

Ba/Ca ratios in teeth reveal habitat use patterns of dolphins

S. Botta^{1,*}, C. Albuquerque², A. A. Hohn³, V. M. F. da Silva⁴, M. C. O. Santos⁵,
C. Meirelles⁶, L. Barbosa⁷, A. P. M. Di Benedetto⁸, R. M. A. Ramos⁹, C. Bertozzi¹⁰,
M. J. Cremer¹¹, V. Franco-Trecu¹², N. Miekeley^{13,†}, E. R. Secchi¹

¹Laboratório de Ecologia e Conservação da Megafauna Marinha – EcoMega, Instituto de Oceanografia, Universidade Federal do Rio Grande, Rio Grande RS 96203-900, Brazil

²Departamento Oceanografia e Limnologia, Universidade Federal do Rio Grande do Norte, Natal 59014-100, RN, Brazil

³National Marine Fisheries Service, National Oceanic and Atmospheric Administration, Beaufort, NC 28516, USA

⁴Laboratório de Mamíferos Aquáticos, Instituto Nacional de Pesquisas da Amazônia, CP 478, Manaus, AM 69011-790, Brazil

⁵Departamento de Oceanografia Biológica, Instituto Oceanográfico, Universidade de São Paulo, São Paulo, SP 05508-120, Brazil

⁶Associação de Pesquisa e Preservação de Ecossistemas Aquáticos – AQUASIS, SESC, Caucaia, CE 61627-010, Brazil

⁷Organização Consciência Ambiental — ORCA, Vila Velha, ES 29101-315, Brazil

⁸Universidade Estadual do Norte Fluminense, CBB/LCA, Campos dos Goytacazes, RJ 28013-602, Brazil

⁹Everest Tecnologia em Serviços Ltda. CP 5074, Vitória, ES 29045-970, Brazil

¹⁰Projeto BioPesca, Centro Universitário Monte Serrat, Santos, SP 11015-530, Brazil

¹¹Departamento de Ciências Biológicas, Universidade da Região de Joinville, Joinville, SC 89219-710, Brazil

¹²Proyecto Franciscana, Sección Etología-Facultad de Ciencias, Iguá 4225, Montevideo, CP 11400, Uruguay

¹³Departamento de Química, Pontifícia Universidade Católica do Rio de Janeiro, Rio de Janeiro, RJ 22453-900, Brazil

ABSTRACT: Teeth and otoliths are metabolically inert structures that preserve a chronology of chemical variations that may be related to the environmental histories experienced by each organism. Because of the natural decrease of barium (Ba) and increase of strontium (Sr) bioavailability in water with increasing salinity, these elements may be especially useful to track habitat use in aquatic organisms. Therefore, we tested whether the Ba/Ca and Sr/Ca ratios in the teeth of dolphins represent a salinity gradient. The main aim was to determine whether these elements can be used as a natural tag for different aquatic environments. Teeth from 2 freshwater dolphins (*Inia geoffrensis* and *Sotalia fluviatilis*) and 2 marine species (*S. guianensis* and *Pontoporia blainvillei*) from Brazil and Uruguay were analyzed using a Laser Ablation Inductively Coupled Plasma-Mass Spectrometer. Intensity ratios of ¹³⁸Ba/⁴³Ca and ⁸⁶Sr/⁴³Ca were measured along a line that covered all growth increments in the dentin from the second year of life onwards. Teeth from the freshwater species had mean Ba/Ca values tenfold higher than marine dolphins, confirming the inverse relationship between salinity (and thus ambient Ba/Ca) and elemental ratios in teeth. Furthermore, Ba/Ca ratios could also differentiate dolphins from lower-salinity estuarine areas from those in areas with minimal freshwater discharge. No significant differences were found for Sr/Ca values. Results presented encouraging indications for the application of this technique as a potential new tool for studying habitat use in aquatic mammals.

KEY WORDS: Barium · Strontium · Laser ablation ICP-MS · Habitat use · Odontocetes

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INTRODUCTION

The physico-chemical characteristics of ecosystems play an important role in determining the distribution patterns of organisms, from primary producers to

top predators, at different spatial and temporal scales (Skov et al. 2008, Fragoso & Smith 2012). Although some aquatic mammals such as migratory whales and pelagic offshore odontocetes can travel long distances to find appropriate foraging grounds, coastal

*Corresponding author: silbotta@gmail.com

†Deceased

cetaceans in general have more restricted home ranges (e.g. Urian et al. 2009, Oshima et al. 2010) associated with the productive estuaries and coastal regions where they feed (Wilson et al. 2013). These often discrete foraging sites are influenced by local oceanographic and climatic dynamics such as upwellings, water mass advection, and rainfall and runoff, among other factors. The local dynamics of the environment will define the physico-chemical signals that are transferred throughout the food web (Elsdon et al. 2008). Therefore, organisms exploiting habitats with geochemical differences may reflect those differences in the characteristic elemental or isotopic compositions of their tissues due to the site-specific elemental and isotopic composition of their diet (Outridge et al. 2003). For this reason, habitat use patterns can be investigated by analyzing chemical elements in organisms.

In recent years, several approaches using naturally occurring chemical elements as tools to track habitat use in aquatic animals have emerged. They have been applied to investigate patterns of habitat use in fish (e.g. Hamer et al. 2006, Albuquerque et al. 2012), sea turtles (e.g. Avens et al. 2013), sea birds (e.g. Jaeger et al. 2010) and aquatic mammals (e.g. Fontaine et al. 2007, Botta et al. 2012). Although natural tracers provide indirect evidence of habitat use, depending on the tissue being analyzed, chemical signals can integrate environmental information on a broad range of time scales, from days (e.g. blood) to years (bone) to the lifespan (teeth) of an individual. Odontocete cetaceans are monophyodontic species as they have only one set of dentition, with no milk or deciduous teeth (Berta et al. 2006). Hence, the dentin is continuously deposited as a series of growth layers from intrauterine life until the individual's death (Hillson 2005, Hohn 2009). This evolutionary trait and the fact that once dentin has been formed it is not subject to strong remodeling and turnover (Hillson 2005) are fundamental for using odontocetes' teeth to understand aspects of their spatial and trophic ecology on a long-term basis.

Some elements, such as barium (Ba) and strontium (Sr), have largely been applied as biochemical tracers in the aquatic environment for the reconstruction of migratory histories between freshwater/estuarine and open marine waters of fish (Elsdon & Gillanders 2005, Albuquerque et al. 2012). Additionally, Ba and Sr levels in coral skeletons were used for tracking environmental Ba/Ca variation related to riverine discharge (i.e. salinity conditions) (Sinclair & McCulloch 2004). Although high Ba/Ca ratios, attributed to the freshwater influence, were found in

bones of cetacean species inhabiting high arctic habitats (Peek & Clementz 2012), to our knowledge these tracers have not yet been applied to describe habitat use patterns in marine mammals. The basis of their application as tracers relies on the fact that the availability of these 2 elements in aquatic marine systems depends mostly on salinity (Jacquet et al. 2005). Rivers are the main source of dissolved and particulate Ba to the oceans and coastal waters (Dehairs et al. 1980, Neff 2002) where the high sulphate content of seawater results in its combination with Ba, forming stable solid barium sulphate (barite) which precipitates, removing Ba^{2+} ions from the water column. Therefore, estuaries are enriched in Ba over more saline coastal waters (Coffey et al. 1997). Rivers also provide the major flux of Sr to the oceans (Palmer & Edmond 1989). Although Sr behaves in a similar way to Ba, it forms an unstable structure, thus a greater proportion dissolves back into the marine water (Gilbert et al. 1994). Hence, salinity is positively correlated with Sr in an increasing gradient from freshwater end-members towards the open ocean (Dorval et al. 2005, Albuquerque et al. 2012). Peek & Clementz (2012) synthesized global marine and terrestrial data on Ba, Sr and Ca concentrations and reported a difference of more than 700-fold between average freshwater and marine water for Ba/Ca levels in contrast to only a 2-fold difference for Sr/Ca values (see Table 1 in Peek & Clementz 2012). Moreover, it was recognized that some estuaries exhibit similar Sr/Ca values between marine and freshwater end-members or minimal salinity-related gradients (Kraus & Secor 2004, Elsdon & Gillanders 2005, Tanner et al. 2013). Indeed, if larger differences are found between freshwater and marine waters for Ba/Ca than for Sr/Ca, freshwater (or terrestrial) versus marine predators should exhibit equivalently more divergent Ba/Ca than Sr/Ca values as a consequence.

The application of these natural tags is dependent on the assumption that Ba and Sr are incorporated into biological structures (e.g. otoliths, bones, teeth) in proportion to environmental levels, thus reflecting the chemical and physical characteristics of the aquatic environment. This assumption has largely been met for otoliths, as water seems to be the main source of Sr and Ba to otolith aragonite (Walther & Thorrold 2006). In mammals, however, the main route of acquisition and deposition of these elements in the hydroxyapatite ($Ca_{10}[PO_4]_6[OH]_2$) structure of the tooth is through assimilated food (Schroeder et al. 1972). As with other alkaline earth metals, both Sr and Ba tend to accumulate in the skeleton (Schroeder

et al. 1972); thus, retained Ba and Sr are incorporated into the hydroxyapatite structure of bone and teeth as a replacement of calcium (Burton et al. 2003). Although Ba and Sr mimic Ca as trace elements due to similar atomic properties, the relative levels of Ba and Sr (i.e. Ba/Ca and Sr/Ca ratios) decrease during metabolism, resulting in Ca biopurification and reduced levels of Ba/Ca and Sr/Ca at higher trophic levels (Elias et al. 1982, Balter 2004, Peek & Clementz 2012). This reduction in elemental ratios towards higher trophic levels, however, seems to occur at fixed ratios (Elias et al. 1982). Therefore, while comparing organisms with similar diets (i.e. similar trophic positions), large differences in environmental Sr/Ca and/or Ba/Ca among areas should still be present as contrasting elemental ratios in consumers along the food web. In fact, differences in Ba/Ca and Sr/Ca ratios in tissues of terrestrial herbivorous mammals and humans were attributed to a variation in Ba and Sr levels in the local geological environment (Burton et al. 2003, Wright et al. 2010).

Moreover, Ba concentrations in bones from marine animals up to 30 times lower than those from terrestrial species were reported and ascribed to the known low Ba availability in the marine environment (Wessen et al. 1977, Burton & Price 1990, Gilbert et al. 1994, Peek & Clementz 2012). This difference has been extensively used in the archaeological field to infer human diet enrichment with products of marine origin based on the potential of Ba/Ca ratios to distinguish marine from terrestrial resources (Burton & Price 1990, Gilbert et al. 1994, Szostek et al. 2009). In spite of the difference in Sr bioavailability between terrestrial and marine habitats, the pattern for Sr levels in consumers from those contrasting environments is not so clear. Wessen et al. (1977) and Gilbert et al. (1994), for example, found no differences in Sr or Sr/Ca values between marine and terrestrial organisms, whereas Schroeder et al. (1972) reported Sr values in fish and seafood 8 times higher than in muscle tissues of terrestrial mammals. Based on the current knowledge about sources of variability in Ba/Ca and Sr/Ca ratios in both marine and freshwater environments, we hypothesized that a significant difference, at least in Ba/Ca ratios, would be found in the hard tissues of aquatic mammals foraging in freshwater or low salinity environments (i.e. estuaries) compared to those inhabiting coastal marine areas.

The objectives of this study were to (1) evaluate the potential of using Ba and Sr assimilated in the teeth of aquatic mammals as natural tags to determine habitat characteristics and use patterns, and (2)

demonstrate that Ba/Ca ratios found in the teeth of dolphins from different populations reflect the influence of freshwater into their coastal habitats along the western South Atlantic Ocean.

MATERIALS AND METHODS

Species and study areas

Ba/Ca and Sr/Ca ratios in teeth from 2 freshwater dolphin species were compared with those found in 2 coastal marine dolphin species. We targeted only resident/small home range species that could reflect local environmental Ba/Ca and Sr/Ca ratios. Amazon River dolphins *Inia geoffrensis* ($n = 17$) and tucuxis *Sotalia fluviatilis* ($n = 24$) that inhabit the Amazon River basin in sympatry (Best & da Silva 1993, da Silva & Best 1994) were chosen as representatives of freshwater habitats. Samples were obtained from dolphin carcasses found along the margins or floating in the Amazon River and its tributaries. Coastal marine Guiana dolphins *Sotalia guianensis* ($n = 97$) and franciscanas *Pontoporia blainvillei* ($n = 99$) were sampled from several locations along their distributions, including coastal regions with diverse freshwater discharge ranging from estuaries (e.g. La Plata River estuary, Uruguay) to regions with minimal freshwater influence (e.g. Ceará, northeastern Brazil). All tooth samples from Guiana dolphins and franciscanas used in this study were obtained from dolphins that were either incidentally killed in fishing gear or found dead washed ashore. Dolphin populations inhabiting some of these locations, such as Guiana dolphins from the Cananéia estuary (São Paulo state; Oshima et al. 2010) and from Babitonga Bay (Santa Catarina state; Hardt et al. 2010), are considered highly resident based on long-term photo-identification data. In the case of the franciscana, molecular (Mendez et al. 2010, Costa-Urrutia et al. 2012) as well as preliminary satellite-tracking (Bordino et al. 2008) data suggest limited home ranges for the species. A summary of the samples and their location is shown in Fig. 1 and Table 1.

Although both Sr/Ca and Ba/Ca exhibit a decreasing trend from herbivores towards upper trophic level predators (Elias et al. 1982, Balter 2004, Peek & Clementz 2012), Burton & Wright (1995) argued that Sr/Ca and Ba/Ca ratios in mammals' bioapatites reflect the mineral components being assimilated through diet rather than an index of trophic position. Thus, for example a diet rich in high-calcium food sources (such as fish) would lead to low Sr or Ba/Ca

ratios in comparison with lower-calcium diets based on plants. The diets of the species used in this study were mostly composed of fish, with some contribution of squid and occasional ingestion of crustaceans

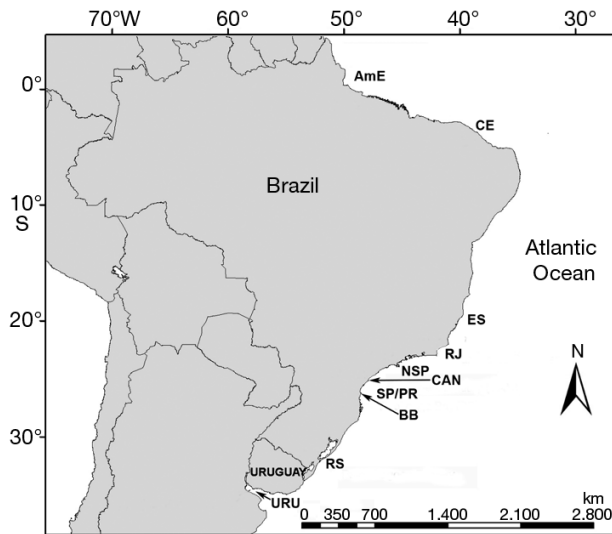


Fig. 1. Sampling locations along the coastline of the western South Atlantic: Amazon Estuary (AmE), Ceará (CE), Espírito Santo (ES), Northern Rio de Janeiro (RJ), Northern São Paulo (NSP), Cananéia estuary (CAN), Southern São Paulo/Northern Paraná (SP/PR), Babitonga Bay (BB), Patos Lagoon estuary and adjacent coastal areas (RS) and La Plata River estuary and adjacent coastal areas, Uruguay (URU)

(Best & da Silva 1993, da Silva & Best 1994, Danilewicz et al. 2002, Rosas et al. 2010). Since the Ca composition of fish and squid is very similar (Sikorski & Kołodziejaska 1986, Vlieg et al. 1991, Lourenço et al. 2009), it was assumed that slight variation in Ca intake did not affect the comparison the elemental ratios of teeth between species.

Age estimation

To prevent the influence of ontogenetic diet shifts on elemental incorporation in teeth, only adults were selected for analysis. Ages of Guiana dolphins and franciscanas were estimated by counting dentinal and or cemental incremental layers or growth layer groups (GLGs; Perrin & Myrick 1980). Teeth were processed for age estimation following Hohn et al. (1989) for Guiana dolphins and Pinedo & Hohn (2000) for franciscanas. For Guiana dolphins, a thick section (1 to 2 mm) of the center of the tooth was cut through the longitudinal axis using a low-speed saw with a diamond-embedded blade. These thick sections, along with the rest of the small-sized franciscanas' teeth were then fixed in 10% formalin and decalcified with RDO® (a commercial mixture of acids). The decalcified tooth sections were then thin-sectioned to 25 µm using a freezing microtome, stained in Mayer's

Table 1. Dolphin species sampled (Guiana dolphins *Sotalia guianensis* and franciscanas *Pontoporia blainvillei*), and main hydrological characteristics of the coastal study areas in Brazil and Uruguay: Amazon Estuary (AmE), Ceará (CE), Espírito Santo (ES), Northern Rio de Janeiro (RJ), Northern São Paulo (NSP), Cananéia estuary (CAN), Southern São Paulo/Northern Paraná (SP/PR), Babitonga Bay (BB), Patos Lagoon estuary and adjacent coastal areas (RS) and La Plata River estuary and adjacent coastal areas, Uruguay (URU). Mean freshwater discharge (FD) of main freshwater inputs are indicated. Main habitat for the species in each area is indicated as coastal (dolphins inhabiting marine coastal areas), estuarine-out (dolphins occupying adjacent coastal areas influenced by estuarine waters) and estuarine-in (dolphins residing inside estuarine waters)

Area	Main freshwater source	FD (m ³ s ⁻¹)	Species	Main habitat	Reference
AmE	Amazon River	180 000	Guiana dolphin	Estuarine-in ^a	Geyer et al. (1996)
CE	Jaguaribe River	22–57	Guiana dolphin	Coastal	Campos et al. (2000)
ES	Doce River	1139	Guiana dolphin Franciscana	Estuarine-out Estuarine-out	Coelho (2006) Coelho (2006)
RJ	Paraíba do Sul River	670	Guiana dolphin Franciscana	Estuarine-out Estuarine-out	Carvalho et al. (2002) Carvalho et al. (2002)
NSP	Baixada Santista estuary	24–127	Franciscana	Coastal	Moser et al. (2005)
CAN	Cananéia estuary	435	Guiana dolphin	Estuarine-in	Schaeffer-Novelli et al. (1990)
SP/PR	Paranaguá estuarine complex	200	Franciscana	Coastal	Mizerkowski et al. (2012)
BB	Babitonga Bay	<20	Guiana dolphin Franciscana	Estuarine-in Estuarine-in	Barros et al. (2010) Barros et al. (2010)
RS	Patos Lagoon	2000	Franciscana	Estuarine-out	Calliari et al. (2009)
URU	La Plata River	23 000	Franciscana	Estuarine-in	Piola & Romero (2004)

^aAlthough Guiana dolphins effectively use this estuary, some of the samples used here were collected in northwestern offshore adjacent waters

hematoxylin, 'blued' in ammonia, dehydrated in glycerin, and mounted on a microscope slide in 100% glycerin. GLG counting was performed without reference to biological data, such as length or sex. Each GLG was considered to represent 1 yr of age (Hohn et al. 1989, Pinedo & Hohn 2000). Dolphins were considered adults and were included in the analysis based on available estimates of age at sexual maturation (information summarized in Danilewicz et al. 2002 and Rosas et al. 2010 for franciscanas and Guiana dolphins, respectively).

No teeth for age estimation were available for Amazon River dolphins; thus, they were classified as adults using published results on length at maturation: >200 cm for males and >180 cm for females (Da Silva 2009). In the case of the tucuxi, no estimate of age at sexual maturation was available, so they were classified as adults at a total length >132 to 137 cm for males and >140 cm for females (Best & da Silva 1984).

Chemical analyses

Sample preparation

Teeth were cleaned of outer soft tissue with a carbide burr, attached to a drill when necessary. Teeth from franciscanas were first glued in a glass slide and then polished by hand with lapping paper until the entire pulp cavity and all the GLGs were visible. Teeth from the remaining species were sectioned using a low-speed saw with a diamond-embedded blade through the longitudinal axis in order to obtain a 2 mm thick section from the center of the tooth, where all the growth layers were exposed. These thick sections were then mounted onto glass slides using fast drying glue. All teeth surfaces were polished with silicon carbide papers (8000 and 12000 grit), washed with and sonicated for 3 min with ultrapure water (Milli-Q, Millipore), and then rinsed 3 times with ultrapure water. The slides were allowed to dry in a horizontal flow cabinet before analysis.

LA-ICP-MS

Teeth were analyzed using a laser ablation system (Nd:YAG 266 nm) coupled to an ELAN 6000 (PerkinElmer, SCIEX) inductively coupled plasma-mass spectrometer (LA-ICP-MS) under the operating conditions provided in the Appendix. The LA-ICP-

MS device was located at the Chemistry Department of the Pontifícia Universidade Católica do Rio de Janeiro (Brazil). Data were acquired for the isotopes ^{43}Ca , ^{138}Ba and ^{86}Sr .

Each tooth was scanned from the enamel to the pulp cavity including all pre- and post-natal dentinal GLGs, always in the crown region of the tooth. Pre-natal and early-life variations in Ba/Ca or Sr/Ca values in teeth resulting from the placental or milk-associated elemental transfer, respectively, and/or differential absorption of elements in the immature gut were reported for some mammals (Humphrey et al. 2008, Austin et al. 2013). In 3 of the species used in this study, lactation lasts less than 1 yr (7 mo in the tucuxi, Best & da Silva 1984; 8.7 to 9.4 mo in the Guiana dolphin, Rosas & Monteiro-Filho 2002; 9 mo in the franciscana, Harrison et al. 1981, Rodríguez et al. 2002). Although lactation may last more than 1 yr in the Amazon River dolphin (da Silva 2009), this was observed in only 1 specimen (Best & da Silva 1993). Exclusive lactation and consequent strong influence on Ba or Sr levels in teeth may occur during the first year of life, decreasing its relative importance with the gradual introduction of solid foods during the weaning process. Therefore, we excluded pre-natal dentin and first dentinal GLG elemental measurements from all scans. Enamel measurements were also excluded due to a different composition and time of deposition of this tissue in comparison with dentin (Hillson 2005). This was performed by measuring these regions (enamel, pre-natal dentin and first dentinal GLG) along the laser scan in a digital image of the tooth (Fig. 2). Measurements were taken with Image J 1.48v software (National Institute of Health). By considering the ICP-MS replicate time of 0.516 s and a laser scanning speed of $40 \mu\text{m s}^{-1}$, counts s^{-1} were assigned to the pre-natal and first GLG region and were excluded from analyses.

Elemental intensities were corrected for argon (Ar) background. The signals observed at each ablation site were corrected for possible differences in ablation yield by using ^{43}Ca as an internal standard (Dolphin et al. 2012). Thus, metal isotope data obtained by LA-ICP-MS were expressed as ratios to ^{43}Ca , hereafter referred to as Ba/Ca and Sr/Ca ratios. Repeatability was estimated as the relative standard deviation (RSD) from 10 consecutive measurements of the standard NIST 1834. The ICP-MS was previously optimized through daily performance for maximum analyte intensities and minimum interferences by oxides and double charged ions. The analysis of repeatability in the standard NIST 1834 resulted in

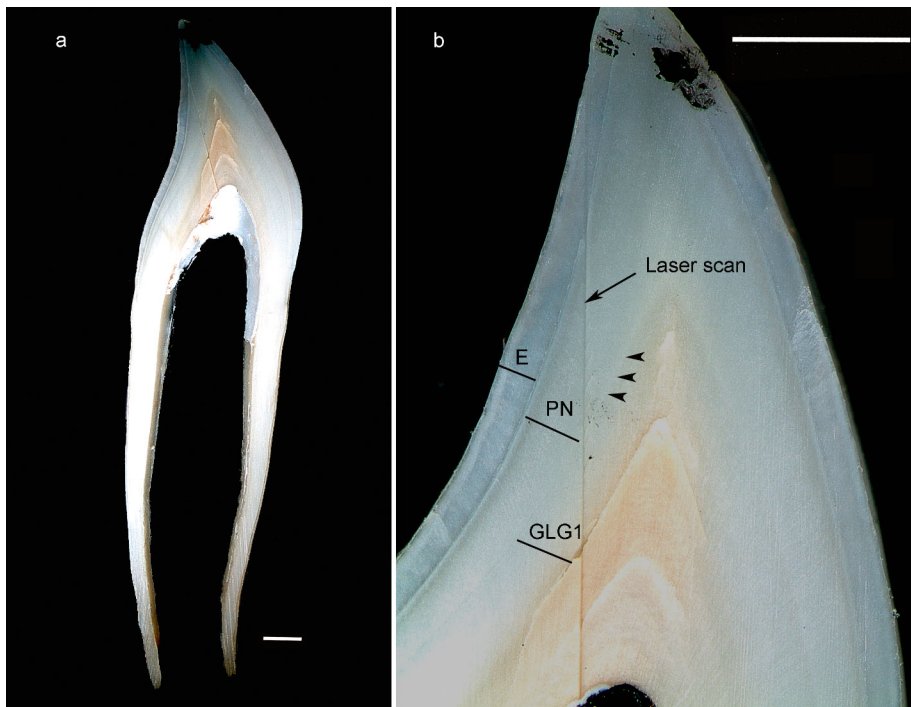


Fig. 2. Longitudinal section of Guiana dolphin *Sotalia guianensis* tooth displaying laser scan (a) detail of the crown region, and (b) areas where enamel (E), pre-natal dentin (PN) and post-natal dentin first growth layer group (GLG1) are indicated. The neonatal line in dentin is indicated by black arrowheads. Scale bars = 1 mm

RSDs of 12.43, 6.91 and 3.99% for ^{138}Ba , ^{86}Sr and ^{43}Ca , respectively.

To evaluate accuracy, we also analyzed a subset of 17 samples (teeth from 5 freshwater Amazon River dolphins, 6 estuarine Guiana dolphins from the Amazon River estuary, AmE, and 6 marine coastal Guiana dolphins from Ceará, CE) through the solution-based ICP optical emission spectrometer (ICP-OES, Perkin-Elmer Optima 4300 DV, Chemistry Department of the Pontifícia Universidade Católica do Rio de Janeiro). In each tooth, dentin powder was obtained by drilling out the material from specific locations along a longitudinal section of the tooth with visible GLGs. The material, weighed to 0.1 mg precision, was then dissolved in 100 μl of high-purity nitric acid (sub-boiled) in closed polypropylene tubes at 80°C on a heating plate. After cooling, the clear solution was then diluted to 10 ml with deionized (DI) water. ICP-OES operational conditions are presented in the Appendix. The results were regressed against the equivalent LA-ICP-MS, thus providing a reference for accuracy and validation of the intensities measured by LA-ICP-MS. Ratios measured in these tooth samples through LA-ICP-MS and ICP-OES were highly and linearly correlated for Ba/Ca ($r^2 = 0.98$, $p < 0.0001$), indicating high accuracy (Fig. 3a), and significantly but not as strongly linearly correlated for Sr/Ca ($r^2 = 0.68$, $p < 0.0001$) (Fig. 3b).

Data analysis and statistics

Data were tested for normality and homogeneity of variances using Kolmogorov-Smirnov and Levene's tests, respectively. Variables were log-transformed to meet parametric statistical assumptions; however, in some cases the assumptions were still not met. Therefore, comparisons were performed using Student's *t*-tests and ANOVAs when data met parametric requirements; otherwise, nonparametric Mann Whitney *U*-tests were employed (Sokal & Rohlf 1981). Mann-Whitney *U*-tests were used to compare elemental ratios between sexes of the studied species in each of the study areas, with the exception of tucuxis (unknown sex) and franciscanas from Espírito Santo (ES), Babitonga Bay (BB) and La Plata River estuary and adjacent coastal areas, Uruguay (URU) (due to small sample sizes). Mann-Whitney *U*-tests were also used for comparing the mean values of elemental ratios found for freshwater versus marine samples. One-way ANOVAs followed by Tukey's HSD tests or Mann Whitney *U*-tests followed by multiple comparisons were used to determine differences among marine areas/species. Spearman's ranked correlations were used to analyze the relationship between the areas' mean freshwater discharge and mean Ba/Ca or Sr/Ca ratios in teeth.

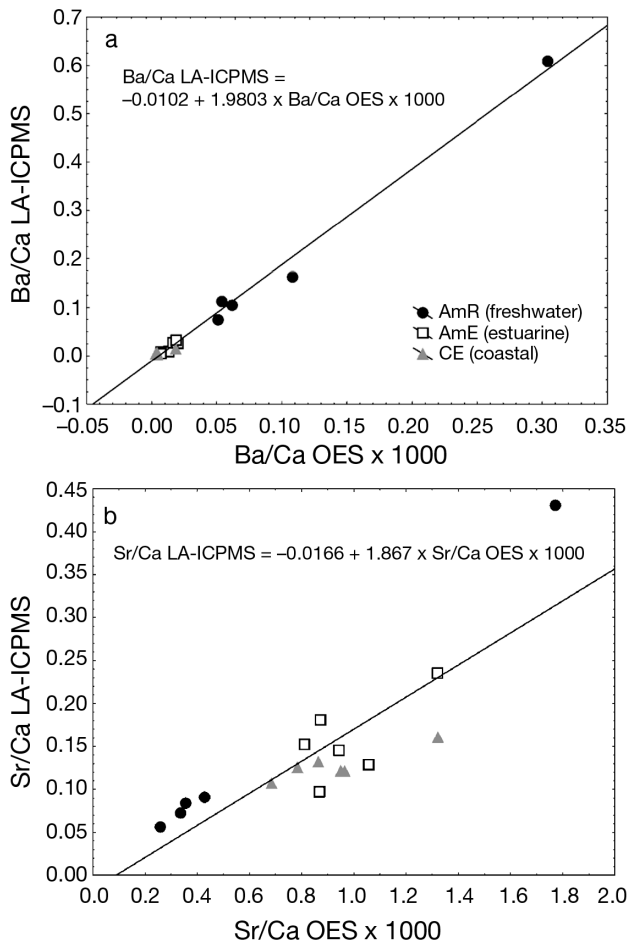


Fig. 3. (a) Ba/Ca and (b) Sr/Ca ratios in teeth of Amazon dolphins *Inia geoffrensis* from the Amazon River (AmR), and Guiana dolphins *Sotalia guianensis* from the Amazon estuary (AmE) and from Ceará (CE), as representatives for freshwater, estuarine and coastal with minimum freshwater runoff environments, respectively. Linear relationships among values obtained by laser ablation inductively coupled plasma-mass spectrometry (LA-ICPMS) and by solution-based ICP optical emission spectrometry (OES) are presented

RESULTS

Elemental differences in dolphin teeth among habitats

Barium

Ba/Ca ratios did not differ between sexes of the studied species in any of the areas (U -tests, $p > 0.05$) with the exception of franciscanas from Northern Rio de Janeiro (RJ), where females exhibited higher values than males (mean Ba/Ca = 0.013 and 0.008 for females and males, respectively; U -test, $p < 0.05$). As this was the only difference, data by sex were combined for further analyses.

Mean Ba/Ca ratios in teeth were significantly higher for freshwater than for marine dolphins (t -test, $p < 0.001$) (Table 2). Significant differences in Ba/Ca ratios were found among marine locations (ANOVA, $F_{9,186} = 13.53$, $p < 0.001$). A post hoc comparison indicated that the largest sampled estuaries (AmE, URU and Patos Lagoon estuary and adjacent coastal areas [RS]) differed significantly from essentially marine sites (CE and Northern São Paulo [NSP]) (Tables 2 & 3). Mean freshwater discharge was significantly correlated to mean Ba/Ca ratios in teeth from marine dolphins (Spearman's $\rho = 0.77$, $p < 0.05$) (Fig. 4).

Differences in Ba/Ca ratios among species were tested in areas where more than one species was sampled. Ba/Ca ratios did not differ between the 2 species of freshwater dolphins (Amazon River dolphin and tucuxis) (t -test, $p = 1.00$). With respect to marine species, comparisons between franciscanas and Guiana dolphins were performed for 3 areas: ES, RJ and BB. Similar Ba/Ca values between those species were found in ES and BB (t -tests, $p = 0.80$ and $p = 0.68$ for ES and BB, respectively) while in RJ, the mean Ba/Ca value in franciscanas' teeth was significantly higher than that from Guiana dolphins (t -test, $p < 0.05$) (Table 2).

When comparing among areas within species, Guiana dolphins from the 3 main estuarine areas (AmE, ES and Cananéia Estuary [CAN]) exhibited higher Ba/Ca ratios than Guiana dolphins from the remaining areas (CE, RJ and BB) (ANOVA, $F_{5,91} = 7.07$, $p < 0.001$). The same pattern was observed for franciscanas, where animals from ES, RS and URU had higher Ba/Ca ratios than those from RJ, NSP, Southern São Paulo/Northern Paraná (SP/PR), or BB areas (ANOVA, $F_{6,92} = 17.19$, $p < 0.001$) (Tables 2 & 4, Fig. 4).

Strontium

No differences were found by sex within species in Sr/Ca ratios in any of the sampled areas (U -tests, $p > 0.05$), and therefore data by sex was combined for further analysis.

Sr/Ca values were not significantly different among freshwater and marine dolphins (U -test, $p = 0.15$) (Table 2). As for marine regions, significant differences were found among some areas (U -tests, $p < 0.001$), though these differences were apparently not linked to habitat characteristics (Tables 2 & 3). Indeed, a small and non-significant correlation was found between local freshwater discharge and mean Sr/Ca found in teeth (Spearman's $\rho = 0.44$, $p = 0.20$, Fig. 5).

Table 2. Mean Ba/Ca and Sr/Ba in teeth of Amazon dolphins *Inia geoffrensis*, tucuxis *Sotalia fluviatilis*, Guiana dolphins *Sotalia guianensis* and franciscanas *Pontoporia blainvillei* from freshwater (Amazon River, AmR) and marine coastal regions in Brazil and Uruguay (see Table 1 for area codes). Significance of differences in elemental ratios (p-values, t-tests) between species in each area where more than one dolphin species were sampled are shown

Area	Ba/Ca (\pm SD)	p	Sr/Ca (\pm SD)	p	n
AmR					
Amazon dolphin	0.203 (0.188)	0.998	0.170 (0.163)	0.614	17
Tucuxi	0.203 (0.142)		0.193 (0.133)		24
Mean freshwater	0.203 (0.161)		0.184 (0.144)		41
AmE					
Guiana dolphin	0.011 (0.004)		0.151 (0.046)		13
CE					
Guiana dolphin	0.005 (0.002)		0.121 (0.017)		17
ES					
Guiana dolphin	0.014 (0.011)	0.793	0.103 (0.017)	<0.001	17
Franciscana	0.015 (0.006)		0.191 (0.023)		4
All	0.014 (0.010)		0.119 (0.040)		21
RJ					
Guiana dolphin	0.007 (0.005)	<0.050	0.077 (0.027)	<0.001	21
Franciscana	0.011 (0.007)		0.172 (0.059)		19
All	0.009 (0.006)		0.122 (0.065)		40
NSP					
Franciscana	0.004 (0.002)		0.089 (0.019)		19
CAN					
Guiana dolphin	0.012 (0.008)		0.127 (0.061)		17
SP/PR					
Franciscana	0.007 (0.004)		0.083 (0.032)		15
BB					
Guiana dolphin	0.008 (0.004)	0.678	0.117 (0.038)	0.903	12
Franciscana	0.008 (0.004)		0.115 (0.019)		5
All	0.008 (0.004)		0.116 (0.033)		17
RS					
Franciscana	0.015 (0.008)		0.124 (0.031)		25
URU					
Franciscana	0.019 (0.011)		0.111 (0.034)		12
Mean marine	0.010 (0.008)		0.117 (0.046)		196

No differences in Sr/Ca values were found between the 2 species of freshwater dolphins (*U*-test, $p = 0.61$) or between franciscanas and Guiana dolphins from BB. However, Guiana dolphins had lower Sr/Ca ratios than franciscanas from both ES and RJ (*U*-tests, $p < 0.001$) (Table 2).

There were some area effects. For Guiana dolphins, Sr/Ca ratios from RJ were significantly different from other areas (*U*-test, $p < 0.001$). For franciscanas, significant differences were found between some of the areas (*U*-test, $p < 0.001$), although no trend was found with increasing freshwater discharge (Tables 3 & 5, Fig. 5).

DISCUSSION

In this study, we demonstrated the potential application of Ba/Ca and Sr/Ca ratios in teeth as natural markers of habitat use in aquatic mammals. While largely reported for fish otoliths (e.g. Elsdon & Gillanders 2003, Albuquerque et al. 2012), no study has examined the relationship between Ba/Ca and Sr/Ca levels in aquatic mammals and the physico-chemical characteristics of their environment. Our large data set, covering distinct coastal areas and species as well as samples from the same species over a broad latitudinal range and varying levels of freshwater discharge, allowed us to make some interpretations about the final elemental ratios found in aquatic mammals' teeth and the ambient concentrations of element/Ca.

Table 3. Multiple comparisons of Ba/Ca and Sr/Ca ratios in teeth dentin of marine dolphins (Guiana dolphins *Sotalia guianensis* and franciscanas *Pontoporia blainvillei*) from Brazil and Uruguay (see Table 1 for area codes). Lower-left p-values from Tukey's HSD tests are for Ba/Ca and upper-right p-values from *U*-tests are for Sr/Ca. * $p < 0.05$; ** $p < 0.001$

	AmE	CE	ES	RJ	NSP	CAN	SP/PR	BB	RS	URU
AmE		1.00	1.00	0.20	**	1.00	**	1.00	1.00	1.00
CE	**		1.00	1.00	0.07	1.00	*	1.00	1.00	1.00
ES	1.00	**		1.00	0.77	1.00	0.19	1.00	1.00	1.00
RJ	0.69	*	0.17		1.00	1.00	0.38	1.00	1.00	1.00
NSP	**	1.00	**	**		1.00	1.00	0.91	0.06	1.00
CAN	1.00	**	1.00	0.84	**		0.33	1.00	1.00	1.00
SP/PR	*	0.99	**	0.54	0.64	0.06		0.24	*	0.96
BB	0.55	0.30	0.16	1.00	*	0.71	0.15		1.00	1.00
RS	0.96	**	0.99	**	**	0.76	**	*		1.00
URU	0.68	**	0.79	**	**	0.36	**	**	1.00	

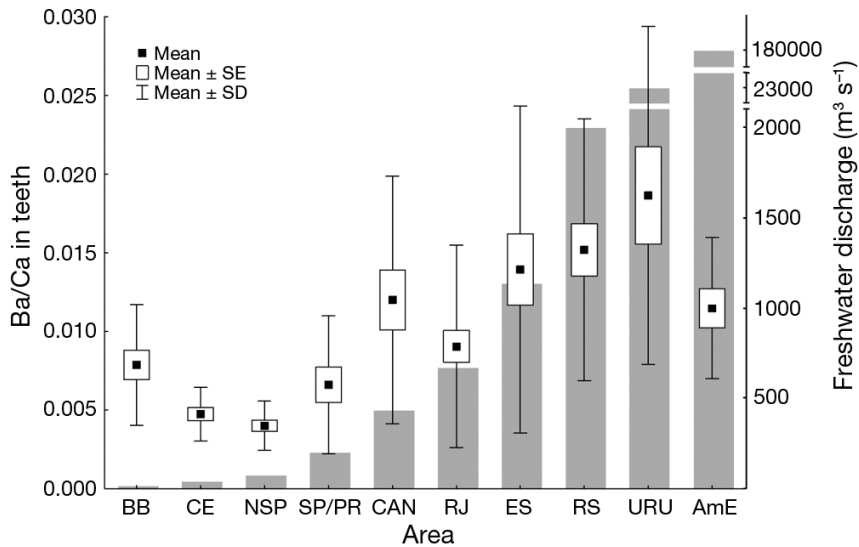


Fig. 4. Mean Ba/Ca ratio in the teeth of Guiana dolphins *Sotalia guianensis* and franciscanas *Pontoporia blainvillei* from marine coastal regions in Brazil and Uruguay, and mean freshwater discharge (grey bars) of the main freshwater input to the area (see Table 1 for details and area codes)

All model species for this study occupied similar trophic levels in their habitats (i.e. ~4th level; Pauly et al. 1998), thus buffering the possible influence of differential Ba/Ca and Sr/Ca fractionation (Balter 2004, Peek & Clementz 2012). Even when stomach content data indicate a difference in trophic level among species, as in the case of the Amazon River dolphin (Best & da Silva 1993) and the tucuxi (da Silva & Best 1994), no difference was found between their Ba/Ca or Sr/Ca ratios. This is evidence that the potential trophic level effect in the observed differences in Ba/Ca or Sr/Ca ratios among sampled areas or species does not hold for our model species. Hence, the element/Ca ratios observed in cetacean teeth probably reflect the basal ambient Ba/Ca or Sr/Ca ratios that cascade up

through local food webs. In that context, therefore, our results strongly support a positive correlation between Ba/Ca in mammalian teeth and freshwater discharge along the southwestern Atlantic Ocean, with decreasing Ba/Ca levels in coastal and marine habitats. These results also illustrate that large differences in basal (i.e. water) levels of Ba should still be detected even after the influence of metabolic processes encountered in food chains. As a consequence, Ba/Ca levels can be used as a proxy for patterns of habitat use in aquatic mammals. Sr/Ca ratios in teeth, on the other hand, did not show clear patterns within the sampled habitats. Future studies should include the examination of elemental availability in the water and its variation in time, and the elemental path-

ways through food chains up to high trophic level predators in order to clarify the fractionation processes underlying the incorporation of Sr and Ba into the teeth of aquatic mammals.

Barium as a tracer of cetacean habitat use

We observed a marked difference in Ba/Ca levels between freshwater and marine species, and a general multispecies pattern of higher Ba/Ca values towards habitats increasingly influenced by freshwater. Ba/Ca ratios were distinctly higher in dolphins from areas with higher freshwater inputs (such as estuaries) than in dolphins inhabiting more coastal

Table 4. Tukey's HSD test p-values of multiple comparisons of Ba/Ca values for teeth dentin of Guiana dolphins *Sotalia guianensis* and franciscanas *Pontoporia blainvillei* from Brazil and Uruguay (see Table 1 for area codes). Upper-right p-values are for Guiana dolphin comparisons and lower-left p-values are for franciscana comparisons. *p < 0.05; **p < 0.001

	AmE	CE	ES	RJ	NSP	CAN	SP/PR	BB	RS	URU
AmE		**	1.00	0.06	–	1.00	–	0.30	–	–
CE	–		**	0.53	–	**	–	0.39	–	–
ES	–	–		*	–	1.00	–	0.20	–	–
RJ	–	–	0.84		–	0.08	–	1.00	–	–
NSP	–	–	**	**		–	–	–	–	–
CAN	–	–	–	–	–		–	0.41	–	–
SP/PR	–	–	*	*	0.38	–	–	–	–	–
BB	–	–	0.59	0.98	0.07	–	0.78		–	–
RS	–	–	1.00	0.48	**	–	**	0.38		–
URU	–	–	1.00	0.17	**	–	**	0.15	0.96	

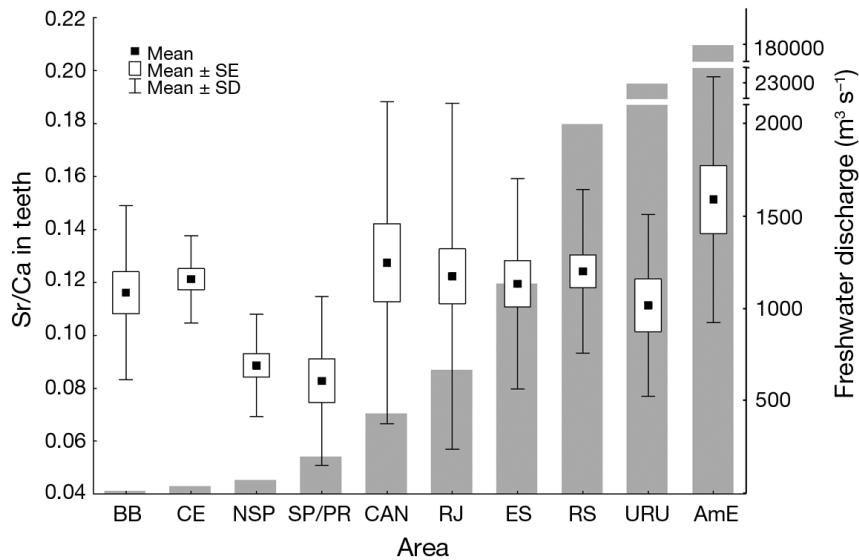


Fig. 5. Mean Sr/Ca in the teeth of Guiana dolphins *Sotalia guianensis* and franciscanas *Pontoporia blainvillei* from marine coastal regions in Brazil and Uruguay, and mean freshwater discharge (grey bars) of the main freshwater input to the area (see Table 1 for details and area codes)

areas. Salinity, however, is not the only variable affecting Ba levels in these coastal waters nor their incorporation into dolphin teeth. For example, differences in catchment rock type and weathering rates may contribute to differences in Ba among estuaries (Coffey et al. 1997). The concentration of Ba in estuaries may also vary over temporal scales due to mixing of fresh and salt-waters (Elsdon & Gillanders 2006). However, local Ba/Ca values reported here should be representative of each sampling location, because (1) our sample size was considerably large, (2) more than 1 species was sampled in some areas, and (3) the tooth sampling strategy encompassed all dentin deposited during the post-weaning period

until death, spanning several years since only adult individuals were considered. Hence, temporal variation in environmental Ba/Ca ratios, and concomitant variation of elemental ratios along food webs, is unlikely to jeopardize the potential application of Ba/Ca ratios as natural markers for tracking habitat use patterns in aquatic mammals, at least on similar spatial and temporal scales as considered in this study.

Dolphins from the 2 coastal areas with the lowest runoff (CE and NSP) exhibited equivalently low Ba/Ca ratios. Similar low Ba/Ca values were also found in franciscanas and Guiana dolphins resident of BB, where low freshwater input (Barros et al. 2010, our Table 1) together with characteristic hydrodynamics result in domination by saline waters (Barros et al. 2010). Further, dolphins' main prey

are marine fishes and cephalopods that occur inside the estuary (Cremer et al. 2012), thus contributing to the explanation of the marine-like Ba/Ca ratios found in the dolphins.

Dolphins sampled in estuarine regions exhibited variable Ba/Ca ratios compatible with the relative mean freshwater discharge within their range. The only exception was for dolphins from AmE, which exhibited lower Ba/Ca ratios than animals from the La Plata River and the Patos Lagoon estuaries, despite the massive Amazon estuary mean freshwater discharge ($180\,000\text{ m}^3\text{ s}^{-1}$; Geyer et al. 1996), which is 8 and 90 times higher than the La Plata River and the Patos Lagoon estuaries, respectively (Piola & Romero

Table 5. Multiple comparisons of Sr/Ca ratios for teeth dentin of Guiana dolphins *Sotalia guianensis* and franciscanas *Pontoporia blainvillei* from Brazil and Uruguay (see Table 1 for area codes). Up-right p-values from U-tests are for Guiana dolphins comparisons and down-left Tukey's HSD p-values are for franciscanas comparisons. *p < 0.05; **p < 0.001

	AmE	CE	ES	RJ	NSP	CAN	SP/PR	BB	RS	URU
AmE		1.00	0.06	**	–	0.46	–	1.00	–	–
CE	–		0.97	**	–	1.00	–	1.00	–	–
ES	–	–		0.07	–	1.00	–	1.00	–	–
RJ	–	–	0.96		–	**	–	*	–	–
NSP	–	–	**	**		–	–	–	–	–
CAN	–	–	–	**	–		–	1.00	–	–
SP/PR	–	–	**	**	0.95	–	–	–	–	–
BB	–	–	0.13	0.20	0.53	–	0.18	–	–	–
RS	–	–	0.08	*	*	–	**	1.00	–	–
URU	–	–	*	**	0.49	–	0.11	1.00	0.86	–

2004, Calliari et al. 2009). Among the main tributaries of the Amazon basin, the Solimões River accounts for 56% of the total discharge of the Amazon at its mouth (Gaillardet et al. 1997). This river is classified as a whitewater river (Sioli & Klinge 1962), which contains high levels of both Ba and Ca (Gaillardet et al. 1997, Kuchler et al. 2000, Queiroz et al. 2009), resulting in low Ba/Ca ratios. Blackwater tributaries such as the Rio Negro, on the other hand, have lower Ba and Ca contents (Gaillardet et al. 1997, Kuchler et al. 2000, Queiroz et al. 2009), leading to a higher Ba/Ca ratio. Therefore, the lower than expected Ba/Ca ratios found in Guiana dolphins from the AmE is probably due to the strong influence of the low Ba/Ca water discharge from the Solimões River.

Although no significant differences were found in franciscanas between the 2 southernmost areas, dolphins from URU had slightly higher average Ba/Ca ratios values than those found in RS. This variation is probably because of the much stronger influence of the La Plata River runoff on coastal areas near Uruguay than that of the Patos Lagoon estuary in Rio Grande do Sul. Also, franciscanas in URU often use the estuarine portion of the La Plata River (Costa-Urrutia et al. 2012), while in RS they have not been seen in the Patos Lagoon estuary since at least 1974 (Pinedo 1998), when local systematic surveys for marine mammals started.

In the remaining estuaries, where franciscanas and Guiana dolphins occur in sympatry, results varied. In ES, both species exhibited the same mean Ba/Ca values, which could be associated with a similar habitat use pattern, although the sample size for franciscanas was small ($n = 4$). In the adjacent estuary to the south (RJ), franciscanas exhibited higher Ba/Ca values than Guiana dolphins. In this area, both species inhabit coastal areas adjacent to the mouth of Paraíba do Sul River (Di Benedetto 2003), which is the main input of freshwater in the area. However, franciscanas feed mainly on benthic/demersal species while Guiana dolphins' main prey includes more pelagic fishes (Di Benedetto & Ramos 2001, 2004). The concentration of dissolved Ba increases with depth due to the positive correlation between pressure and barite solubility (Neff 2002), thus higher Ba/Ca ratios are expected for benthic feeders (Peek & Clementz 2012), which might explain the higher Ba/Ca ratios found in franciscanas.

Although franciscanas from SP/PR inhabit coastal waters adjacent to the Cananéia estuary, they exhibited lower Ba/Ca ratios in their teeth than Guiana dolphins from CAN. The latter are resident in this estuary while franciscanas are only occasionally seen

using the area (Santos et al. 2007). In fact, all franciscanas analyzed in this study were incidentally caught in fishing nets operating in coastal marine waters.

Strontium as a tracer of cetacean habitat use

In contrast to Ba/Ca, there was no evidence that differences in ambient water significantly influenced Sr/Ca levels in the teeth of dolphins from the various habitats. Similarly, previous attempts to evaluate seafood consumption of ancient human populations based on Sr/Ca ratios in bone also concluded that those elemental ratios could not be used to differentiate marine and terrestrial resources (Sealy & Sillen 1988, Gilbert et al. 1994, Burton & Price 1999). On the other hand, Gilbert et al. (1994) reported noticeable differences in Ba/Ca values in marine (including marine mammals) and terrestrial food sources. These results are consistent with the 700-fold difference in Ba/Ca found between freshwater and marine water, while only a 2-fold difference was found for Sr/Ca (Peek & Clementz 2012), and that Sr/Ca may show only minimal salinity-related gradients (Kraus & Secor 2004, Elsdon & Gillanders 2005, Tanner et al. 2013). Biopurification processes along the food web could be overprinting basal environmental differences, especially in high trophic level predators. Consequently, freshwater (or terrestrial) versus marine predators should exhibit equivalent less divergent or even a lack of any difference in Sr/Ca values (as seen in our results) than Ba/Ca.

CONCLUSIONS

We have shown for the first time that Ba/Ca ratios in teeth from different dolphin species may be diagnostic of the salinity of their habitats. The teeth of freshwater dolphins presented Ba/Ca ratios more than 10-fold higher than those of marine species. Furthermore, Ba/Ca decreased with decreasing freshwater discharge in different areas along the coasts of Brazil and Uruguay. Although several factors could affect final Ba concentrations between elemental uptake and final deposition in teeth, environmental Ba availability at least plays a significant role in determining the amount of Ba/Ca deposited in mammals' bioapatites. Sr/Ca ratios, on the other hand, may not be useful as a natural marker for habitat use in marine mammals. Spatial variation in Sr/Ca ratios seem to be completely masked in apatite structures of predators in high trophic levels, even when large

ambient differences between fresh and marine waters are found. Although further studies are needed in order to validate and quantify the extent of the relation among ambient water/diet and elemental deposition in teeth to clarify the fractionation processes underlying the incorporation of Sr and Ba into the teeth of aquatic mammals, results presented here encourage the application of this technique as a potential new or complementary tool for studying habitat use of cetaceans. Ba/Ca ratios in particular clearly differentiate freshwater from marine species. Given some overlap of values found in different areas in the marine environment, we recommend the use of Ba/Ca ratios in combination with other natural tracers (e.g. C, O or S stable isotope compositions) to better elucidate dolphin habitat use. Nevertheless, results presented here strongly suggest that the use of Ba/Ca ratios alone or combined with other chemical tracers such as oxygen isotope ratios (Yoshida & Miyazaki 1991) provides a powerful tool for predicting habitat type (i.e. freshwater vs. marine) in both extant and fossil aquatic or aquatic-dependent mammals.

Additionally, due to the chronological deposition of discrete layers of dentin in cetaceans' teeth until death, long time-integrated and chronologically arranged Ba/Ca values could be obtained in order to analyze ontogenetic changes in those ratios by sampling dentin mineralized at different life stages. The advantage of obtaining time series of age-related measurements of trace metals and other elements is being increasingly utilized for answering different questions about the ecology of marine mammals (e.g. Evans et al. 1995, Newsome et al. 2009). Furthermore, the relatively new technique of laser ablation sampling coupled to an ICP-MS provides a useful method to profile the distribution of trace elements in incremental hard tissues such as teeth (Evans et al. 1995, Outridge & Evans 1995). Additional advantages of LA-ICP-MS over solution-based methods are fine spatial resolution (1 to 50 μm) and minimal sample preparation, contamination and amount of sample required for analysis (Dolphin et al. 2012). Therefore, by integrating the analysis of Ba/Ca (and/or Sr/Ca in the case of a strong environmental gradient in their values) in teeth and the time-recording property of dentin deposition, it would be theoretically possible to reconstruct habitat use of marine mammals. A combination of traditional approaches with this new tool, each having their distinct advantages and limitations, could provide important new insights into the study of habitat use patterns of these aquatic mammals.

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Appendix. Instrumental conditions for elemental analysis of dolphin teeth dentin by (A) laser ablation inductively coupled plasma mass spectrometer (LA-ICP-MS) and (B) ICP optical emission spectrometer (ICP-OES). Cal.range: range of calibration curve. Limit of detection (LOD) in mg l^{-1} can be converted to mg kg^{-1} by multiplication with the corresponding dilution factor

(A) LA-ICP-MS ICP-MS operating conditions	Laser operating conditions
Spectrometer: Elan 6000, PerkinElmer	LSX-100, CETAC
RF-power (W): 1500	Mode: Q-switched
Dwell time (ms): 40	Repetition rate (Hz): 20
Sampling time (min): 0.8–1	Rastering speed ($\mu\text{m s}^{-1}$): 40
Cell gas flow ($\text{dm}^3 \text{min}^{-1}$ Ar): 0.9	Defocus (μm): 20
Replicates: 200–800	Energy (mJ pulse^{-1}): 1.26
Scanning Mode: peak-hop	Pulse width (ns): 8–12
Isotopes measured: ^{43}Ca , ^{138}Ba , ^{86}Sr	Pre-ablation time (s): 5

(B) ICP-OES experimental conditions	Element	View	λ (nm)	R^2 (cal. range, $\mu\text{g l}^{-1}$)	LOD (mg l^{-1})
Power (W): 1500	^{138}Ba	Axial	455.403	0.99996 (1–10)	0.00005
Ar plasma (l min^{-1}): 15	^{43}Ca	Radial	422.673	0.99995 (500–3000)	0.010
Ar auxiliary (l min^{-1}): 0.2	^{86}Sr	Axial	421.552	0.99987 (1–10)	0.00005
Ar carrier (l min^{-1}): 0.6					
Sample aspiration rate (ml min^{-1}): 1.5					

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