Asia; 2) *Lipotes*, now extinct but formerly endemic to the Yangtze River (Chang Jiang) of China; and 3) *Inia* found in the Amazon and Orinoco basins of South America (Best and da Silva, 1989; 1993).

Intriguingly, “river dolphins” display a suite of convergent morphological specializations that have been widely observed in the descriptive and systematic literature, creating confusion about the relationships of these taxa with other odontocetes. This suite of features includes many traits that diverge from the majority of delphinoids, such as flexible necks with unfused cervical vertebrae; wide, paddle-like flippers; reduced or absent dorsal fins; reduced orbits and eyes; and elongate rostra with lingual accessory cusps on the posterior dentition (Arnason and Gullberg, 1996; Cassens et al., 2000; Hamilton et al., 2001; Heyning, 1997; Rice, 1998; Simpson, 1945; Messenger and McGuire, 1998; de Muizon, 1988a, 1994). Many of these traits have also been identified in fossil odontocetes besides “river dolphins” and could be interpreted as plesiomorphic characters. For example, elongate rostra and complex posterior dentition have been identified in eurhinodelphinids and squalodontids (Fordyce, 1994). However, the molecular phylogenetic framework underpinning the paraphyly of “river dolphins” suggests that at least some of these commonalities are homoplasious adaptations to freshwater habitats.

It is not clear if the morphology underpinning sound generation and reception shows similar convergence. In terms of sound reception, the tympanic bullae and periotics of “river dolphins” have also been noted for their disparity relative to marine odontocetes (e.g., Kasuya, 1973), but detailed comparisons in a quantitative framework are lacking.

Here, we investigate one specific morphological component of sound reception by focusing on the periotic, an element that is often recovered from extant field collections during carcass preparation because it is dense, robust and relatively well protected from initial scavenging, despite being easily lost from decaying carcasses (Schäfer, 1972; Fordyce and de Muizon, 2001). These taphonomic properties also permit the periotic to be well represented in the fossil record of odontocetes (Uhen and Pyenson, 2007), and we thus incorporate data from both extant and fossil odontocetes in this dataset. Previous work (Ketten and Wartzok, 1990; Ketten, 1992) has suggested specific, linear periotic dimensions associated with acoustic signals. We selected many of these measurements, together with traditional ones (Kasuya, 1973),

and included additional new ones (Table 1) to test if external and internal periotic morphology differed between freshwater and marine odontocetes. We included fossils to test environmental assignments based on sedimentological evidence against our morphological data. In this study, we introduced fossil taxa and sedimentological data to provide important temporal and paleoenvironmental contexts that can better constrain evolutionary hypotheses about the origin of the periotic traits.

2. Materials and methods

2.1. Materials

Our dataset includes linear measurements (Table 1) from the periotics of extant and extinct odontocetes that correlate with known acoustic frequencies (see Table 2, Fig. 1). We collected a total of 18 measurements from 114 specimens that covered the following taxonomic breadth: fossil and extant Delphinidae ($n = 28$); fossil Delphinoidea indeterminate ($n = 1$); fossil and extant Iniidae ($n = 16$); fossil Inioida indeterminate ($n = 7$); fossil Kentriodontidae ($n = 24$); extant Monodontidae ($n = 1$); fossil Odobenocetopsidae ($n = 2$); extant and fossil Phocoenidae ($n = 20$); fossil Physeteridae ($n = 1$); fossil and extant Platanistidae ($n = 3$); fossil Platanistoidea ($n = 2$); and fossil and extant Pontoporiidae ($n = 9$) (see Table S1 for detailed taxonomy of less inclusive taxa).

2.1.1. Institutional abbreviations

IDSM: Instituto de Desenvolvimento Sustentavel Mamiraua, Tefe, Brazil; MACN: Museo Argentino de Ciencias Naturales, Buenos Aires, Argentina; MLP: Museo de La Plata, La Plata, Argentina; MNHN: Museum national d'Histoire naturelle, Paris, France; MPC: Museo Paleontológico de Caldera, Atacama Region, Chile; SGO-PV: Area Paleontología, Museo Nacional de Historia Natural, Santiago, Chile; UFSC: Laboratorio de Mamíferos Aquáticos, Departamento de Ecologia e Zoologia, Universidade Federal de Santa Catarina, Florianópolis, Brazil; USNM: Department of Paleobiology and Division of Mammals, Department of Vertebrate Zoology, National Museum of Natural History, Smithsonian Institution, Washington, District of Columbia, U.S.A.

2.2. Methods

2.2.1. Computed volumetric tomography

We evaluated the internal cochlear morphology of the periotics (Supplementary Table 1) in our dataset using computed tomography (CT), a technology that permits high-resolution and precise imaging of small and obscured anatomical structures without modifying or destroying original voucher specimens. Specifically, in this study, we used volumetric CT with a cone beam array (I-Cat, from Imaging Science International), which provided resolution of the internal periotic morphology without gaps and with 0.2 mm precision. CT scanning with the cone beam method allowed us to scan an entire set of periotics oriented in the same plane (fixed to a wood plate with wax), thereby avoiding the effects of reflection (i.e., mirroring), and damage to the specimens. Lastly, this method produced a relative density spectrum for the specimens. The resultant DICOM images were analyzed and rendered in OsiriX (Rosset et al., 2004); we used the open polygon tool to collect all of the measurements. The measurements are described in Table 1 (see also Fig. 1).

2.2.2. Environment

To test for environmental correlations, we pre-classified the data matrix with groupings based on the observed habitat environment, as follows: riverine; fully marine; and coastal-estuarine (including the categories of coastal, shallow and epicontinental sea with freshwater input; see S1 and S2). In the case of the fossil specimens, the environmental categories were defined compiling locality, horizon, geologic

Table 1
Description of measurement made in the periotics. See also Fig. 2. The measurements follow Kasuya (1973), Ketten and Wartzok (1990) and Ketten (1992), with the exception of new measurements proposed in the present work, indicated with *. The measurements taken through cone-beam tomography images are indicated with **.

Measurement number	Description
1	Total length of periotic
2	Width of pars cochlearis from anteriormost point to cochlear window
3	Width of internal acoustic meatus
4	Length of internal acoustic meatus
5	Length of anterior process: from anterior incisure to the anteriormost point of the anterior process
6	Length of anterior process: from parabular process to the anteriormost point of the anterior process
7	Maximum diameter (antero posterior) of the malleus fossa
8	Total width of the pars cochlearis
9	Distance from fossa incudis to the apex of the pars cochlearis (medially)*
10	Total width of the periotic in the median region from the epitympanic hiatus to the apex of the pars cochlearis (medially)
11	Height of pars cochlearis from vestibular window to the apex of the pars cochlearis (medially)
12	Cochlear window diameter (medio laterally)
13	Width of the fossa for stapedial muscle
14	Depth of the fossa for stapedial muscle*
15	Maximum thickness of pars cochlearis
16	Minimum thickness of pars cochlearis on its apex
17	Width of epitympanic hiatus
18	Depth of acoustic internal meatus
19	Height of pars cochlearis in transversal view**
20	Maximum diameter of the cochlea, in transversal view**
21	Maximum thickness of the pars cochlearis, in transversal view*
22	Maximum dorsoventral expansion of the cochlear canal from the apex to the base, in transversal view**

unit, sedimentologic and paleoecologic data available for each specimen in the literature (e.g., Achurra, 2004; Achurra et al., 2009; Cione et al., 2005a, b; Visaggi and Godfrey, 2010; Ward and Andrews, 2008; Whitmore and Kaltenbach, 2008; see also S1 and S2). For the extant specimens, the distribution data of the species were compiled from literature (Reeves et al., 2002; Shirihi and Jarrett, 2009; see S1). Although data on the environmental context for fossil taxa may reflect a variety of taphonomic biases (Uhen and Pyenson, 2007), we assumed that the depositional environment for these specimens was effectively similar to their original habitat.

2.2.3. Taxonomy

To compare environmental signals of the dataset with phylogenetic history, we also grouped our data taxonomically by family and genus levels following Steeman et al. (2009) and Geisler et al. (2011; see Table S1). Although supra-familial relationships among extant cetaceans, especially among odontocetes, have not yet reached a consensus, we decided to use Steeman et al. (2009) and Geisler et al. (2011) as a proxy for phylogeny. We allotted our taxa among the following stem and crown groups (stem-Inioidea, Iniidae, Pontoporiidae, Delphinidae, Kentriodontidae, Phocoenidae, Platanistidae, stem-Platanistoidea, *Odobenocetops* + *Delphinapterus*) and superfamily level (Platanistoidea, Inioidea, Delphinoidea).

2.2.4. Statistical analysis

The measurements were analyzed in two different ways: first as raw data; and then divided by the total width of the median portion of the periotic (Fig. 1, Table 1; measurement 10), to control for size disparity. We then conducted multivariate analysis of variance and canonical variate analysis (MANOVA and CVA, respectively) using PAST 2.11 (Hammer et al., 2001). We used the CVA biplot option in PAST to interpret the canonical axes as they scale CVA loadings by the pooled within-group covariance matrix (see SI.3 and SI.4).

The main measurements in the multivariate analysis biplots were then analyzed in a paired comparison analysis, with a Kruskal–Wallis ranked test for significance, which assessed the differences among the selected measurements. We also compared pairs of variables that had the largest values in the CVA loadings (Fig. SI.3.2) and accounted for the most variance in canonical axes. This step allowed us to compare morphological differences between groups in a simpler way than allowed by the multivariate analyses.

3. Results

3.1. Morphology

Our observations of periotics revealed five qualitative groupings that are summarized in this section. These periotic groupings are described among three main anatomical portions (sensu Mead and Fordyce, 2009): the periotic processes (anterior and posterior); the pars cochlearis; and the inner morphology of the cochlea, which were clearly recognized, along with the cochlear duct, from cone-beam tomography imaging. The cochlear duct itself is located inside the pars cochlearis and it is positioned with its base at the ventral surface of the periotic and the apex at the dorsal surface of the periotic, connecting to the acoustic meatus (see Fig. 1 for periotic orientations). When possible, we discussed the inner cochlear duct morphology and orientation in connection to the external pars cochlearis morphology (sensu Mead and Fordyce, 2009:111–133, and references therein).

In general, we were able to determine that external pars cochlearis morphology consistently discriminated riverine from marine taxa. For example, the riverine odontocetes studied herein (e.g., *Inia*, *Platanista*, and an iniid from the Ituzaingó Formation of Argentina [MACN 9231]) presented a consistently rounded, slender and high pars cochlearis morphology (Fig. 2). By comparison, marine and estuarine taxa (e.g., *Sotalia guianensis* and *Pontoporia*, Fig. 2) exhibited a dorsal-ventrally globose and thick pars cochlearis. These external, morphological distinctions paralleled cone-beam tomography results (see S1), which showed that the larger cochlear duct sizes directly corresponded to larger external pars cochlearis sizes (see Fig. 3).

The first morphological category is characterized by the periotics of *Platanista*. These periotics were larger, in absolute size, than every other odontocete periotic in this dataset. Notably, the pars cochlearis in *Platanista* was oval in shape, with a rounded medial surface and rectilinear anterior and posterior surfaces, which can be observed clearly in ventral and dorsal views (Fig. 2). The internal acoustic meatus was circular in shape, as seen medially, which is a condition only observed in other platanistoid periotics (e.g., the extinct *Notocetus*). The anterior process in *Platanista* was elongate and robust, while the posterior process was reduced and narrow. The anterior process showed a noticeable anteromedial deviation. The lateral surface (in ventral view) was expanded, as in *Inia geoffrensis* and the Ituzaingó Formation iniid (MACN 9231; Fig. 2).

Table 2

Comparisons of periotic morphology, habitat preference and frequency preferences. CT) Computed tomography; MRI) Magnetic resonance. The frequencies included are the best frequency and frequency range, respectively.

Taxa	Description	Pars cochlearis dorsoventral/mediolateral profile	N turns/cochlear duct type	Emitted frequency (kHz)	Acoustic properties of the echolocation beam	Data source
Delphinidae						
<i>Stenella attenuata</i>	Marine	?	2.5 ^a Type II	40–140 40–60 and 120–140	Bimodal	Wartzok and Ketten (1999), Schotten et al., 2004
<i>Tursiops truncatus</i>	Marine	Globose/rounded	2.25 ^a Type II	34.5–131.9 67 and 114	Bimodal	Nakamura (1999 in Morisaka and Connor, 2007), Wartzok and Ketten (1999)
<i>Sotalia fluviatilis</i>	Riverine	Globose/rounded	2 ^b	88.35 (SD = 3.01)	?	CT scan, May-Collado and Wartzok (2010)
<i>Sotalia guianensis</i>	Estuarine	Globose/rounded	1.75 ^b	80–95; 64–105	?	Cone beam CT, Kamminga (1988), Kamminga et al. (1993), Sauerland and Dehnhardt (1998)
Delphinidae indet.	Marine	Globose/rounded	1.5 ^b Type I	?	?	Cone beam CT
Phocoenidae						
<i>Phocoena spinipinnis</i>	Estuarine	Slender/pointed	1.5 ^b Type I	?	?	Cone beam CT
<i>Phocoena phocoena</i>	Estuarine	Slender/pointed	1.5 ^a Type I	118–128; 139	Narrow-band	Ketten (1992), Au et al. (1999, 2006)
<i>Neophocaena phocaenoids</i>	Coastal/riverine	Slightly flattened	?	125; 87–145	One-peak	Li et al. (2005), Nakamura (1999 in Morisaka and Connor, 2007)
Monodontidae						
<i>Delphinapterus leucas</i>	Coastal/estuarine	Slightly flattened	2.25 ^b	71 and 112; 46.6–125.7	Bimodal	Cone beam CT
Pontoporiidae						
<i>Pontoporia blainvillei</i>	Estuarine	Globose/rounded	1.75 ^b Type I	130 (SD = 10)	Narrow-band	Cone beam CT, von Fersen et al. (2000)
Iniidae						
<i>Inia geoffrensis</i>	Riverine	Slightly flattened	1.5 ^a Type II 2 ^b	Emitted 16–170 (best Freq. 60–80); 85–100auditory sensitivity 75–90	Narrow-band (narrow sonar beam 20–30°)	Cone beam CT, Evans (1973), Kamminga and Weirsmas (1981), Weirsmas (1982)
Platanistoidea						
Squalodontoid	Marine	Slightly flattened	1.75 ^b Type I	?	?	Luo and Marsh (1996)
cf. <i>Notocetus vanbenedeni</i>	Marine	Slightly flattened	1.75 ^b Type I	?	?	Cone beam CT (referred specimen)
Platanistidae						
<i>Platanista gangetica</i>	Riverine	Slightly flattened	?	15–60/20–100	?	Herald et al. (1969)
Physeteridae						
Physeteridae indet.	Marine	Globose/rounded	1.75 ^b	?	?	Cone beam CT
<i>Physeter catodon</i>	Marine	Globose/rounded	1.75 ^a	15, 5–24	One peak	Madsen et al. (2002)

^a Taken from Wartzok and Ketten (1999).^b Present work.

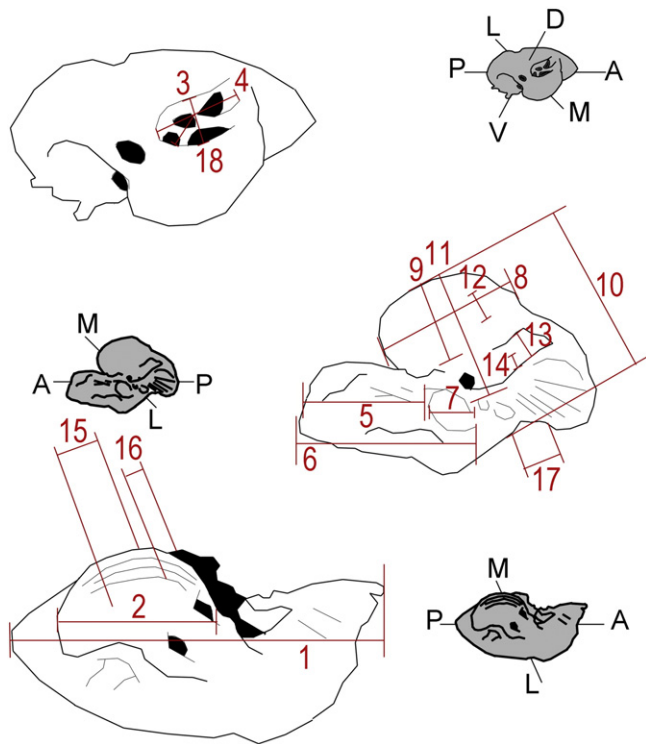


Fig. 1. Schematic drawings of a generalized odontocete periotic showing measurements used herein. The anatomical positioning of the views are indicated in the shadowed schemes: A) anterior, P) posterior, L) lateral, M) medial, D) dorsal and V) ventral. For a detailed description of the measurements, see Table 1.

The second morphological category is characterized by *Inia*, which presents an extreme reduction and anteroposteriorly orientation of the anterior and posterior processes. The pars cochlearis was rounded (in ventral view) and more slender (in medial view), with a marked, mediolaterally oriented sulcus. The internal acoustic meatus was circular as in *Platanista*, but it was not visible in the medial view. Compared with fossil Iniidae from our dataset, *Inia* only differed by an absence of the pars cochlearis sulcus and the oval shape of the internal acoustic meatus. MACN 9231 showed a widely exposed facial canal.

The third morphological category, in contrast to *Platanista* and *Inia*, was an overall more slender morphology, characterized by *Phocoena*. The anterior process here was narrow and both processes were largely separated from the pars cochlearis, whose lateral surface was almost absent, in contrast to *Platanista* and iniids.

The fourth category, characterized by *Brachydelphis mazeasi*, a fossil inioid, had a relatively small periotic, with a diminished anterior process comparable to *Pontoporia* and *Pliopontos*. *Brachydelphis* differed from these latter taxa by having a broad and rounded posterior process and a pars cochlearis triangular in shape with a clear, medially pointed apex. The anterior process was pointed in its anterior extremity and bent medially, with it being closer to the pars cochlearis. The lateral surface of the anterior process was also somewhat expanded, in contrast to phocoenid and pontoporiid morphology.

The fifth and last morphological category is represented by *Delphinodon dividum*, which is broadly similar to extant delphinids in periotic morphology. Here the anterior process is elongate and rectangular and deflected in an anteromedial direction. The pars cochlearis of *D. dividum* was oval in shape, similar to *Platanista* (see above,

Fig. 2). The internal acoustic meatus was oval in shape, with the endocranial opening of the facial canal exposed dorsally. Its lateral surface was straight and slightly expanded, with the tympanic hiatus anterior–posteriorly elongated when compared to *Brachydelphis*, for example.

3.2. Cochlear duct versus pars cochlearis shape

The volumetric tomography performed for nine specimens of different groups (see Table 2 and SI.1) provided access to the cochlear and general inner ear bony morphology in a non-destructive manner. The cochlear shape varied from a globose pars cochlearis and expanded cochlear duct to a more compressed cochlea with a similarly flattened pars cochlearis (Fig. 3). We noted that a bulbous profile of the pars cochlearis generally corresponded to a more dorsoventrally expanded cochlear duct, as observed in *Sotalia guianensis*. Equally, we noted that a slender pars cochlearis profile corresponded to a dorsoventrally compressed cochlear duct in *Inia*. The relationship between both features was more diffuse in *Phocoena*. These patterns are largely quantified in the results from our analyses, which showed that the main variables in the environmental morphometric analysis were pars cochlearis proportions, as detailed below (see 3.3, Figs. 4–6). Measurements from the internal portion of the cochlea, including the cochlear duct (i.e., the maximum diameter of the cochlear duct in the base and the maximum distance between the apical and basal portion of the cochlear duct; see Wartzok and Ketten, 1999), were strongly correlated with measurements taken at the external portion, the pars cochlearis. Specifically, the inner measurements corresponded to 59–74% of the external bony measurements, including different species from different clades and geologic ages (see Fig. 2).

The pars cochlearis diameter or width was not recovered as an accurate representative of the maximum diameter of the cochlea. Nevertheless, the differences between thickness and the width of the cochlear duct, as observed in the external pars cochlearis morphometrics, hinted at a positive correlation, where *Inia*, the only riverine species examined with tomography, showed one of the smallest cochlear duct heights in the dataset (Fig. 7). On the other hand, the fully marine species were distributed at the opposite extreme of the scatterplot in Fig. 7, showing the largest cochlear duct heights. Estuarine or coastal species were located between these extremes, yet they appeared to group closer to marine taxa.

3.3. Morphometry

The main features of the periotic morphology that varied across taxa and environment were the relative proportions among the pars cochlearis, and the anterior and posterior processes. Previous authors have noted that the relative orientation of the latter features, along with their associated foramina, were valuable for discriminating among odontocete taxa at the generic level (Kasuya, 1973; Barnes, 1985; de Muizon, 1988a). Here, we have determined that some of these characteristics also clustered into discrete groups based on known environmental preferences. For example, the pars cochlearis shape was strongly correlated with environment: cochlea from extant “river dolphins” was taller, more rounded, and slender than those from oceanic delphinoids (Fig. 4).

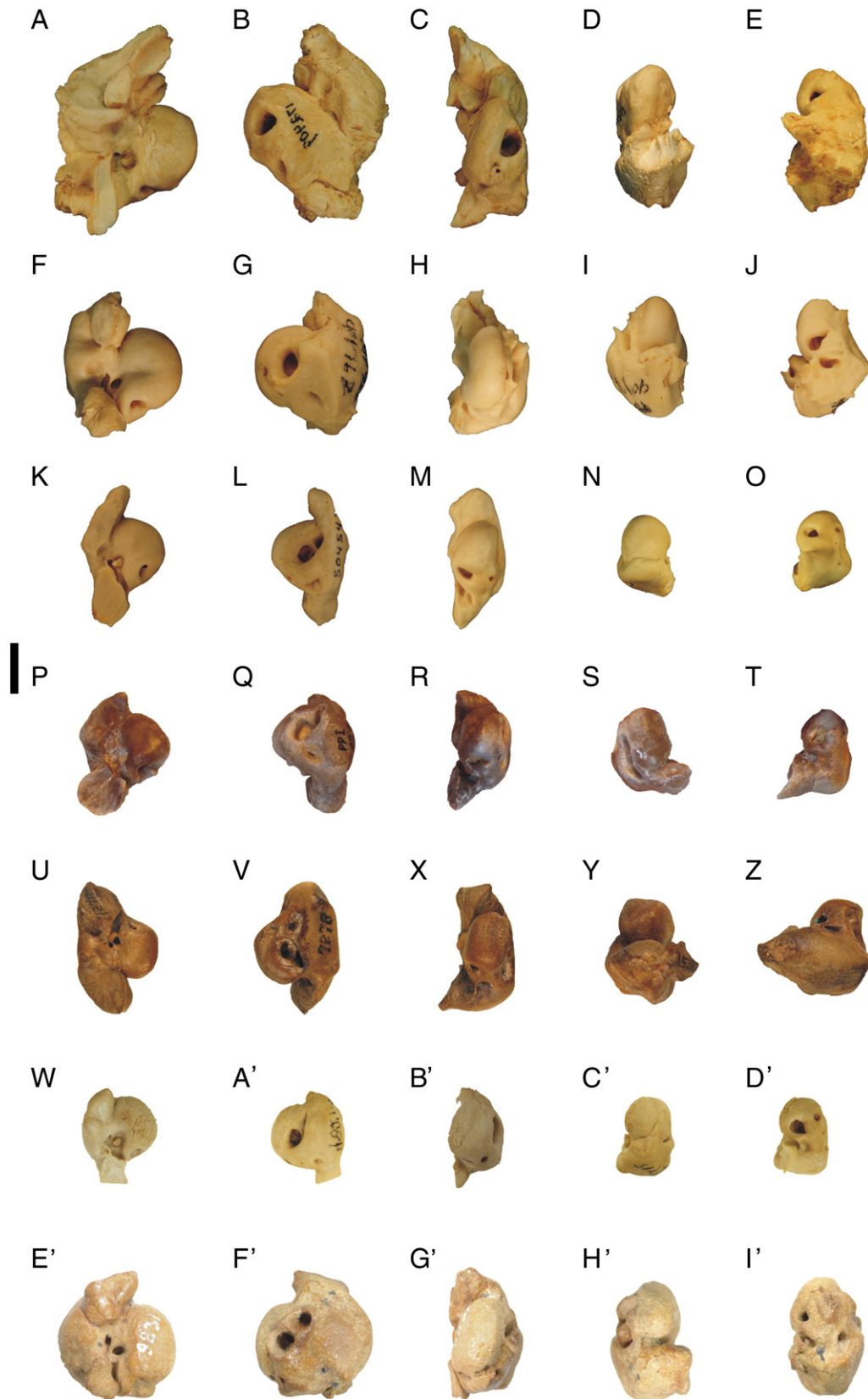
3.3.1. Morphometry and taxonomy

Significant results were not recovered for genus-level taxonomic groups in our CVA results. Nevertheless, groupings at family-suprafamilial levels (Fig. 5) recovered some of the groups as

Fig. 2. Periotics of selected taxa in the dataset (for a complete list of taxa see S1). The periotics are shown, from left to right, in ventral, dorsal, medial, posterior and anterior views. Each row shows a species, as follows: A–E) *Platanista gangetica* USNM 176409; F–J) *Inia geoffrensis* USNM 396166; K–O) *Phocoena phocoena* USNM 504543; P–T) *Brachydelphis mazeasi* (MHNH PPI 121; U–Z) *Delphinodon dividum* (USNM 7278); W–D’) *Pontoporia blainvillei* (USNM 482715). E’–I’) Iniidae from Ituzaingó Formation, Entre Rios, Argentina (MACN 9231) Scale bar = 1 cm.

significant, although the first canonical axis only explained 64% of the variation, and both of the axes explained together less than 80% of the total variation. Notably, Delphinidae were significantly different from all groups (except Platanistidae), with Kentriodontidae,

Pontoporiidae, Iniidae, and Phocoenidae following Delphinidae in descending order of significant differences in the pairwise comparisons. Regardless, the most important measurements in both canonical axes were the diameter of the malleus fossa (measurement 7) and the length



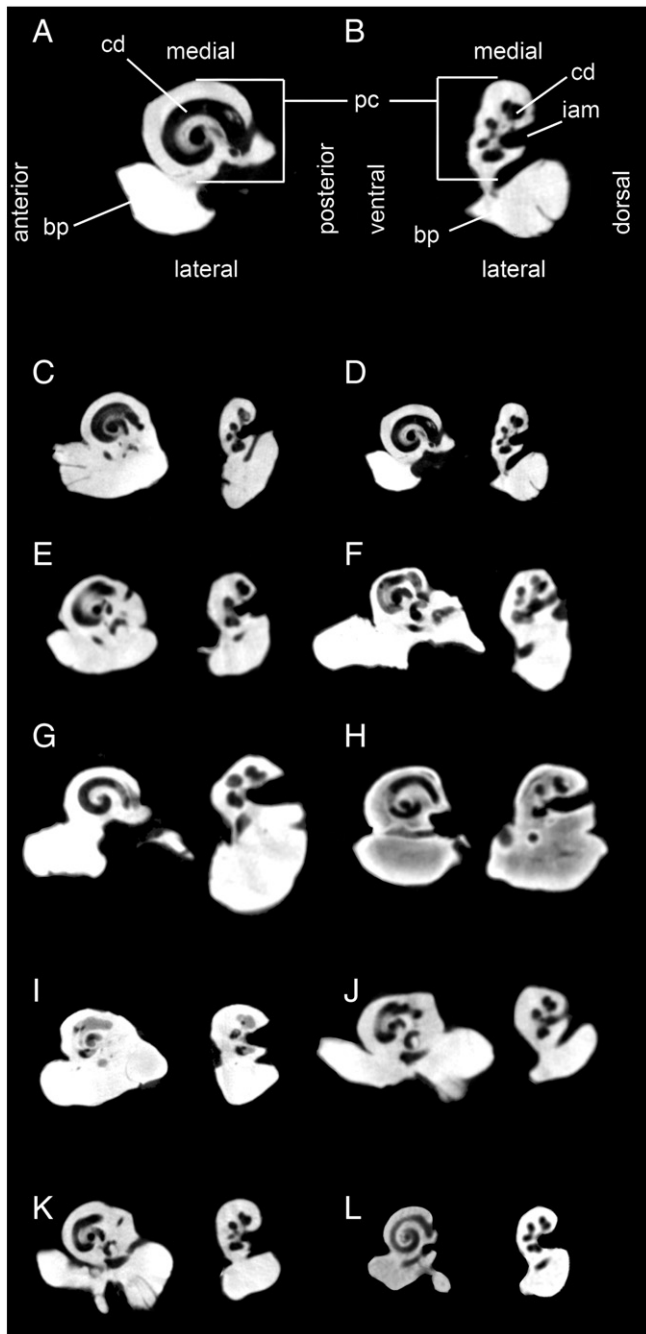


Fig. 3. Cone beam tomography slices of ten periotic specimens at coronal (A) and transversal (B) cuts. C) *Inia geoffrensis* MCN-M32, D) *Phocoena spinipinnis*, E) *Sotalia guianensis* UFSC 1293, F) cf. *Notocetus vanbenedeni* MLP 76-IX-25, G) Platanistoidea indet. MPEF-PV 517, H) Delphinidae indet. MLP 76-IX-2-7, I) *Delphinapterus leucas* MLP1484, J) Physeteridae indet., K) *Pontoporia blainvillei* UFSC 1093, L) *Sotalia fluviatilis* USNM 504316. Anatomical abbreviations: cd) cochlear duct, i.a.m.) internal acoustic meatus, pc) pars cochlearis, bp) body of periotic.

of the anterior process (measurements 5 and 6), although the latter was less important than the former.

3.3.2. Morphometry and environment

In the test for correspondence of these morphometric features with their environmental origin, the CVA results showed significant differences between each pairwise comparison (see SI.3, Table SI.3.1) with each environmental group (i.e., morphometry versus fully marine, coastal/estuarine, and riverine; see S1). The first canonical axis explained 87.5% of overall variance, where the most important

measurements were: length of periotic (measurement 1); width and height of the pars cochlearis (measurements 2, 8, and 11); length of acoustic internal meatus (measurement 4); and width of the periotic (measurement 10). Thus, the majority of these measurements were related to the pars cochlearis shape. Variance for the second canonical axis was represented by the internal acoustic meatus (measurement 3) and the width of the pars cochlearis (measurement 8), but only 12.5% of the variance in the data was explained by this axis. Moreover, there was little differentiation among the groupings on this axis.

For the size-independent dataset, CVA results were similar to the raw data, with the first canonical axis explaining 87.1% of the data and dominated by the following measurements (Fig. SI.3.2): periotic length (measurement 1); malleus fossa size (measurement 7); pars cochlearis height (measurement 11); and anterior process length (measurement 5). Additionally, the most relevant measurement in this size-corrected analysis was the maximum thickness of the pars cochlearis (measurement 15), which explained approximately 20% of the first canonical axis alone. For the second canonical axis, only 12.9% of the variance was explained, with little difference among the possible groupings. For this latter axis, the important measurements were width of the fossa for the stapedial muscle (measurement 13), the maximum and minimum thicknesses of the pars cochlearis (measurements 15, 16), the epitympanic hiatus width (measurement 17), and the internal acoustic meatus depth (measurement 18), which combined accounted for approximately 56% of the variance along the second canonical axis.

We also compared inner ear morphology, from tomography data, against environmental classifications because previous authors have suggested that inner ear measurements are functionally relevant to hearing (Wever et al., 1971; Ketten, 1992; Wartzok and Ketten, 1999; Miller et al., 2006). The morphometric analyses of these measurements (Table 1) recovered the pars cochlearis external width, height, and thickness as significant variables. As expected, these linear dimensions essentially correlated with inner cochlear shape and length. More importantly, these analyses group specific taxa into cochlear types proposed previously (Wever et al., 1971; Ketten and Wartzok, 1990; Ketten, 1992).

In this sense, the paired comparisons (measurement versus measurement) revealed that the most significant differences between the environmental groups ($p < 0.01$) were thickness versus height and width of pars cochlearis (Fig. 6). These results are in agreement with the aforementioned functional scheme, where the more elongate and thinner pars cochlearis morphology reflected the cochlear duct morphologic types I and II (see Section 4.3 for further discussion). Our results also demonstrated that the shape of internal acoustic meatus was significantly correlated with environmental type: namely, its width (measurement 3) explained the variance of the coastal and estuarine group and its length (measurement 4) correlated with the riverine group. The significance of the internal acoustic meatus, however, was secondary to the aforementioned, primary measurements.

3.3.3. Environmental classification of fossil taxa

We generated a priori environmental categorizations for fossil specimens by searching the source literature for associated sedimentologic and paleoecologic data (as detailed in Section 2, S1 and S2). Although these data inform only about the environment of final deposition (thanatocoenosis), we view these data as reasonable approximations of the original source habitat (biocenosis) for these extinct taxa based on the general fidelity of extant death assemblages to their source communities (e.g., live-dead studies detailed by Pyenson, 2010; 2011). Using the periotic CVA scores, 94 out of 110 total fossil odontocetes were correctly assigned habitat classifications consistent with their geological context.

Nevertheless, the post hoc predictions of the CVA results can also work as tests for the likely original habitats of extinct odontocetes. The analyses conducted herein permit the categorization of fossil taxa to specific environmental types. For example, *Delphinodon dividum*, a

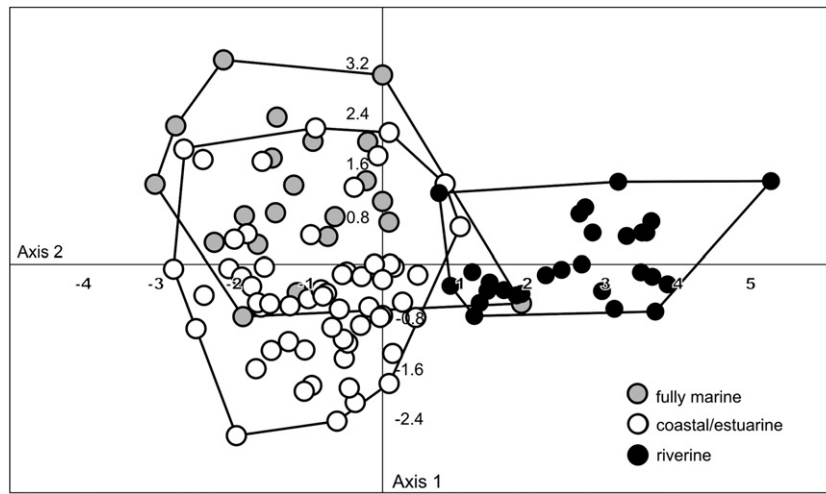


Fig. 4. Scatterplot of the CVA analysis performed in PAST (Hammer et al., 2001) of the three environmental groups, where only the marine–riverine and riverine–estuarine pairs were significantly different ($p < 0.01$) (see also S1 and S2).

kentriodontid from the Miocene of the western Atlantic Ocean, was similar to *Delphinapterus* in having CVA classify it as a riverine taxon, dictated mostly by its slender pars cochlearis profile. Another fossil taxon, *Odobenocetops*, an enigmatic walrus-convergent odontocete known from the Pliocene of Peru (de Muizon, 1993) was also an outlier, with results spread across the total distribution of the coastal/epicontinental/estuarine specimens. Such a difference could easily be interpreted as a consequence of its relatively large size, but the size-independent matrix showed that it nonetheless was located at the extremes of the scatterplot distribution.

Other specimens misclassified (i.e., a priori classification differing from statistical predictions) were: *Lophocetus pappus* and *Lophocetus calvertensis* from the Calvert Fm.; *Brachydelphis mazeasi* from Pisco Fm. (given: coastal; predicted: fully marine); *Phocoena phocoena* (given: marine; predicted: riverine); and *Kentriodon* sp. from the Calvert Fm. (given: fully marine; predicted: coastal/estuarine). Finally, *Platanista* showed conflicting results between its given (riverine) category and its predicted (fully marine) one. The jackknife results from the predicted categories added 10 more misclassified taxa: *Inia geoffrensis*; *Neophocaena phocaenoides*; three specimens referred to Pontoporiidae indet. (two as fully marine and one as riverine); and two additional *L. pappus* specimens.

4. Discussion

4.1. Acoustic parameters and morphology

The volumetric tomography data (nine specimens of different groups) were slightly different from the overall patterns observed by Ketten (1992) and Wartzok and Ketten (1999). These authors stated that higher frequency hearing (type I) would be associated to more compressed cochleae, which have fewer turns and a greater expansion of the outer osseous spiral lamina along the length of the basilar membrane (Fig. 3, Table 2). This functional implication is possible in light of basilar membrane morphometrics (e.g., the width to thickness ratio) and the outer spiral lamina extension as acceptable proxies of stiffness of this membrane (von Bekesy, 1960; Wever et al., 1971; Ketten, 1984) at a given scale (for a review see Miller et al., 2006) permitting a relative definition of echolocation types. These features allow the morphologic discrimination of the two main types of echolocation observed in living species.

In riverine and coastal species, there is a low frequency cut off in the echolocation beam, which produces a narrow-banded click structure, while fully marine species present both, high and low peak frequency, producing a bimodal sound structure (e.g., *T. truncatus* and *S. attenuata*;

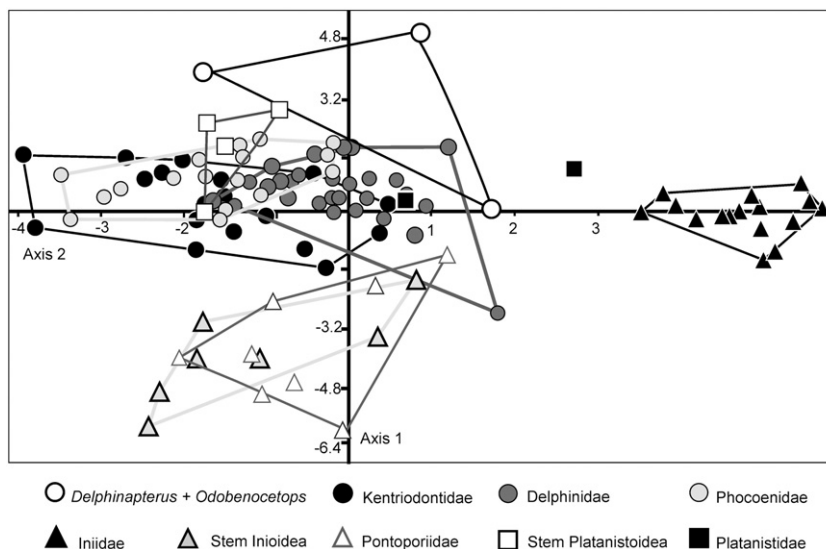


Fig. 5. Scatterplot of the CVA analysis of the taxonomic groups, divided in stem and crown taxa of the different super-families of Odontoceti (see also S1).

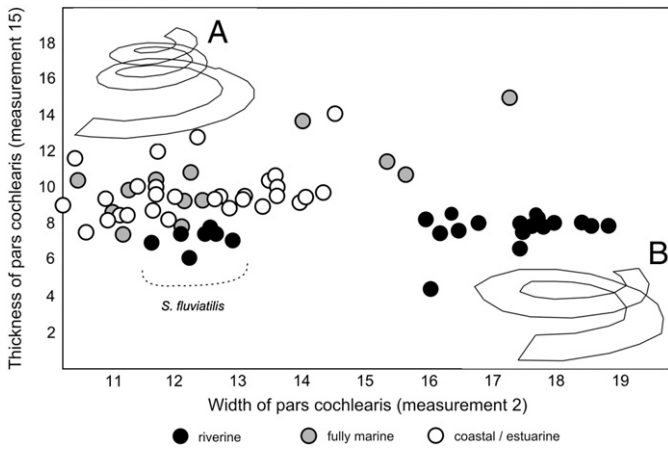


Fig. 6. Scatterplot of thickness versus width of pars cochlearis and schematic drawing of a cochlear duct in *Sotalia guianensis* (A) and *Inia* (B).

Table 2, Miller et al., 2006). This is slightly different from the scheme proposed by Wartzok and Ketten (1999) of cochlear types, where type I cochlea and echolocation high peak frequency (100 kHz) would be associated with a more broad, turned and contracted cochlea, that have the base to apex axis oriented rather ventromedially than

dorsoventrally. Type II cochleae would have the base to apex axis oriented dorsoventrally, with a more expanded cochlea associated with broader echolocation ranges including a lower peak frequency (~40 to 70 kHz). When the acoustic parameters used for echolocation in living odontocetes are compared with morphology, taxonomy and environment (Table 2), it is notable that the acoustic properties sort mainly by emitted frequencies in two ways: first, with a low-peak frequency, giving a bimodal structure to the echolocation beam observed in marine species (not measured here) as *T. truncatus* and *S. attenuata* and other delphininids (see also Morisaka and Connor, 2007; Wartzok and Ketten, 1999); and, second, with riverine species emitting only a high peak frequency (Miller et al., 2006).

To explain these two different patterns, Morisaka and Connor (2007) suggested that the narrow-band echolocation clicks (emission) pattern and its low frequency cut off was linked to the loss of whistle (emission) in *Pontoporia*, *Cephalorhynchus* and the Phocoenidae family. Nevertheless, here we only could confirm that there is a relationship between environment and the shape of the cochlear duct and pars cochlearis (i.e., part of the hearing system). In this sense, it is also plausible to suggest that the morphological differences (type I and type II, hearing) are functionally related to the low frequency cut off, where the frequency range is reduced to one (instead of two) narrow-band higher frequency beam (emission) in accordance with the possible loss of the cochlear portion responsible for hearing at “lower” frequencies. In any case, the emitted frequencies may be not very different than the actual acoustic sensitivity (hearing). For example, in *Inia* the auditory sensitivity is at a lower frequency than the one emitted, but only by a small amount (~10 kHz; see Table 2).

4.2. Ear morphology correspondence to echolocation type and habitat

The correspondence between odontocete cochlear morphology (both inner and external) and echolocation type was proposed in the literature 40 years ago, with some reinterpretation in subsequent, but singular studies (Wever et al., 1971; Ketten, 1992; Wartzok and Ketten, 1999; Miller et al., 2006). Our study is the first to integrate a morphometric dataset (including extant and extinct taxa) within a robust statistical context. We found that the inner cochlear and the external pars cochlearis morphology were tightly correlated and we propose that these features can be used as ecomorphologic indicators of environmental preference, for both extant and extinct taxa. We nonetheless caution that the accuracy of these correlations depend on appropriate and independent contexts (i.e., sedimentological data). The robust results showing the discrimination of riverine versus marine and coastal-estuarine classifications for fossil and extant odontocetes strongly suggest the potential for periotic features to serve as valuable indicators of environmental preference, primarily based on the correspondence of these structures to the frequency range of hearing and the particular acoustic properties of water in these environments.

Our study fits squarely in previous sets of observations that notes the external and osteological convergences among the so-called “river dolphins” (Simpson, 1945; Rice, 1998; Hamilton et al., 2001; Nikaido et al., 2001; Geisler et al., 2011). In parallel, there has also been indication that functional aspects of “river dolphin” echolocation has converged on similar solutions for producing and receiving sound in an environment unlike the putative oceanic conditions of their ancestors. The freshwater systems inhabited by extant “river dolphins” differ in acoustic and optic properties from marine ones, including: water temperature, which alone can affect the propagation of echolocation signals (Wartzok and Ketten, 1999); turbidity; and complex, physical obstacles that are seasonally present in the flooded forest environments of Amazonia, for example (Martin and da Silva, 2004; Martin et al., 2004). It is plausible that such environmental differences in the acoustic properties of riverine (i.e., high temperature, low visibility, small areas full of obstacles) versus marine (i.e., lower temperature, high visibility,

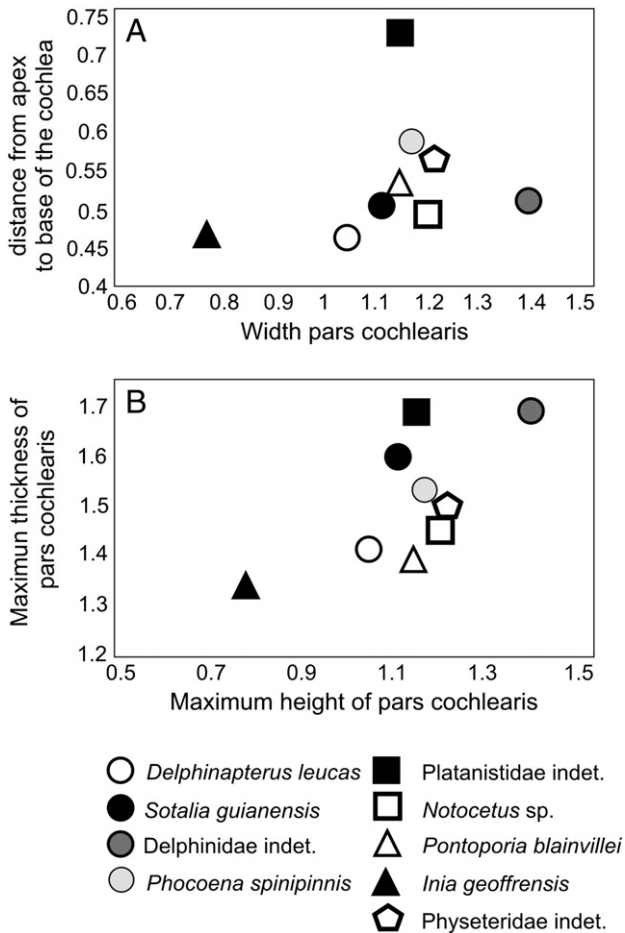


Fig. 7. Comparisons between A) pars cochlearis (external; x) and cochlear duct proportions (internal; y); and B) cochlear duct maximum height (x) versus maximum thickness (y). For descriptions of the measurements, see Table 1.

wide-open areas) habitats have led to the different ecomorphologies of marine and riverine dolphin hearing systems. Alternatively, different prey types could also be a factor in sound production differences, but there is no evidence for this supposition currently (see Madsen et al., 2004).

Interestingly, the non-functional measurements, here considered as control measurements (e.g., anterior process size [5, 6]), were not important in the environmental analysis, with the only exception of the total length of the anterior process, although they were influential for discriminating taxonomic groups. de Muizon (1988a) originally observed that the absolute and relative size of the processes correlated with taxonomic groups, but measurements related to other functional structures, such as the size of the malleus fossa and internal acoustic meatus proportions, were secondarily important in most of our analyses.

4.3. Periotic morphology and environmental classification

In some instances, CVA results grouped the periotic shapes of some taxa in conflict with their actual environmental type. For example, our results classified *Delphinapterus leucas* as riverine, despite its actual coastal to estuarine habitat. This result is interesting not only because *Delphinapterus* inhabits an intermediate environment (e.g., some populations in the St. Lawrence Seaway, which is mixed coastal and estuarine), but it also possesses a unique echolocation type. Behavioral studies of show *Delphinapterus* emitting click trains (rather than isolated clicks; Madsen et al., 2004) at very high frequencies, but with a bimodal structure (70 and 112 kHz), a feature that is congruent with their cochlear duct morphology (Type I, 2.25 turns; Table 2). Some have attributed this unusual sound emission pattern to their ability to survive in pack ice-dominated environments (Madsen et al., 2004). *Delphinodon dividum* was similar to *Delphinapterus* in the multivariate analysis. We suggest that such findings should be considered in any further paleobiological interpretations for this taxon. Likely, *Delphinodon* would be rather coastal-estuarine than fully marine or at least would have a more similar kind of echolocation to coastal-estuarine taxa here analyzed. In *Odobenocetops* the overall very distinct morphology as shown by the CVA results (Figs. SI.3.1, SI.4.1) may indicate some unknown functional significance.

Phocoena was pre-classified as marine (given its present distribution) although Ketten (1992) and Wartzok and Ketten (1999) reported narrow banded beam characteristics (frequency > 100 kHz; Akamatsu et al., 1994) more typical of riverine species. Morphological observations did not reveal this pattern at the outset, although morphometric and statistical analyses were able to recover a morphological affinity of *Phocoena* with riverine species. In this case, the riverine classification of *Phocoena* can be attributed to the correspondence of the external periotic morphology with the type I cochlear morphology and hence, its narrow-banded echolocation (see Section 4.3). Nevertheless, the environmental misclassification does not have a simple explanation. It could be related to the more coastal preference of most *Phocoena* species. Alternatively, it may be argued that these differences show a disconnection between echolocation acoustics and hearing anatomy, but the overall accordance with the rest of the dataset suggests otherwise. Regarding *Platanista*, the conflicting results (misclassification as fully marine) may stem from its plesiomorphic features, which reflect a closer marine ancestry with fossil platanistoids, which are entirely known from marine deposits (see Fordyce, 1994; but see Geisler et al., 2011, for a different interpretation).

4.4. Evolutionary scenarios for the marine–freshwater transition in odontocetes

Our results provide one set of tools that may assist other lines of evidence in resolving outstanding questions about the pattern of odontocete invasions in freshwater ecosystems during the Neogene. Generally, environmental transformations that occurred during the

Neogene have been proposed as drivers of large-scale cetacean evolutionary change (Fordyce, 1980; Nikaido et al., 2001; Steeman et al., 2009). Specifically, Steeman et al. (2009) tested competing hypotheses to explain extant cetacean diversification: whether abiotic drivers, such as physical restructuring of the oceans, played a major role in the radiation of extant cetaceans (Fordyce, 1980; 2003) or if extant cetacean diversity was driven by rapid changes into an open adaptive zone, such as the initial radiation in stem Cetacea (Nikaido et al., 2001). Steeman et al. (2009) obtained strong correlations between molecular clock estimations, which restricted extant lineage diversification to two periods of major oceanic restructuring (35–31 and 13–4 Ma), the latter inferred through time-series curves of global ocean productivity proxies, temperature (Zachos et al., 2001) and sea-level fluctuations (Miller et al., 2005). It is important to note that Steeman et al. (2009), however, did not directly incorporate fossil diversity data to test their competing scenarios, nor did they rigorously constrain their molecular clock calibrations with fossil data following now-established best practices (Parham et al., 2012).

In this light, the multiple origins of “river dolphins” provide a compelling series of case studies where known marine to freshwater transitions ought to be linked to patent physical, environmental changes during their evolutionary histories. South America provides important datasets towards addressing this issue. The orogeny of the Andes, the re-direction of major river systems, and closing and opening of major ocean seaways during the Neogene all provide ample instances of geologic-scale changes that would have factored into the diversification of lineages at the interface of marine and freshwater environments (Latrubesse et al., 2007; Hoorn, 2009; Hoorn et al., 2010). Extant lineages in this continent (*Inia*, *Pontoporia*) provide a valuable source of extant molecular datasets to understand recent divergences among populations (Hamilton et al., 2001; Banguera-Hinestroza et al., 2002), but deeper divergences from ancestral marine odontocetes remains outside of the scope of these data. However, this continent preserves an abundant and rich record of fossil odontocetes from different sedimentary basins (Cozzuol, 1988; 1996; Cione et al., 2005a, b; 2009; Gutstein et al., 2009).

In South America, the timing of the marine to freshwater transition in odontocetes likely occurred between 13 and 4 Ma (middle Miocene through early Pliocene). Marine transgressions across the Brazil Craton during the middle Miocene (Latrubesse et al., 2007; Hoorn et al., 2010) would have created an entirely new set of habitats in a region that currently consists of terrestrial Neotropical forest ecosystems, extending from the northern portion of South America to as far south as Argentina, forming a body of water termed the Paranean Sea (Donato, 2006; Latrubesse et al., 2007, 2010; Cione et al., 2010; Hoorn et al., 2010). The timing of this inland continental flooding of South America coincides with the most species-rich period of the Inioidea (Cozzuol, 2010), a group that encompasses the extant Amazon River dolphin (*Inia*) and many fossil genera spread from the North Atlantic to the South Pacific (late Miocene to early Pliocene), including the freshwater systems of northeastern Argentina (late Miocene), as well as the estuarine extant species, *Pontoporia* (Allen, 1941; de Muizon, 1983, 1988a, b; Cozzuol, 1988, 1996, 2010; Pyenson and Hoch, 2007; Godfrey and Barnes, 2008; Gibson and Geisler, 2009; Gutstein et al., 2009). Subsequent to the middle Miocene eustatic sea-level maxima (Hamilton et al., 2001; Miller et al., 2005), inland flooding subsided, likely isolating some lineages, although it is unclear if such a mechanism explains the specific freshwater origin for *Inia*, which remains the singular representative of this once diverse clade in the Amazon–Orinoco River systems.

Interestingly, another species endemic to the Amazon River system, the tucuxi (*Sotalia fluviatilis*) has been interpreted as a recent re-entrant to freshwater systems because its nearest relatives (*S. guianensis* and other delphinids) are fully marine taxa (Cunha et al., 2005; Caballero et al., 2007). Also, compared to *Inia*, it inhabits a region further downstream and hears at an intermediate frequency range 80–95 kHz (although it is not clear whether it has also a bimodal or narrow-band

echolocation beam; Kamminga et al., 1993). Molecular divergence time estimates between *S. fluviatilis* and its sister taxon, *S. guianensis*, proposed a Pleistocene split (~1.2–1.0 Ma), hinting that eustatic sea-level changes during this interval might also play a role in driving the evolution of this delphinid freshwater invasion.

5. Conclusions

Our results demonstrate that there is correspondence between echolocation acoustics, morphology and environmental type in odontocetes. The external morphology of the pars cochlearis was related to the inner cochlear types. Additionally, both pars cochlearis and acoustic meatus shape reflected the different acoustic properties of the environments that their respective taxa inhabit, which allowed us to differentiate between riverine versus marine (i.e., fully marine or coastal or estuarine) environments. Although previous work had shown that pars cochlearis dimensions partitioned odontocetes into cochlear morphotypes (sensu Ketten, 1992), our study used morphometry and robust statistical analyses to discriminate between morphologies and their representative environmental categories. These results also outline one possible source of evidence (i.e., periotic morphology) for deciphering environmental preferences of extinct odontocetes. This timing is in accordance with regional (Hamilton et al., 2001) and recently more global (Steeman et al., 2009) analyses that emphasize the primacy importance of physical (i.e., geologic and oceanographic, respectively) drivers in the evolutionary history of cetaceans.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.palaeo.2014.01.026>.

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