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**USO DO HABITAT E IDENTIFICAÇÃO DE UNIDADES
POPULACIONAIS DE PEQUENOS CETÁCEOS DO ATLÂNTICO
SUL-OCIDENTAL ATRAVÉS DE ISÓTOPOS ESTÁVEIS E
ELEMENTOS TRAÇO**

SILVINA BOTTA

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Orientador: Eduardo Resende Secchi
Co-orientadora: Aleta A. Hohn

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Ciencia es todo aquello sobre lo cual
siempre cabe discusión.

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INDICE

	Página
RESUMO.....	1
ABSTRACT.....	3
PREFÁCIO.....	5
1.INTRODUÇÃO.....	5
1.1. A identificação de estoques de toninhas e botos-cinza.....	5
1.2. O uso do habitat.....	9
1.3. Informação contida nos dentes revelada por traçadores químicos.....	13
1.3.1. Isótopos estáveis de carbono e nitrogênio.....	14
1.3.1.1. <i>Análise de isótopos estáveis: conceitos básicos e terminologia</i>	15
1.3.1.2. <i>Os isótopos estáveis como traçadores no meio aquático</i>	18
1.3.2. Bário e estrôncio.....	21
1.4. Objetivos e hipóteses.....	24
2. MATERIAL E MÉTODOS: LINHAS GERAIS.....	25
2.1. Obtenção de material.....	25
2.2. Estimativa das idades.....	25
2.3. Análises químicas.....	26
2.3.1. Análise de isótopos estáveis.....	26
2.3.2. LA-ICP-MS.....	28
2.4. Análises estatísticas.....	29
3. SÍNTESE DOS RESULTADOS.....	30
4. CONCLUSÕES.....	37
5. LITERATURA CITADA.....	38
6. FIGURAS.....	57

Anexo I. Ecological stocks of Guiana dolphins (<i>Sotalia guianensis</i>) along the Brazilian coast as inferred from stable isotopes.....	64
Anexo II. Evidence of ecological separation in franciscana dolphins (<i>Pontoporia blainvillei</i>) based on stable isotopes.....	113
Anexo III. Isotopic variation in delphinids from the subtropical western South Atlantic.....	159
Anexo IV. Ba/Ca and Ba/Sr ratios in teeth reveal habitat use patterns of dolphins.....	192
Apêndice 1.....	233

RESUMO

O objetivo geral desta tese foi identificar unidades populacionais (estoques ecológicos) e determinar padrões de uso de habitat de pequenos cetáceos no Atlântico Sul Ocidental por meio de traçadores químicos. A análise de isótopos estáveis foi utilizada como ferramenta para identificar estoques ecológicos de botos-cinza (*Sotalia guianensis*) e toninhas (*Pontoporia blainvillei*) e o uso do habitat de delfínídeos. Adicionalmente, as razões de Ba/Ca, Sr/Ca e Ba/Sr foram utilizadas, pela primeira vez, para caracterizar os padrões de uso de habitats por espécies de cetáceos ao longo de gradientes de salinidade. As análises químicas foram realizadas em dentes, utilizando um espectrômetro de massa acoplado a um analisador de elementos, no caso dos isótopos e a técnica de ablação a laser seguida da análise num espectrômetro de massa e plasma indutivamente acoplado, no caso do Ba e Sr. Quatro estoques ecológicos de botos-cinza foram identificados ao longo da costa do Brasil: (1) grupo Norte composto por animais de áreas costeiras adjacentes ao estuário do Amazonas (AmE) e indivíduos do Ceará (CE); (2) botos do Espírito Santo (ES); (3) animais do Norte do Rio de Janeiro (NRJ); e (4) indivíduos coletados na Baía da Babitonga (SC). Os animais coletados no Sul de São Paulo/Norte do Paraná (SP/PR) não constituíram um grupo separado. Os valores médios de $\delta^{13}\text{C}$ e $\delta^{15}\text{N}$ das toninhas sugeriram a existência de ao menos cinco estoques ecológicos habitando as seguintes regiões: (1) ES; (2) NRJ; (3) Norte de São Paulo (NSP), SP/PR e SC; (4) Rio Grande do Sul e Uruguai (RS e URU); e (5) Sul de Buenos Aires e Norte da Patagônia (ARG). Com base nos valores de $\delta^{15}\text{N}$ encontrados em ambas as espécies foi comprovada a existência de um gradiente latitudinal de enriquecimento em ^{15}N nas costa Atlântica da América do Sul, provavelmente influenciado pelas contribuições diferenciais de nutrientes das distintas massas d'água que

operam ao longo da mesma. A análise de isótopos estáveis de sete espécies de delfínídeos revelou valores altamente relacionados ao habitat e a ecologia trófica. As espécies apresentaram valores de $\delta^{13}\text{C}$ decrescentes entre espécies costeiras>de plataforma média e externa>oceânicas. Por outro lado, as espécies conformaram guildas tróficas evidenciadas pelos seus valores de isótopos de C e N. As razões Ba/Ca e Ba/Sr tiveram valores dez vezes mais altos em espécies de água doce (*Inia geoffrensis* e *Sotalia fluviatilis*) do que nas espécies marinhas. Os valores de Sr/Ca não variaram entre elas. Nas espécies marinhas, populações de toninhas, botos-cinza e *Tursiops* sp. residentes em áreas tipicamente estuarinas (p.ex. AmE, URU e ARG) apresentaram valores mais altos destas razões do que aquelas populações de áreas com menor influência de águas continentais (CE, NRJ, NSP, SP/PR e SC). O perfil de variação de Ba/Ca e Ba/Sr ao longo da vida obtido para nove indivíduos de *Tursiops* sp. do RS revelou três padrões temporais: perfis homogêneos e de valores baixos; perfis com variações temporais e valores mais altos do que os perfis homogêneos; e perfis com picos de valores extremamente altos, refletindo possivelmente, marcadas variações interanuais no regime de chuvas na região.

PALAVRAS-CHAVE: isótopos estáveis, bário, uso do habitat, Atlântico Sul Ocidental, cetáceos, identificação de estoques.

ABSTRACT

The main objective of this thesis was to identify population units (ecological stocks) and to determine the patterns of habitat use of small cetaceans in the Western South Atlantic by means of chemical tracers. Stable isotope analysis was used as tool for identifying ecological stocks of Guiana dolphins (*Sotalia guianensis*) and franciscanas (*Pontoporia blainvillei*) and for studying habitat use of delphinids. Additionally, the use of Ba/Ca, Sr/Ca and Ba/Sr ratios for characterizing habitat use patterns through aquatic salinity gradients was first assessed in cetaceans. Teeth chemical analyses using a mass spectrometer coupled to an elemental analyzer in the case of stable isotopes and with a laser ablation coupled to a mass and plasma inductively coupled spectrometer for the Ba, Ca and Sr analyses. Four ecological stocks of Guiana dolphins were identified along the Brazilian coast: (1) a northern group composed by dolphins from adjacent coastal areas of the Amazon estuary (AmE) and individuals from Ceará (CE); (2) dolphins from Espírito Santo (ES); (3) animals from northern Rio de Janeiro (NRJ); and (4) individuals collected in Babitonga Bay (SC). Guiana dolphins from southern São Paulo/northern Paraná (SP/PR) could not be grouped apart. Mean $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values in franciscana teeth, suggested the existence of five ecological stocks inhabiting the following areas: (1) ES; (2) NRJ; (3) northern São Paulo (NSP), SP/PR and SC; (4) Rio Grande do Sul and Uruguay (RS and URU); and (5) southern Buenos Aires and northern Patagonia (ARG). Based on $\delta^{15}\text{N}$ values found in both species, a latitudinal trend in ^{15}N enrichment along the Atlantic coast of South America was observed, probably due to varying nutrient contributions from different water masses. Stable isotope analysis of seven delphinids revealed values highly related to their habitat and trophic ecology. Mean $\delta^{13}\text{C}$ values decreased from coastal

species>mid and outer continental shelf species>offshore. On the other hand, C and N isotopes values indicated the existence of trophic guilds among some species. Ba/Ca and Ba/Sr ratios were tenfold higher in freshwater species (*Inia geoffrensis* and *Sotalia fluviatilis*) than in marine species. Sr/Ca values did not differ between them. Among marine species, resident populations of franciscanas, Guiana dolphins and *Tursiops* sp. from typical estuarine areas (e.g. AmE, URU and ARG) presented higher ratios than populations from areas with minimal freshwater discharge (CE, NRJ, NSP, SP/PR and SC). Ba/Ca and Ba/Sr age-related profiles from nine individuals of *Tursiops* sp. from RS revealed three temporal patterns: low values with minimal temporal variation; intermediate values with peaks and troughs; and high values with peaks and troughs, the latter probably reflecting interannual variations in precipitation rates.

KEY WORDS: stable isotopes, barium, habitat use, western South Atlantic, cetaceans, stock identification.

PREFÁCIO

A costa oeste do Oceano Atlântico Sul possui uma grande diversidade de cetáceos incluindo várias espécies de odontocetos. Algumas espécies por serem restritas às regiões costeiras, são mais conhecidas e estudadas, porém são também as mais vulneráveis às atividades antrópicas, principalmente às capturas acidentais em redes de pesca. Outras espécies que são encontradas em regiões mais inacessíveis sobre as plataformas média, externa e talude são menos conhecidas, e informações básicas sobre distribuição, uso do habitat e ecologia trófica entre outras, são ainda inexistentes para esta região do Atlântico. O avanço nas técnicas químicas hoje disponibilizaram o uso de traçadores químicos para estudos em variados campos da biologia e nos mais diversos tipos de organismos. Assim, nesta tese buscou-se explorar a potencialidade de duas destas novas metodologias (composição de isótopos estáveis e elementos traço) para a identificação das populações e o estudo do uso do habitat pelos pequenos cetáceos da costa oeste do Atlântico Sul.

A Tese foi estruturada em quatro artigos independentes redigidos em língua inglesa (Anexos I-IV), precedidos por uma primeira parte, redigida em língua portuguesa. Nesta, é apresentada uma introdução, os objetivos e hipóteses do estudo, uma breve descrição dos materiais e métodos, uma síntese dos resultados e as conclusões.

1. INTRODUÇÃO

1.1. A identificação de estoques de toninhas e botos-cinza

A toninha (*Pontoporia blainvillei*) é o único membro da família Pontoporiidae, relacionada filogeneticamente com os golfinhos de rio, porém que ocupa o ambiente

marinho. A distribuição da mesma abarca a região costeira do Atlântico Sul Ocidental, desde Itaúnas (18°25'S), no Espírito Santo (Siciliano 1994) até o Golfo San Matías (41°09'S) na Argentina (Crespo et al. 1998).

A espécie ocorre principalmente em águas rasas, normalmente até a isóbata de 30m, nadando em pequenos grupos de 2-5 indivíduos (Danilewicz et al. 2009). Sua dieta é basicamente composta de teleósteos, cefalópodes e crustáceos, estes sendo mais importantes nos juvenis do que nos adultos (Basso 2005, Bittar & Di Benedetto 2009,). Este hábito costeiro faz com que exista uma importante sobreposição com atividades antrópicas, dentre as quais a pesca é a que mais afeta à sua conservação. Capturas acidentais, principalmente em redes de pesca de emalhe, têm uma alta frequência ao longo de toda a sua distribuição (Corcuera 1994, Secchi et al. 1997, Di Benedetto & Ramos 2001, Bertozzi & Zerbini 2002; Rosas et al. 2002, Secchi et al. 2003b, Cappozzo et al. 2007, Franco-Trecu et al. 2009, Secchi 2010). Assim, a espécie encontra-se atualmente incluída na categoria de "Vulnerável" no Livro Vermelho de Espécies Ameaçadas da União Mundial para a Conservação da Natureza (IUCN 2010) e "em perigo" no Livro Vermelho da fauna brasileira ameaçada de extinção (MMA 2008).

Com a finalidade de avaliar o efeito das capturas acidentais a nível local e definir estratégias de conservação que assegurassem a sobrevivência das suas populações, propôs-se a separação da espécie em quatro estoques, os quais ocorrem em áreas denominadas Áreas de Manejo para a Toninha/Franciscana (*Franciscana Management Areas*, FMAs I, II, III e IV – ver Secchi et al. 2003a). A FMA I inclui as águas costeiras do Espírito Santo e Rio de Janeiro; a FMA II abrange a região desde São Paulo até Santa Catarina; a FMA III compreende as águas costeiras do Rio Grande do Sul e Uruguai; e a FMA IV limita-se às

águas costeiras da Argentina desde Buenos Aires até Chubut. Porém, a falta de informações suficientes nos dois extremos da sua distribuição fez com que as toninhas que habitam Espírito Santo e o norte do Rio de Janeiro, separadas por um hiato na distribuição (Siciliano et al. 2002) tenham sido incluídas num único estoque e aquelas que habitam as costas da Argentina foram separadas em outro estoque, utilizando a divisão política com Uruguai como limite entre esta FMA e a FMAIII. Esta subdivisão tem caráter adaptativo e recomenda-se que outros limites ou áreas sejam propostos à medida que novas informações sejam obtidas (Secchi et al. 2003a).

O boto-cinza (*Sotalia guianensis*) foi recentemente reconhecido como uma espécie diferente do tucuxi (*S. fluviatilis*) com base em dados morfológicos (Monteiro-Filho et al. 2002) e moleculares (Cunha et al. 2005, Caballero et al. 2007). A espécie distribui-se em águas costeiras, desde Nicarágua até Santa Catarina, sul do Brasil (Simões-Lopes 1988, Borobia et al. 1991, Carr & Bonde 2000, Flores & da Silva 2009).

Esta espécie costeira também sofre altas taxas de capturas acidentais ao longo da sua distribuição no Brasil (Siciliano 1994, Di Benedetto 2003, Netto & Barbosa 2003, Monteiro-Neto et al. 2004, Netto & Di Benedetto 2008), assim como também são alvo de capturas deliberadas na região Norte do país (Secchi 2009). Porém, apesar da sua ampla distribuição, muitos aspectos da biologia e ecologia da espécie permanecem desconhecidos (Santos et al. *no prelo*) razão pela qual o estado de conservação das suas populações ainda não pode ser avaliado formalmente e a espécie ainda encontra-se como "Dados Insuficientes" na classificação da IUCN (IUCN 2010).

Dados moleculares indicaram a existência de ao menos seis unidades de manejo (MUs, *sensu* Moritz 1994) no Brasil: Pará, Ceará, Rio Grande do Norte, Bahia, Espírito

Santo e o grupo de distribuição mais austral composto pelos animais que habitam as regiões sul e sudeste do Brasil (Cunha et al. 2010). Dados morfométricos avaliados em botos-cinza do Espírito Santo, norte e sul do Rio de Janeiro, e São Paulo/Paraná também apresentaram diferenças significativas entre estas possíveis populações (Ramos 2001). Outras características como assobios (Azevedo & Van Sluys 2005) e carga de contaminantes (Lailson-Brito et al. 2010), por outro lado, evidenciaram que uma certa estruturação populacional existe na região sul-sudeste do Brasil.

Contudo, nenhum dos estudos foi o suficientemente abrangente para amostrar a totalidade das populações, faltando principalmente dados das regiões nordeste e norte do Brasil. Desta forma, atualmente considera-se prioritária a delimitação das populações com a finalidade de distinguir possíveis unidades de manejo ou unidades evolutivamente significativas (Moritz 1994, Waples 1995), recomendando-se o uso de várias linhas de evidência para uma melhor compreensão sobre a estrutura populacional desta espécie (Cunha et al. 2005, Caballero et al. 2007).

A identificação de unidades populacionais discretas é crucial para a avaliação do potencial efeito de impactos naturais e antropogênicos (Dizon et al. 1992, Moritz 1994), e posterior estabelecimento de medidas de conservação dos pequenos cetáceos. O conceito de estoque como “Unidade Evolutivamente Significativa” (*Evolutionary Significant Unit* ou *ESU sensu* Moritz 1994) lida com a estrutura populacional numa escala histórica, sendo definida como populações que têm estado historicamente isoladas, caracterizadas por níveis baixos ou inexistentes de intercâmbio genético (Dizon et al. 1992, Moritz 1994). Porém, para atingir objetivos de manejo a curto prazo de populações ameaçadas, muitas vezes é necessário definir unidades de manejo num sentido menos restrito, podendo-se utilizar, além de dados genotípicos e fenotípicos, outras fontes de informação. Dados sobre

distribuição e padrões de movimentos, parâmetros populacionais, comportamento e diferenças ecológicas como alimentação, parasitas, razões de isótopos estáveis e assinaturas de ácidos graxos podem ser utilizados para distinguir unidades discretas a serem manejadas (Dizon et al. 1992, Wang 2002, Taylor 2005). Desta forma, podem ser definidas unidades de manejo que equivalem ou não a estoques como ESUs mas que, por outro lado, são úteis para definir e implementar estratégias de manejo a curto prazo (Secchi et al. 2003a). Assim, o termo estoque que foi utilizado nesta tese refere-se a unidades de manejo que, eventualmente, pode representar uma ESU.

1.2. O uso do habitat

A distribuição dos cetáceos é resposta de fatores demográficos, evolutivos e ecológicos, relacionados às características do habitat e à presença de distúrbios antropogênicos (Forcada 2002). Porém, o fator determinante é a distribuição e a densidade das suas presas principais (Hastie et al. 2004). Os estuários são regiões altamente produtivas (Lalli & Parsons 1997) que oferecem alimento e abrigo a muitas populações de cetáceos, sendo comum encontrar populações residentes ou com fidelidade a esses sítios. Além do mais, a especialização a um local e às suas presas pode acabar isolando geneticamente essas populações, inclusive de populações adjacentes como ocorre com os golfinhos-nariz-de-garrafa do Indo Pacífico (*Tursiops aduncus*) de Port Stephens na Austrália (Möller et al. 2007), os golfinhos-nariz-de-garrafa (*T. truncatus*) de várias regiões estuarinas da costa oeste do Atlântico Norte (Rosel et al. 2009) e as toninhas da Baía Samborombón na Argentina (Mendez et al. 2010).

Ao longo da costa leste da América do Sul, indivíduos residentes foram identificados por meio da foto-identificação em vários estuários. Populações residentes de botos-cinza

nos estuários de Caravelas, no Sul da Bahia (Rossi-Santos et al. 2007), Baía de Guanabara, no Rio de Janeiro (Azevedo et al. 2007), Cananéia, no sul de São Paulo (Oshima et al. 2010), e na Baía de Babitonga, norte de Santa Catarina (Hardt 2005). Várias populações de golfinhos-nariz-de-garrafa (*Tursiops* sp.) são residentes de desembocaduras de rios e estuários no sul do Brasil, entre elas, a população que habita o estuário da Lagoa dos Patos (Dalla Rosa 1999, Fruet et al. 2011). As toninhas também são animais associados a águas turvas deste tipo de ambiente, podendo ser encontradas dentro do complexo de estuários de Paranaguá (Santos et al. 2009), na Baía da Babitonga (Cremer & Simões-Lopes 2005) e no estuário do Rio da Prata (Praderi 1986).

Pouco se conhece sobre o uso do habitat de espécies que habitam a plataforma continental e o talude do Atlântico Sul Ocidental. Porém há uma importante diversidade de delfínídeos nesta região (Zerbini et al. 2004). Golfinhos do gênero *Stenella* podem ser encontrados tanto em águas da plataforma interna (e.g. golfinho-pintado-do-Atlântico, *S. frontalis*) como em águas da plataforma média e externa (e.g. golfinho-pintado-pantropical, *S. attenuata*), outros habitam águas oceânicas da plataforma e do talude (e.g. golfinho-rotador, *S. longirostris*) (Moreno et al. 2005). O golfinho-de-dentes-rugosos (*Steno bredanensis*) é descrito na literatura como de hábitos oceânicos, porém no Brasil são comuns as avistagens desta espécie em águas costeiras (e.g. Lodi & Hetzel 1998). A falsa-orcas (*Pseudorca crassidens*) e o golfinho-de-Fraser (*Lagenodelphis hosei*) são tipicamente habitantes de águas oceânicas, porém conteúdos estomacais destas espécies provindos de animais encalhados no sul do Brasil revelaram a presença de presas costeiras (Pinedo & Rosas 1989, Pinedo et al. 2001, Santos & Haimovici 2001, Moreno et al. 2003), o que sugere movimentos longitudinais. Certas espécies possuem populações costeiras e

oceânicas, como é o caso do golfinho-nariz-de-garrafa (*T. truncatus*) da costa oeste do Atlântico Norte (Rosel et al. 2009) e as orcas (*Orcinus orca*) na costa leste do Pacífico Norte (Ford 2002). Poucas informações existem sobre orcas nas costas brasileiras, porém tanto animais costeiros (e.g. Dalla Rosa et al. 2005, Santos & da Silva 2009) como oceânicos (Secchi & Vaske Jr 1998) já foram registrados. No sul do Brasil, o golfinho-nariz-de-garrafa ocupa regiões costeiras como desembocaduras de rios e estuários (Castello & Pinedo 1977, Simões-Lopes & Fabian 1999, Dalla Rosa 1999, Fruet et al. 2011), porém há registros da espécie na plataforma externa (Zerbini et al. 2004) e ilhas oceânicas (Ott et al. 2009).

Ao contrário das espécies costeiras, para as quais estudos de foto-identificação permitem o seguimento e a identificação de populações residentes, hábitat mais inacessíveis inviabiliza ou tornam excessivamente dispendiosa a aplicação desta técnica. Informações sobre o uso de habitat destes cetáceos são baseadas em avistagens a partir de plataformas de oportunidade e evidências indiretas dos ambientes de forrageio através do estudo dos conteúdos estomacais de animais encalhados (Barros & Clarke 2009). Esta técnica, porém, traz vieses devido ao diferente grau de digestibilidade das presas, superestimando a importância daquelas de digestão mais lenta (e.g. bicos de cefalópodes e crustáceos) (Jobling & Breiby 1986, Santos et al. 2001), à ingestão secundária de presas e ao curto intervalo de tempo de alimentação que esta técnica evidencia (Hobson et al. 1994, Dehn et al. 2006).

A distribuição e abundância das presas, por serem os são fatores determinantes dos padrões de distribuição dos cetáceos, podem servir para prever as variações espaço-temporais da distribuição dos predadores (e.g. Wells et al. 1999, Forcada 2002). Assim,

características fixas do habitat como a batimetria, declividade e distâncias da costa, rios e estuários podem influenciar na produtividade da área disponibilizando maior oportunidade de captura de presas (e.g. Ballance 1992, Hooker et al. 1999, Parra 2006). Análises e modelos estatísticos permitem avaliar a influência que estas múltiplas variáveis ambientais têm sobre a distribuição e abundância espaço-temporal das espécies (Azevedo-Gutierrez 2009). Por exemplo, áreas de maior declividade foram associadas com maiores densidades de golfinhos-nariz-de-garrafa no estuário da Lagoa dos Patos (DiTullio 2009), assim como águas rasas foram também evidenciadas como preferenciais nas zonas costeiras adjacentes de esta e outras populações de golfinhos como *Sousa chinensis* e *Orcaella heinsohni* em Queensland, Austrália (Parra et al. 2006). Por outro lado mudanças em variáveis ambientais como temperatura e salinidade também são responsáveis por variações temporais na distribuição dos cetáceos. Tanto em regiões onde ocorrem sazonalmente eventos oceanográficos como frentes e ressurgências, como em estuários onde as condições podem variar segundo a descarga de água doce e outros fatores, puderam ser comprovadas mudanças nas distribuições de cetáceos de acordo com as variações ambientais ocorridas (e.g. Fielder & Reilly 1994, Di Tullio 2009).

Assim, até o momento técnicas tradicionais como a foto-identificação que permite a captura-recaptura de animais associando-os a determinadas áreas, avistagens oportunistas, estudos dedicados a identificação de áreas de distribuição correlacionando-as com variáveis ambientais e o uso de traçadores naturais, como a composição de presas, vêm sendo aplicadas para o estudo do uso do habitat dos cetáceos do Atlântico Sul Ocidental. Outras abordagens como o uso de transmissores satelitais são aplicados apenas em grandes baleias (e.g. baleia-jubarte, *Megaptera novaengliae*, Dalla Rosa et al. 2008) e a alguns grupos de

toninhas da Argentina (Bordino et al. 2008). Esta técnica, além de ser dispendiosa, envolve uma série de procedimentos logísticos que requerem grande experiência, de forma que são aplicados apenas a uns poucos indivíduos da população. Atualmente novas metodologias complementares estão sendo cada vez mais utilizadas para o estudo do uso do habitat nestes e em outros organismos aquáticos, tais como isótopos estáveis de elementos (e.g. carbono, nitrogênio, estrôncio e alguns metais) e ácidos graxos (e.g. Herman et al. 2005, Krahn et al. 2007).

1.3. Informação contida nos dentes revelada por traçadores químicos

Os dentes dos cetáceos são compostos por tecidos protéicos e calcificados: a camada de esmalte, na superfície exposta do dente ou coroa, a dentina, que ocupa todo o interior do dente até a cavidade pulpar e o cimento, que recobre a raiz do dente fixando-o à mandíbula (Figura 1). Tanto a dentina quanto o cimento apresentam camadas de crescimento ou GLGs (*Growth Layer Groups*, Perrin & Myrriick 1980) visíveis, as quais, dependendo da espécie, são utilizadas em maior ou menor medida para a estimativa da idade do indivíduo. Na maioria dos delfínídeos e na toninha cada GLG, encontrada tanto na dentina pós-natal (depositada após o nascimento) como no cimento, representa 1 ano de idade (Hohn et al. 1989, Pinedo & Hohn 2000). A dentina é formada por minerais depositados numa matriz de fibras colágenas. O principal constituinte mineral é a hidroxiapatita $[Ca_{10}(PO_4)_6(OH)_2]$ e as fibras colágenas são formadas por fibroblastos, osteoblastos e odontoblastos (Langvatn 1995). Assim, os diversos componentes químicos depositados no dente, tanto na matriz orgânica como na porção inorgânica podem ser analisados para responder perguntas específicas da história de vida do organismo.

As principais vantagens de utilizar traçadores químicos extraídos de bioapatitas são a "marcação" do animal desde a vida intra-uterina até a morte, a permanência da marca devido à inércia química do material depositado, e a possibilidade de extrair informações de forma ordenada e cronológica podendo ser associadas a idades ou anos específicos (Elsdon et al. 2008). Dois tipos de traçadores químicos extraídos dos dentes dos exemplares analisados foram utilizados nesta tese: isótopos estáveis de carbono e nitrogênio e elementos traço (bário e estrôncio). As composições de isótopos estáveis foram utilizadas como ferramenta para a identificação de estoques de botos-cinza do Brasil (Anexo I) e de toninhas ao longo de toda a sua distribuição (Anexo II) e para estudar o uso do habitat de várias espécies de delfínídeos (Anexo III). O potencial uso do Ba e Sr, amplamente utilizados como traçadores do uso do habitat em peixes (Elsdon & Gillanders 2006, Albuquerque et al. 2010), foi pela primeira vez explorado em cetáceos (Anexo IV). Ambas as técnicas envolvem uma série de fundamentos teóricos e definições que são necessários compreendê-los para se interpretar corretamente as informações que elas aportam. Desta maneira, nas seções seguintes, as principais noções sobre a análise de isótopos estáveis e dos elementos Ba e Sr são apresentadas, assim como uma revisão da sua utilização como traçadores, com ênfase nos cetáceos.

1.3.1. Isótopos estáveis de carbono e nitrogênio

A análise de isótopos estáveis em tecidos biológicos vem sendo aplicada com sucesso para o estudo de diversos campos das ciências, tais como a ecologia trófica (Hobson et al. 1997, Hammill et al. 2005, Lusseau & Wing 2006, Muto & Soares 2011), fisiologia (Gannes et al. 1998) e oceanografia (Ostrom et al. 1997). O uso destes traçadores para a identificação de estoques é recente, porém em crescimento, principalmente para diferenciar

populações de peixes (e.g. Stephenson et al. 2001, Ashford et al. 2008, Newman et al. 2010) e mamíferos aquáticos (e.g. Born et al. 2003, Outridge et al. 2003, Barros et al. 2010). Adicionalmente, migrações (e.g. Summers et al. 2006, Quillfeldt et al. 2010), uso do habitat e ecologia trófica (e.g. Lewis et al. 2006, Garcia et al. 2007, Pinela et al. 2010, Ricchialdelli et al. 2010) também podem ser estudados por meio desta técnica.

1.3.1.1. Análise de isótopos estáveis: conceitos básicos e terminologia

Os isótopos estáveis mais comumente utilizados são os de carbono e nitrogênio. A razão (R) de dois isótopos estáveis, um pesado e um leve ($^{13}\text{C}/^{12}\text{C}$ e $^{15}\text{N}/^{14}\text{N}$) é quantificada nas amostras e expressa em relação àquela encontrada num padrão internacionalmente aceito, com a notação δ : $\delta X\text{‰} = [(R_{\text{amostra}}/R_{\text{padrão}}) - 1] * 1000$, onde X é ^{13}C ou ^{15}N . Uma amostra que possui mais quantidade do isótopo pesado com relação ao padrão é comumente chamada de enriquecida (valores positivos de δ) e aquelas com quantidades proporcionalmente menores do isótopo pesado, de empobrecido (valores negativos de δ). Na natureza, devido a sua diferença de massa, os isótopos leves e pesados se comportam de forma diferente em muitos processos ambientais e fisiológicos, e os leves encontram-se em maior quantidade do que os pesados. O fracionamento isotópico é o processo pelo qual um isótopo é discriminado em relação ao outro, ou é absorvido preferencialmente, de modo que o produto possui um valor de δ diferente daquele da fonte. Assim, o fracionamento resulta no enriquecimento ou empobrecimento do isótopo pesado da amostra em estudo (produto) em relação a sua fonte (substrato).

Para os ecossistemas, as composições de isótopos de C e N dependem dos intercâmbios e fracionamentos que ocorrem durante os ciclos biogeoquímicos (Peterson & Fry 1987). A incorporação de carbono pelas plantas C_3 , por exemplo, envolve um fracionamento de

aproximadamente 21‰ entre a atmosfera (-7‰) e a planta (-28‰). Por outro lado, a incorporação de carbono nas plantas C₄ envolve um pequeno fracionamento de apenas 6‰, de modo que estas são em geral mais enriquecidas em ¹³C do que as plantas C₃. No oceano, o intercâmbio entre a atmosfera e a superfície marinha ocorre em equilíbrio. A retirada de carbono para formar carbonatos envolve um pequeno fracionamento ($\delta^{13}\text{C}_{\text{bicarbonatos}}=0\text{‰}$). Porém, a fixação de carbono pelas algas por meio da fotossíntese envolve um grande fracionamento, resultando em valores de $\delta^{13}\text{C}$ entre -19 a -24‰ (Peterson & Fry 1987) (Figura 2).

O ciclo do nitrogênio envolve uma maior diversidade de processos que afetam a distribuição e os valores isotópicos no ambiente marinho. A fixação de N atmosférico não acontece com importantes fracionamentos, de forma que o $\delta^{15}\text{N}$ do N fixado no oceano é de aproximadamente -1‰. O escoamento terrestre e a precipitação atmosférica podem ser afetados por processos biológicos e aportes antropogênicos ao longo do trajeto ao mar e nos estuários que podem alterar o $\delta^{15}\text{N}$ final do aporte. A denitrificação é o maior mecanismo de perda de N fixado no oceano, ocorrendo tanto na coluna d'água como nos sedimentos em baixas concentrações de oxigênio. Este processo discrimina fortemente o ¹⁵N, enriquecendo por tanto, o restante de nitrato. Quando isto ocorre em concentrações baixas de oxigênio, se produz uma elevação do $\delta^{15}\text{N}$ do nitrato, sendo esta a razão pela qual o nitrato oceânico tem $\delta^{15}\text{N}$ mais elevado do que aquele derivado da fixação de N atmosférico (Peterson & Fry 1987, Sigman et al. 2009). Estes e outros processos e fracionamentos que ocorrem durante o ciclo do nitrogênio no oceano encontram-se esquematizados na Figura 3.

Os alimentos são a principal fonte de carbono e nitrogênio nos consumidores, assim suas composições isotópicas são essencialmente determinadas por aquelas presentes nas

suas dietas (Peterson & Fry 1987, Kelly 2000). Desta forma, para interpretar a composição isotópica em tecidos animais é necessário entender o papel de três fontes principais de variação isotópica: (1) a composição isotópica da dieta; (2) os fracionamentos entre a dieta e os tecidos do consumidor; e (3) o tempo que o valor isotópico das fontes demora para se refletir nos tecidos do consumidor (taxa de reposição ou *turnover*) (Newsome et al. 2010).

O fracionamento entre a dieta e os tecidos dos animais varia dependendo do resultado de diferentes rotas metabólicas entre tecidos (e.g. lipídeos, proteínas, carboidratos), a taxa de crescimento do animal, a qualidade nutricional da dieta, a variação na composição de aminoácidos ou lipídeos dos tecidos e a interação entre estes fatores e a ecologia e fisiologia do organismo (Newsome et al. 2010).

O fracionamento do nitrogênio entre níveis tróficos varia tipicamente entre +2 e +5‰ (DeNiro & Epstein 1981, Minagawa & Wada 1984, Kelly 2000, Post 2002) e é atribuído, principalmente, à excreção de uréia e outros compostos nitrogenados que são empobrecidos em ^{15}N (Minagawa & Wada 1984). Assim, o $\delta^{15}\text{N}$ encontrado nos consumidores é, geralmente, utilizado como indicador da posição trófica que os mesmos têm na teia alimentar (DeNiro & Epstein 1981, Minagawa & Wada 1984, Kelly 2000, Post 2002).

O fracionamento do carbono entre a dieta e os tecidos do consumidor é, geralmente, considerado em aproximadamente 0,5-1‰ (DeNiro & Epstein 1978). Porém, depende do conteúdo de carboidratos, lipídeos ou proteínas do tecido analisado. Assim, o fracionamento varia de acordo com a proporção destes três componentes no tecido analisado. Tecidos moles como músculo, fígado e pele são enriquecidos em ^{13}C em 1-2‰ em relação ao valor médio dos tecidos da dieta, enquanto o colágeno dos tecidos ósseos em geral possui valores de $\delta^{13}\text{C}$ 4-5‰ mais altos do que aqueles encontrados na dieta (Koch

2007). É importante, portanto, levar em consideração o tecido analisado ao se compararem valores obtidos de diferentes fontes. Este mínimo fracionamento que ocorre entre níveis tróficos é a razão pela qual o $\delta^{13}\text{C}$ é, em geral, utilizado para inferir a composição isotópica dos produtores que sustentam a rede trófica.

Finalmente, devido à diferente taxa de renovação ou *turnover* de cada tecido, estes proporcionam informação integrada de diversos lapsos de tempo. Por exemplo, tecidos de rápida renovação como pele, sangue e músculo integram os valores obtidos da dieta das últimas semanas ou meses. Os tecidos considerados metabolicamente inertes ou de crescimento contínuo (e.g. vibrissas, cerdas bucais, dentes), oferecem, por outro lado, informação a mais longo prazo, incluindo informação da vida toda do animal, como no caso dos dentes (Walker & Macko 1999, Kelly 2000).

1.3.1.2. Os isótopos estáveis como traçadores no meio aquático

A composição de isótopos de carbono e nitrogênio dos cetáceos é basicamente o produto dos valores isotópicos encontrados nas suas presas. Porém, a interação de diversos fatores tais como as fontes de nutrientes, a composição de produtores e algumas características oceanográfica determinam a composição de isótopos nos ambientes marinhos afetando os valores isotópicos encontrados nas diferentes redes tróficas. Assim, diferenças e gradientes isotópicos entre os diversos ambientes onde os cetáceos se alimentam (e.g. pelágico vs bentônico, costeiro vs oceânico vs estuarino, Clementz & Koch 2001) podem ser utilizados para evidenciar diferenças tróficas entre populações ou para estudar os padrões de uso do habitat.

As redes tróficas de ambientes costeiros (incluindo estuários) são sustentadas por uma ampla variedade de produtores, cada um dos quais possui valores isotópicos diferentes,

principalmente de $\delta^{13}\text{C}$, por exemplo, plantas C_3 terrestres ($\delta^{13}\text{C}=-23$ a -30‰), fanerógamas marinhas ($\delta^{13}\text{C}=-3$ a -15‰ ; -26‰ em algumas espécies que crescem em baixas salinidades), macroalgas (-8 a -27‰), plantas de marismas C_3 (-23 a -26‰), plantas de marismas C_4 (-12 a -14‰), algas bentônicas (-10 a -20‰), fitoplâncton marinho (-18 a -24‰) (Fry & Sherr 1984, Michener & Kaufman 2007).

Os valores de $\delta^{15}\text{N}$ não diferem tanto entre os produtores (Connolly et al. 2004), porém são afetados por contribuições de origem antrópica, geralmente enriquecidas em ^{15}N (Macko & Ostrom 1994, McClelland et al. 1997, Dillon & Chanton 2007), e processos oceanográficos como ressurgências costeiras que disponibilizam nitratos de origem profunda enriquecidos em ^{15}N (Miyake & Wada 1967, O'Reilly et al. 2002, Bode et al. 2003, 2004, Sigman et al. 2009).

No oceano aberto, a fonte primária de alimento é limitada ao fitoplâncton, de tal forma que as variações encontradas nas composições de isótopos de carbono das redes tróficas são devidas principalmente a fatores tais como a temperatura do ambiente e a morfologia e taxa de crescimento do fitoplâncton (Michener & Kaufman 2007). O $\delta^{15}\text{N}$, por outro lado, depende principalmente das fontes de nitrogênio utilizadas (gás N_2 , amônia e nitrato), o fracionamento isotópico durante a assimilação, do tamanho do pool de nutrientes e do grau em que os produtores consomem o nitrogênio disponível (Montoya 2007). A fixação de N_2 gera matéria orgânica com baixos valores de $\delta^{15}\text{N}$ (-3 a 3‰). Os produtores que utilizam nitrato como fonte de nitrogênio têm preferência por incorporar o isótopo mais leve, tornando-os, em geral, menos enriquecidos em ^{15}N do que o nitrato disponível. Porém, a medida que todo o nitrato é consumido, o fitoplâncton passa a ter um valor de $\delta^{15}\text{N}$ similar ao do nitrato assimilado (Montoya 2007).

A interação de todos estes fatores que atuam sobre as composições de isótopos estáveis nos ecossistemas marinhos resultam em variações espaciais tanto latitudinais quanto longitudinais nos valores isotópicos encontrados em predadores como os cetáceos e outros componentes de níveis tróficos elevados (Graham et al. 2010). Estas "paisagens isotópicas" ou *isoscapes* (Graham et al. 2010, Jaeger et al. 2010) foram utilizados para identificar populações de por exemplo, baleias jubarte (*Megaptera novaeangliae*) no Pacífico Norte (Witteveen et al. 2009), baleias minke (*Balaenoptera acutorostrata*) do Atlântico Norte (Born et al. 2003) e narvais (*Monodon monoceros*) da Groenlândia (Dietz et al. 2004). Apesar das diferenças entre as populações serem possivelmente devidas ao nível trófico ocupado, como por exemplo entre as orcas (*Orcinus orca*) residentes e transeuntes do Pacífico Norte, especializadas na captura de salmão e mamíferos marinhos, respectivamente (Ford et al. 1998), diferenças espaciais nos valores isotópicos são utilizadas para a identificação de populações explorando recursos de níveis tróficos equivalentes em diferentes regiões. Por exemplo, diferenças em valores de $\delta^{13}\text{C}$ devido ao aporte diferencial dos produtores presentes na região contribuíram para identificar populações de água doce e marinhas de focas-do-porto (*Phoca vitulina*) do Canadá (Smith et al. 1996). Por outro lado, Aurióles-Gamboa et al. (2009) encontrou diferenças marcantes nos valores de $\delta^{15}\text{N}$ entre leões marinhos do gênero *Zalophus* sp. do Golfo da Califórnia e Ilhas Galápagos, atribuindo as diferenças a padrões oceanográficos atuando sobre os níveis basais de isótopos de nitrogênio. Valores altos de $\delta^{15}\text{N}$ resultantes de redes tróficas enriquecidas em ^{15}N por processos de ressurgências foram registrados, por exemplo, em baleias-da-Groenlândia (*Balaena mysticetus*) das regiões costeiras do Mar de Beaufort (Schell et al. 1989), leões-marinhos-da-Califórnia (*Zalophus californianus*) do Golfo da

California (Aurioles-Gamboa et al. 2009) e em consumidores em geral do ecossistema de ressurgência das costas da Galícia na Espanha (Bode et al. 2003) .

Um dos gradientes mais explorados e conhecidos de isótopos de carbono é a diminuição do valores de $\delta^{13}\text{C}$ entre as regiões costeiras, com alta contribuição de produtores bentônicos à rede trófica, e oceânicas, onde a produção é principalmente pelágica (France 1995, Burton & Koch 1999, Takai et al. 2000, Clementz & Koch 2001, Lesage et al. 2001). Barros et al. (2010) e Herman et al. (2005) utilizaram estes gradientes para identificar populações oceânicas e costeiras de golfinhos-nariz-de-garrafa (*Tursiops truncatus*) e orcas (*Orcinus orca*), respectivamente. Outros autores também estudaram estes gradientes para estudar as relações tróficas e o uso do habitat de diferentes espécies de delfínidos das costas da Moorea na Polinésia Francesa (Kiszka et al. 2010), noroeste da África (Pinela et al. 2010) e da Terra do Fogo, na Argentina (Ricciardelli et al. 2010).

1.3.2. Bário e estrôncio

A variação natural de certos elementos químicos nos ambientes aquáticos vêm sendo utilizadas para estudar os padrões de movimentos e uso do habitat principalmente em peixes (e.g. Elsdon & Gillanders 2006, Albuquerque et al. 2010). Elementos tais como o bário e o estrôncio são depositados na matriz calcária dos otólitos em relação direta com as características químicas e físicas do ambiente em que vivem. Desta forma, a análise química das camadas de crescimento nestas estruturas proporcionam informação cronologicamente ordenada dos diferentes ambientes utilizados pelo animal ao longo da sua vida (Elsdon & Gillanders 2002, Fowler et al. 2005). A disponibilidade destes dois elementos no ambiente aquático depende, em grande parte, da salinidade (Surge & Lohmann 2002, Jacquet et al. 2005). A água marinha possui alto conteúdo de sulfatos o

qual resulta na combinação do mesmo com o Ba numa forma estável de sulfato de Ba (barita) que precipita, removendo íons Ba^{2+} da coluna d'água. O estrôncio, embora se comporte de forma similar ao Ba, forma estruturas mais instáveis e, por consequência, uma maior proporção de Sr dissolvido retorna ao ambiente (Kitano 1975 *apud* Gilbert et al. 1994, Jacquet et al. 2005). Assim, alta biodisponibilidade de Sr e baixa de Ba existem na água marinha relativa a água doce (Burton & Price 1990). Esta relação positiva do Sr e negativa do Ba com a salinidade pode ser detectada em estruturas duras como os otólitos de peixes (Elsdon & Gillanders 2006, Albuquerque et al. 2010).

A utilização desta técnica em peixes tem sido cada vez mais freqüente e aperfeiçoada, enquanto em mamíferos o uso se restringe apenas ao campo bioarqueológico (Gilbert et al. 1994, Sponheimer et al. 2005, Szostek et al. 2009). A análise do conteúdo de Ba e Sr em relação ao Ca nos dentes e ossos de grupos ancestrais de humanos encontrados em escavações arqueológicas vem sendo aplicada para estudar a sua dieta. A principal via de aquisição destes elementos é a água e o alimento, sendo incorporados na matriz de hidroxiapatita de dentes e ossos em substituição ao Ca (Burton et al. 2003). Por serem elementos não essenciais eles não estão sob controle homeostático, porém sofrem uma certa discriminação em relação ao Ca (Szostek et al. 2009). Assim, as razões Sr/Ca e Ba/Ca são utilizadas para estimar a posição trófica discriminando, principalmente, organismos herbívoros e carnívoros (Burton et al. 2003). Por outro lado, os baixos valores de Ba/Ca e Ba/Sr encontrados em organismos marinhos em comparação com os terrestres são bons indicadores da contribuição dos alimentos de origem marinha na dieta de populações ancestrais que ocuparam regiões costeiras (Burton & Price 1990, Gilbert et al. 1994, Szostek et al. 2009).

Portanto, apesar da biopurificação do Ca contrária à acumulação do Ba e Sr, a disponibilidade desses dois últimos elementos em ambientes com diferentes salinidades, ficarão registradas em concentrações diferenciadas nos dentes e ossos dos mamíferos, de forma similar ao que ocorre em otólitos. Com base nestes antecedentes, esperar-se-ia que os dentes de cetáceos que habitam ambientes de água doce *versus* estuários *versus* áreas costeiras com pouca descarga de água doce possam ser discriminados pela quantidade média de Ba e Sr presentes nos seus dentes. Por outro lado, devido à deposição cíclica das camadas de crescimento na dentina, a amostragem de cada uma delas poderia ser um indicador de mudanças temporais no uso do habitat (movimentos) como de alterações das condições ambientais no caso de populações residentes.

No caso dos dentes dos odontocetos, a amostragem de material a partir das camadas de crescimento de maneira individualizada para a realização de análises químicas é complicada devido à difícil visualização destas camadas, principalmente aquelas depositadas mais recentemente nos animais de idade avançada. Porém, a técnica de ablação a laser (*Laser ablation* - LA) outorga a oportunidade de obter micro-amostras cronologicamente ordenadas de material que, por meio do acoplamento com um espectrômetro de massa e plasma (ICP-MS), podem ser analisadas separadamente. O LA-ICP-MS é hoje amplamente utilizado para analisar materiais carbonáticos diversos como conchas (e.g. Gillikin et al. 2006, Belloto & Miekeley 2007), corais (e.g. Alibert et al. 2003, Jupiter et al. 2008), otólitos (e.g. Campana et al. 1994, Hamer et al. 2006, Albuquerque et al. 2010) e, inclusive, esmalte dentário de humanos e outros primatas (e.g. Kang et al. 2004, Dolphin et al. 2005, Humphrey et al. 2008). Porém, os antecedentes do seu uso em dentes

de mamíferos aquáticos se limitam a poucos trabalhos realizados nos anos 1990 (Evans & Outridge 1994, Outridge & Evans 1995).

1.4. Objetivos e hipóteses

O objetivo geral desta tese é identificar unidades populacionais e determinar padrões de uso de habitat de pequenos cetáceos no Atlântico Sul Ocidental por meio de traçadores químicos.

Objetivos específicos

Cada objetivo visa responder ao menos uma hipótese ecológica, conforme abaixo discriminado:

1.4.1. Avaliar a utilização de isótopos estáveis como ferramenta para a identificação de estoques ecológicos de pequenos cetáceos (Anexos I e II).

Hipótese 1- A composição de isótopos estáveis de carbono e nitrogênio presentes nos dentes dos exemplares de botos-cinza e toninhas são específicas para cada região, permitindo assim a identificação de estoques ecológicos destas espécies ao longo das suas distribuições.

1.4.2. Estudar a composição de isótopos estáveis em diferentes espécies de cetáceos para evidenciar o padrão de uso do habitat (Anexo III).

Hipótese 2 - Espécies e/ou populações que pertencem a diferentes guildas tróficas e forrageiam em ambientes diferentes poderão ser distinguidas com base nas composições de isótopos estáveis presentes nos seus dentes.

1.4.3. Avaliar a possibilidade de utilizar elementos como Ba e Sr como indicadores do uso do habitat em cetáceos (Anexo IV).

Hipótese 3 - Espécies de cetáceos que habitam águas doces versus estuários versus áreas costeiras com pouca descarga de água doce poderão ser diferenciados com base nas quantidades e proporções desses elementos presentes nos dentes.

Hipótese 4 - Variações temporais nas concentrações de Ba e Sr ao longo das camadas de crescimento nos dentes dos cetáceos amostrados em águas estuarinas e áreas adjacentes poderão ser evidências de mudanças ambientais e/ou movimentos entre diferentes ambientes.

2. MATERIAL E MÉTODOS: LINHAS GERAIS

2.1. Obtenção de material

Amostras de toninhas, golfinhos-nariz-de-garrafa e outras espécies de delfínídeos depositadas na coleção do Laboratório de Tartarugas e Mamíferos Marinhos (IO-FURG) foram triadas e selecionadas. Para a obtenção de amostras de dentes de toninhas, botos-cinza e outras espécies de pequenos cetáceos de outras regiões estabeleceram-se parcerias com dez instituições/grupos de pesquisa do Brasil, Uruguai e Argentina (Apêndice 1). Em cada caso, certificou-se que as amostras tivessem sido coletadas sob as correspondentes licenças de coleta de material biológico emitidas por autoridades competentes de cada país. A totalidade dos dentes foram obtidos de animais encontrados encalhados ou capturados acidentalmente em redes de pesca.

2.2. Estimativa das idades

Todos os exemplares recebidos tiveram suas idades estimadas no Laboratório de Tartarugas e Mamíferos Marinhos (IO-FURG). A técnica mais utilizada de estimativa de

idade em pequenos cetáceos é a leitura das GLGs na dentina e/ou no cimento de um corte de dente. Cada GLG, segundo a literatura especializada, representa um ano na vida do animal, para as espécies consideradas neste estudo (Hohn et al. 1989, Pinedo & Hohn 2000).

A metodologia incluiu, como primeiro passo, a descalcificação do dente em RDO[®] (ácido de mistura comercial). No caso dos delfinídeos (*Tursiops truncatus* e *Sotalia guianensis*), o dente foi previamente cortado em serra metalográfica e apenas a porção central foi descalcificada. Após a descalcificação, o dente foi cortado com micrótomo de congelamento obtendo lâminas de 25 micrômetros de espessura. Os cortes obtidos foram corados com hematoxilina de Mayer, passados por amônia diluída e, após serem enxaguados, foram deixados em glicerina 50% por meia hora. Finalmente, foram colocados em glicerina 100% até o momento da montagem em lâminas de microscopia. A montagem foi realizada em glicerina pura, selando a lamínula com esmalte ou selador comercial.

As leituras e contagem das GLGs (Figura 4) em cada lâmina foram feitas sem ter acesso à informação biológica (p.ex. sexo e comprimento total) do exemplar. Três leituras independentes foram realizadas pelo mesmo observador, com intervalos de tempo suficientes para garantir leituras independentes.

2.3. Análises químicas

2.3.1. Análise de isótopos estáveis

Para a realização das análises químicas, os dentes foram previamente limpos de restos de tecidos moles por meio de uma ferramenta abrasiva rotatória e enxaguados com água destilada. Após secagem em estufa, os dentes das toninhas, por serem de tamanho pequeno, foram pulverizados em gral e pistilo. Os dentes dos delfinídeos, por serem maiores, foram

cortados longitudinalmente no eixo buco-lingual com serra metalográfica para expor as camadas de crescimento na dentina, obtendo-se pó das mesmas por meio de uma broca. O pó de dentina obtido por meio das duas metodologias foi acondicionado em recipientes do tipo *Eppendorf*®.

Com o objetivo de eliminar o carbono inorgânico da dentina pulverizada, as amostras foram acidificadas com ácido clorídrico a 30%. Depois da secagem das amostras em estufa a 60°C por 1 hora, aproximadamente 5 mg da dentina pulverizada e acidificada de cada exemplar foi acondicionada em cápsulas de estanho. Por meio da análise no espectrômetro de massa (*IRMS*; *GV Instruments*, Manchester, UK) acoplado com um analisador de elementos (*Elemental analyzer*) as concentrações de isótopos de C e N foram obtidas. Os isótopos de carbono e nitrogênio foram determinados por meio de combustão única utilizando um sistema de forno duplo composto por um forno de oxidação a 1020°C e um forno de redução a 650°C. Através do processo de combustão, o carbono é convertido em CO₂ e o nitrogênio em N₂. Estes gases são quimicamente secos e diretamente injetados na fonte do IRMS.

Assim, a razão isotópica (R) de cada elemento (¹³C/¹⁴C e ¹⁵N/¹⁴N) na amostra e no padrão foi calculada para estabelecer os padrões isotópicos dos indivíduos por meio da fórmula:

$$\delta X = [(R_{\text{amostra}}/R_{\text{padrão}}) - 1] * 1000$$

Desta forma, as razões isotópicas naturais encontradas nos tecidos dos indivíduos (¹³C/¹²C e ¹⁵N/¹⁴N) são expressas pela notação delta (δ) (δ¹³C e δ¹⁵N, respectivamente), em partes por mil (‰) dos padrões internacionais: PDB (*Peedee Belemnite limestone*) para carbono e ar atmosférico para nitrogênio.

2.3.2. LA-ICP-MS

Para a realização das análises de LA-ICP-MS selecionaram-se exemplares adultos de boto-do-Amazonas (*Inia geoffrensis*), tucuxi (*Sotalia fluviatilis*), boto-cinza, toninha e golfinho-nariz-de-garrafa da maioria das áreas amostradas. Os dentes foram cortados longitudinalmente com serra metalográfica para expor a totalidade das GLGs. No caso do pequeno dente das toninhas, os mesmos foram colados em lâminas de vidro e polidos com papel de lixa até expor as camadas de crescimento.

Os cortes de dentes foram lavados, secos e colados numa lâmina de vidro com cola instantânea. As suas superfícies foram polidas com papel de lixa (nº8000), lavados com água Mili-Q e enxaguados três vezes com água deionizada. Após a secagem em capela, os cortes foram analisados no LA-ICP-MS. O equipamento utilizado é composto por um sistema de ablação a laser Nd-YAG (CETAC, LSX 100) (Figura 5) e um ICP-MS (ELAN 6000, PE-Sciex) operando em potência de 1250 watts. Os dentes foram percorridos pelo laser desde o limite entre a dentina e o esmalte até a cavidade pulpar, garantindo assim que a totalidade das camadas de crescimento fossem amostradas. Os isótopos medidos foram: ^{43}Ca , ^{138}Ba , ^{208}Pb , ^{87}Sr , ^{86}Sr , ^{66}Zn , ^{25}Mg e ^{55}Mn . O Ca foi utilizado como referência interna, pelo qual as intensidades dos elementos restantes obtidas, após serem corrigidas pelos valores de fundo do argônio (gás utilizado para o transporte da célula de ablação até o ICP-MS), foram relacionadas ao Ca.

Para analisar a variação temporal dos elementos, nove indivíduos da espécie de maior tamanho (*Tursiops* sp.) tiveram suas razões Ba/Ca e Ba/Sr relacionadas às idades. Um dente de um golfinho-de-dentes-rugosos (*Steno bredanensis*) foi também analisado para poder comparar os perfis obtidos ao desta espécie oceânica. A largura das GLGs foram

medidas na lâmina corada do dente utilizado para a estimativa de idades, assegurando-se de que as medições fossem tomadas na mesma região onde foi realizado a varredura do laser. Utilizando a velocidade do laser (40µm/s) e o tempo de amostragem de cada microamostra (0,516 s) cada valor obtido foi relacionado à idade específica, obtendo-se assim, a média de Ba/Ca e Ba/Sr para cada idade (Figura 6).

2.4. Análises estatísticas

Nos Anexos I e II, o teste-*t* de Student foi utilizado para verificar a existência de diferenças significativas nos valores médios de $\delta^{13}\text{C}$ e $\delta^{15}\text{N}$ nos dentes de toninhas e botos-cinza entre os sexos em cada uma das áreas. Análises de correlação foram realizadas para evidenciar a existência de uma relação entre os valores de $\delta^{13}\text{C}$ e $\delta^{15}\text{N}$ e as idades/comprimentos totais dos indivíduos em cada área. Análises de variância (ANOVA) seguidos de testes *a posteriori* de Tukey (*Tukey's HSD test*) foram feitas para verificar a existência de diferenças significativas nos valores médios de $\delta^{13}\text{C}$ e $\delta^{15}\text{N}$ entre as áreas amostradas.

No Anexo I, como complemento aos resultados obtidos da ANOVA foi realizado uma análise de árvores de classificação para explorar a potencialidade dos isótopos estáveis como variáveis preditivas da área de origem dos botos-cinza.

No anexo II, mapas de isótopos (*isomaps*) foram construídos para a melhor visualização das tendências latitudinais nos valores de $\delta^{13}\text{C}$ e $\delta^{15}\text{N}$. Os mesmos foram construídos a partir da interpolação dos valores georeferenciados dos indivíduos amostrados, por meio da ferramenta *Spatial Analyst* do software ArcMAPTM 9.3.1 (ESRI).

Análises de variância (ANOVA) seguidos de testes de Tukey foram realizadas no Anexo III, para evidenciar as diferenças nos valores médios de $\delta^{13}\text{C}$ e $\delta^{15}\text{N}$ entre as espécies de delfínídeos amostradas. Posteriormente, uma análise de agrupamentos foi realizada, utilizando distâncias Euclidianas e o método de ligação completa.

No Anexo IV, testes *t* de Student foram utilizados para evidenciar diferenças significativas nos valores médios de Ba/Ca, Sr/Ca e Ba/Sr entre as espécies de água doce e as marinhas. Análises de correlação foram realizadas para verificar a existência de uma relação entre os valores das razões e a idade/comprimento total dos indivíduos em cada área/espécie. ANOVAs seguidos de testes *a posteriori* de Tukey foram realizados para verificar se havia diferenças significativas nos valores médios de Ba/Ca e Ba/Sr entre as áreas marinhas agrupando as espécies, bem como em separado. Posteriormente foram realizadas análises de agrupamentos a partir dos valores médios de Ba/Ca e Ba/Sr encontrados em cada área (agrupamentos das áreas) e aqueles encontrados em cada espécie amostrada em cada área (agrupamentos das espécies por área).

3. SÍNTESE DOS RESULTADOS

As composições de isótopos estáveis de carbono e nitrogênio nos dentes dos botos-cinza (Anexo I) classificaram os animais em quatro grupos principais: (1) grupo Norte composto por animais do estuário do Amazonas e áreas costeiras adjacentes (AmE) e indivíduos do Ceará (CE), com os menores valores de $\delta^{15}\text{N}$; (2) outro grupo formado pelos botos do Espírito Santo (ES) com valores maiores $\delta^{15}\text{N}$ porém menores que nas restantes áreas; (3) animais do Norte do Rio de Janeiro (NRJ) enriquecidos em ^{15}N porém com baixos valores de $\delta^{13}\text{C}$; e (4) o grupo composto pelos indivíduos coletados na Baía da Babitonga (SC), apresentando altos valores de $\delta^{15}\text{N}$ e sendo mais enriquecidos em ^{13}C do

que os animais do NRJ. Os indivíduos coletados no Sul de São Paulo/Norte do Paraná (estuário de Cananéia e áreas costeiras adjacentes, SP/PR) não foram classificados em um grupo separado, sendo, entretanto, mais similares ao NRJ.

Os animais coletados em SP/PR foram provenientes tanto de encalhes (dentro e fora do estuário) como de capturas acidentais pela frota pesqueira de Cananéia que atua na área costeira adjacente ao estuário. Analisando as composições de isótopos dos animais de procedência mais precisa, evidenciou-se que os animais que haviam sido encontrados flutuando ou encalhados dentro do estuário possuíam valores de $\delta^{13}\text{C}$ e $\delta^{15}\text{N}$ menores dos que aqueles procedentes de captura acidental (fora do estuário). Esta mistura na procedência dos animais pode ter influenciado a sua classificação, evidenciando valores de isótopos muito variáveis que se sobrepuseram aos valores das áreas adjacentes.

Esta separação ecológica, teve concordância com evidências moleculares (Cunha et al. 2010) e morfológicas (Ramos 2001), exceto pelos botos-cinza do grupo Norte. Indivíduos do AmE e CE são genética e morfológicamente diferentes (Cunha et al. 2010, Fetuccia 2006), demonstrando assim, que na ausência de outras evidências, e ante um ambiente isotopicamente homogêneo, o uso único de traçadores isotópicos como ferramenta para identificar estoques, pode ser inadequado.

A análise das composições de isótopos estáveis nos dentes das toninhas provenientes de toda a distribuição da espécie (Anexo II), demonstraram a separação de cinco grupos: (1) animais do ES, com os menores valores de $\delta^{15}\text{N}$; (2) toninhas do NRJ, com valores intermediários de $\delta^{15}\text{N}$ e os mais baixos valores de $\delta^{13}\text{C}$; (3) toninhas do Norte de São Paulo (NSP), SP/PR e SC com valores intermediários de $\delta^{15}\text{N}$ e valores de $\delta^{13}\text{C}$ superiores aos encontrados em NRJ; (4) toninhas do Rio Grande do Sul e Uruguai (RS e URU),

apresentando altos valores de $\delta^{15}\text{N}$ e os valores de isótopos mais enriquecidos em ^{13}C de toda a distribuição; e (5) toninhas do Sul de Buenos Aires e Norte da Patagônia (ARG) possuindo as composições mais enriquecidas em ^{15}N registradas neste estudo.

Os agrupamentos encontrados com base nas composições de isótopos estáveis de C e N estiveram de acordo com a separação proposta por Secchi et al. (2003a), com a exceção da FMA I. As toninhas incluídas dentro desta área de manejo (ES e NRJ) apresentaram valores de isótopos significativamente diferentes. Esta diferenciação ecológica está de acordo com evidências moleculares (Cunha et al. *in prep*) e morfométricas (Ramos et al. 2002), indicando que estas duas áreas devem ser tratadas no futuro como áreas de manejo diferentes.

Considerando os valores de isótopos de N encontrados em botos-cinza e toninhas, um marcado gradiente latitudinal de aumento dos valores de $\delta^{15}\text{N}$ foi evidenciado. Diferenças isotópicas entre as fontes de nutrientes aportadas tanto pelas contribuições continentais como pelas massas d'água presentes na região (Montoya 2007) foram postuladas como a causa mais provável das diferenças encontradas. Condições mais oligotróficas encontradas nas regiões entre AmE e ES, onde as fontes principais de nitrogênio são empobrecidas em ^{15}N (Sigman et al. 2009) são substituídas por áreas fertilizadas em nitratos de águas profundas com valores altos de $\delta^{15}\text{N}$ (Sigman et al. 2009). As composições de isótopos de carbono, por outro lado, tiveram uma menor variação em comparação às do nitrogênio, apresentando valores típicos de cadeias tróficas costeiras (Burton & Koch 1999) na maior parte das áreas. As exceções foram os valores empobrecidos em ^{13}C encontrados em ambas as espécies no NRJ e os altos valores de $\delta^{13}\text{C}$ encontrados nas toninhas amostradas nas duas regiões tipicamente estuarinas (RS e URU). Duas hipóteses foram consideradas para

explicar os baixos valores de $\delta^{13}\text{C}$ encontrados no NRJ: aportes continentais empobrecidos em ^{13}C (Michener & Kaufman 2007) ou fontes de carbono empobrecidas em ^{13}C provenientes das ressurgências costeiras (O'Reilly et al. 2002) que afetam as costas desta região (Rodrigues & Lorenzzetti 2001). As áreas RS e URU, sendo influenciadas por grandes estuários (Rio da Prata e Lagoa dos Patos), que possuem áreas de marismas ricas em plantas C_4 (Isacch et al. 2006), as quais, enriquecidas em ^{13}C (Michener & Kaufman 2007), contribuiriam com o aumento dos valores de $\delta^{13}\text{C}$ nas redes tróficas das áreas costeiras adjacentes.

Assim, as diferenças geográficas em valores de isótopos estáveis encontradas ao longo das distribuições destas duas espécies de cetáceos indicaram a separação de estoques ecológicos das mesmas (*Hipótese I*).

O habitat utilizado para forrageio e o nível trófico ocupado por várias espécies de delfínídeos do Atlântico Sul Ocidental foi evidenciado por distintas composições de isótopos estáveis nos seus dentes (Anexo III). Assim, espécies costeiras como os golfinhos-nariz-de-garrafa e as orcas, tiveram os valores mais altos de $\delta^{13}\text{C}$, enquanto espécies de plataforma média e externa, como o golfinho-de-dentes rugosos, o golfinho-pintado e o golfinho-comum-de-bico-curto apresentaram valores intermediários de $\delta^{13}\text{C}$ entre os costeiros e o golfinho-de-Fraser, a única espécie oceânica analisada, que apresentou o valor mais baixo de $\delta^{13}\text{C}$. Este gradiente de enriquecimento em ^{13}C do oceano aberto para as áreas costeiras é reconhecido como um dos padrões mais observados nos ambientes aquáticos marinhos (e.g. France 1995, Burton & Koch 1999, Clementz & Koch 2001, Riccialdelli et al. 2010). Por outro lado, um gradiente costa-oceano de diminuição nos valores de $\delta^{15}\text{N}$ também foi observado. Tanto o golfinho-nariz-de-garrafa quanto a orca

apresentaram valores enriquecidos em ^{15}N , evidenciando uma predação sobre espécies demersais costeiras, no caso dos botos, e uma predação sobre presas de níveis tróficos mais elevados, inclusive pequenos cetáceos, no caso das orcas. Os valores isotópicos comuns encontrados nas espécies de plataforma média e externa sugerem que estas espécies formam uma guilda trófica. As falsas-orcas apresentaram valores de carbono iguais às espécies de plataforma média e externa. Por outro lado, os valores de $\delta^{15}\text{N}$ demonstraram um padrão bimodal, onde um grupo de animais provenientes de um encalhe massivo tiveram valores de $\delta^{15}\text{N}$ significativamente menores do que outros dois animais encalhados individualmente. Estes resultados concordaram com informações obtidas de conteúdos estomacais de espécimes encontrados na região, onde alguns indivíduos continham peixes e lulas costeiros (Pinedo & Rosas, 1989), enquanto um grupo de falsas orcas encalhadas em massa na região apresentaram apenas lulas oceânicas nos seus estômagos (Andrade et al. 2001).

Este trabalho demonstrou que espécies que conformam guildas tróficas podem ser identificadas pelos seus valores de isótopos, assim como também as suas áreas de forrageio ao longo de gradientes ambientais (*Hipótese 2*).

As razões Ba/Ca e Ba/Sr encontradas nos dentes dos exemplares amostrados foram aproximadamente 10 vezes mais altas em espécies de água doce (boto-do-Amazonas, *Inia geoffrensis* e tucuxi, *Sotalia fluviatilis*) do que em espécies marinhas (boto-cinza, toninha e golfinho-nariz-de-garrafa) (Anexo IV). A razão Sr/Ca não foi significativamente diferente entre os animais encontrados nestes dois ambientes. Entre os cetáceos amostrados em diferentes ambientes de água marinha, aqueles encontrados nas principais áreas estuarinas, AmE, RS e URU, apresentaram os valores mais altos de Ba/Ca e Ba/Sr, sendo nas últimas

duas áreas ainda mais altas do que nos indivíduos do AmE, apesar de se esperar valores maiores nesta última, devido à extraordinária descarga de água doce que acontece na mesma (Geyer et al. 1996). A influência da Corrente Norte do Brasil resulta no transporte da pluma do Amazonas para o noroeste (Santos et al. 2008), criando assim, condições oceanográficas diferentes em ambos os lados da boca do estuário. O forrageio dos botos-cinza na área de maior salinidade pode ser a causa dos baixos valores de Ba/Ca e Ba/Sr encontrados. A análise de agrupamentos demonstrou a separação destas áreas estuarinas num subgrupo, relacionados também com os animais do ES.

As áreas com menor influência de água doce conformaram um outro agrupamento, onde NRJ, SP/PR e SC, áreas com moderada descarga de água doce formaram um grupo separado das duas áreas com mínima descarga de água doce (CE e NSP) (ver Tabela 1, Anexo IV).

Por outro lado, diferenças no uso do habitat puderam ser verificadas entre espécies simpátricas, como as toninhas e os botos-cinza do ES e SP/PR. As toninhas do NRJ foram agrupadas com aquelas espécies de áreas com significativa influencia de águas continentais, enquanto os botos-cinza se agruparam com aquelas espécies provenientes de áreas com menor aporte de água doce. Nesta região as toninhas são avistadas o ano todo na região da desembocadura do rio Paraíba do Sul, enquanto os botos-cinza são mais comumente observados no outono/inverno (Di Benedetto et al. 2001). Por outro lado, um agrupamento contrário a este foi observado para as toninhas e botos-cinza do SP/PR. Nesta região, os botos-cinza ocupam as águas do interior do estuário de Cananéia (Oshima et al. 2010) enquanto as toninhas são raramente avistadas dentro do mesmo (Santos et al. 2007).

Desta forma, a influência de diferentes regimes de salinidade no hábitat dos cetáceos pode ser detectada nas razões Ba/Ca e Ba/Sr encontradas nos dentes destes organismos (*Hipótese 3*).

A análise de variação temporal de Ba/Ca e Ba/Sr em nove golfinhos-nariz-de-garrafa do RS, revelou três padrões diferentes de mudanças. Dois animais apresentaram perfis homogêneos onde valores baixos destas razões se mantiveram ao longo da vida dos mesmos. Estes perfis foram similares àquele apresentado pelo golfinho-de-dentes-rugosos, sugerindo a possibilidade de estes animais habitarem um ambiente diferente. Ao menos um desses animais, foi identificado como pertencente a um provável grupo oceânico de golfinhos-nariz-de-garrafa, com base em morfometria craniana e valores de isótopos estáveis de carbono e nitrogênio (Botta et al. *in press*, Anexo III). O grupo mais numeroso apresentou perfis com variações de valores após o primeiro ano de vida, oscilando entre valores acima dos encontrados nos botos com perfil homogêneo. Finalmente, um grupo de três indivíduos apresentou os maiores valores, a partir do primeiro ano de vida, chegando a valores consideravelmente maiores de todo o resto dos animais amostrados. Estes dois padrões de oscilação de valores de Ba/Ca e Ba/Sr provavelmente estão relacionados à variações ambientais, como por exemplo descarga de água doce em função da precipitação as quais refletem na disponibilidade de Ba na coluna d'água e, conseqüentemente, nos dentes de animais residentes na região. Os três animais que apresentaram os valores mais altos destas razões, por exemplo, experimentaram ao menos três eventos de "El Niño" considerados fortes enquanto viveram (1980-2008).

A variação temporal de Ba/Ca e Ba/Sr, refletem as mudanças de uso do habitat que pode ser observada em indivíduos de uma mesma espécie mesmo ocorrendo na mesma

região (*Hipótese 4*). A possibilidade de avaliar mudanças ambientais através de marcadores químicos em dentes de indivíduos residentes ressalta a importância de continuar explorando esta alternativa em estudos futuros.

4. CONCLUSÕES

A partir desta pesquisa pode-se concluir que:

- ✓ Traçadores como os isótopos de carbono e nitrogênio são uma ferramenta útil para diferenciar estoques ecológicos de pequenos cetáceos costeiros residentes ao longo de gradientes geográficos nos valores de $\delta^{13}\text{C}$ e $\delta^{15}\text{N}$ (Anexos I e II).
- ✓ Existe um gradiente latitudinal de enriquecimento em ^{15}N na costa Atlântica da América do Sul, provavelmente influenciado pelas contribuições diferenciais de nutrientes das diferentes massas d'água que operam ao longo da mesma (Anexos I e II).
- ✓ Gradientes longitudinais costa-oceano em valores destes isótopos são refletidos nos valores encontrados em espécies que ocupam diferentes habitats marinhos (Anexo III).
- ✓ Predadores simpátricos podem ser agrupados em guildas tróficas de acordo com valores similares de $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ (Anexo III).
- ✓ As razões de Ba/Ca e Ba/Sr presentes nos dentes dos cetáceos refletem os ambientes aquáticos em que eles habitam (Anexo IV).
- ✓ Perfis de variações temporais de Ba/Ca e Ba/Sr podem ser utilizados para a caracterização do uso do hábitat de cetáceos, caracterizar a influência da descarga de água doce no habitat de populações residentes de cetáceos e/ou para identificar movimentos entre áreas com diferentes níveis de salinidades (Anexo IV).

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6. FIGURAS

Figura 1. Corte de dente de toninha (*Pontoporia blainvillei*) para estimativa de idades.

Figura 2. Distribuição dos valores de $\delta^{13}\text{C}$ nos ecossistemas. Os valores estão expressados em partes por mil (‰). Os valores nas setas indicam fracionamento (modificada de Peterson & Fry 1987).

Figura 3. Processos que afetam a distribuição dos valores de $\delta^{15}\text{N}$ no oceano (modificada de Sigman et al. 2009).

Figura 4. Corte de dente de boto-cinza (*Sotalia guianensis*) utilizado para a estimativa da idade do indivíduo.

Figura 5. Esquema mostrando as partes que compõem o sistema de ablação a laser (modificado de Sinclair 1999).

Figura 6. Esquema representativo do processo de obtenção de valores médios de Ba/Ca por idades: lâmina utilizada para estimativa da idade (a) onde foram medidas as camadas de crescimento (números 1-7, PN=dentina pré-natal); dente utilizado para a análise com LA-ICP-MS indicando o local do *scan* do laser (b); perfil de variação temporal de valores de Ba/Ca (c).

Figura 1.

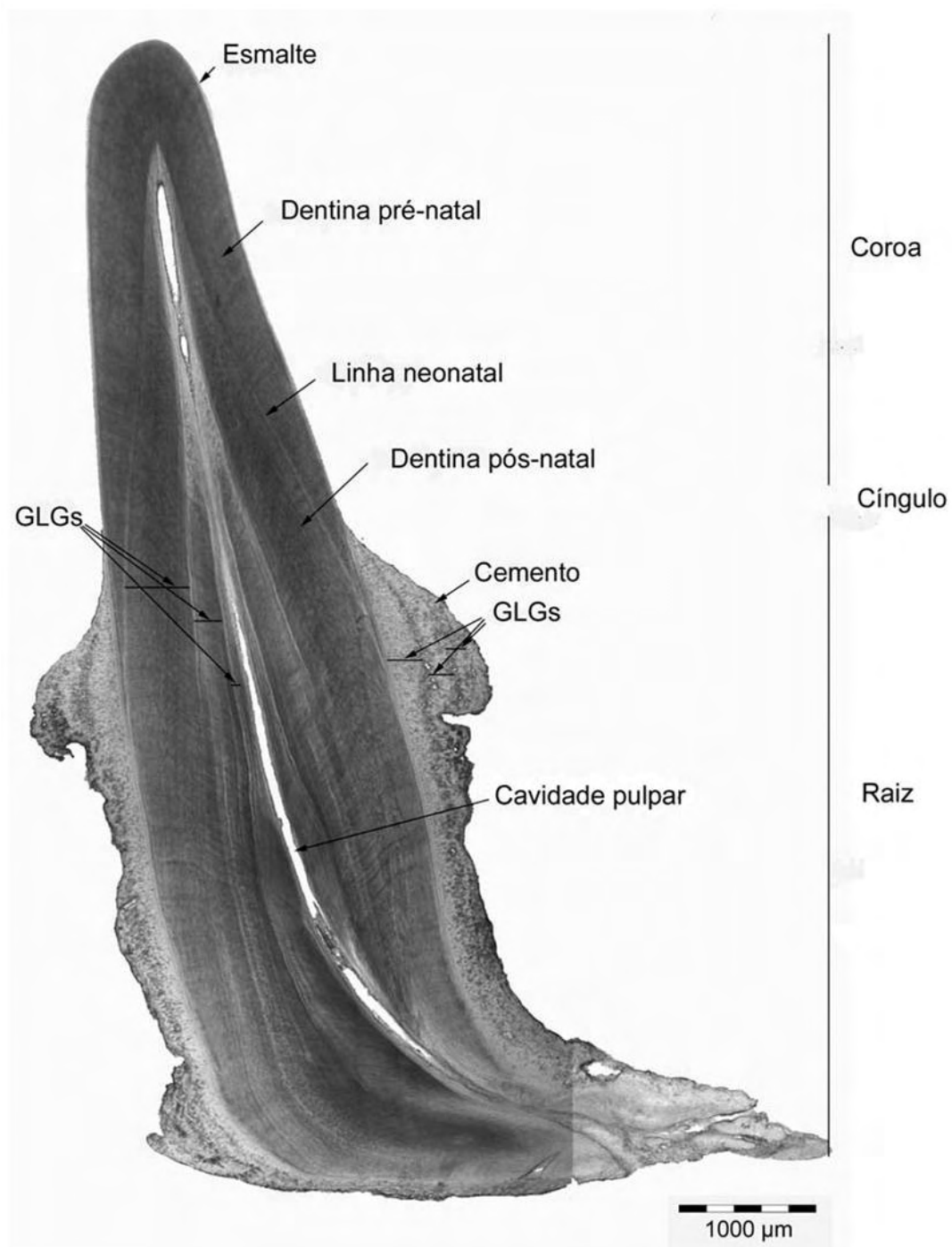


Figura 2

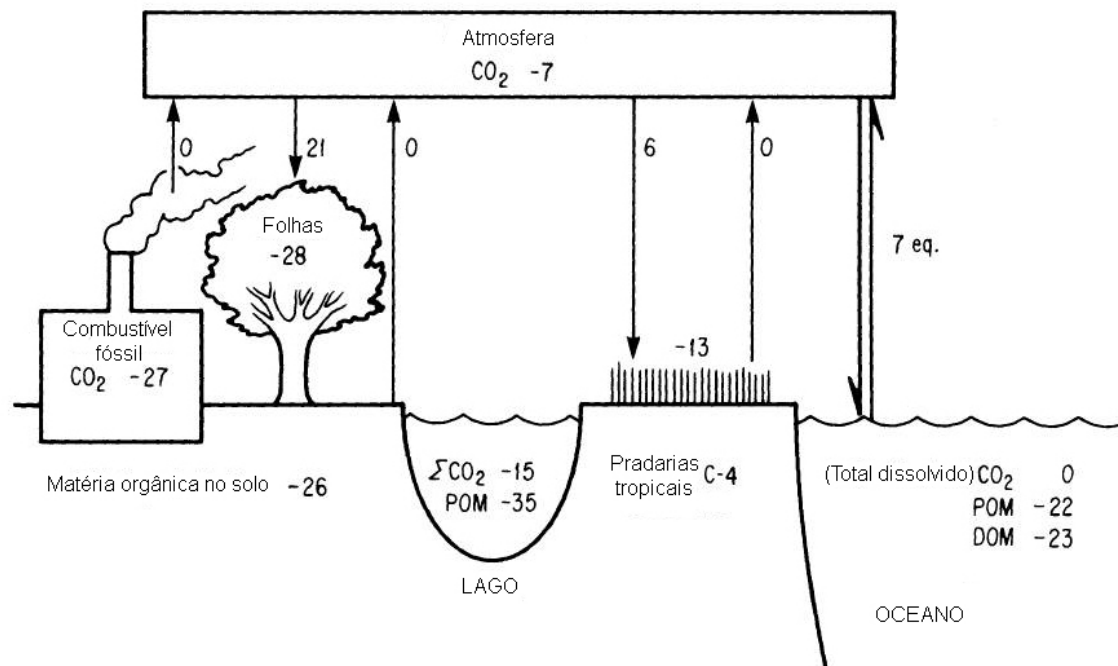


Figura 3.

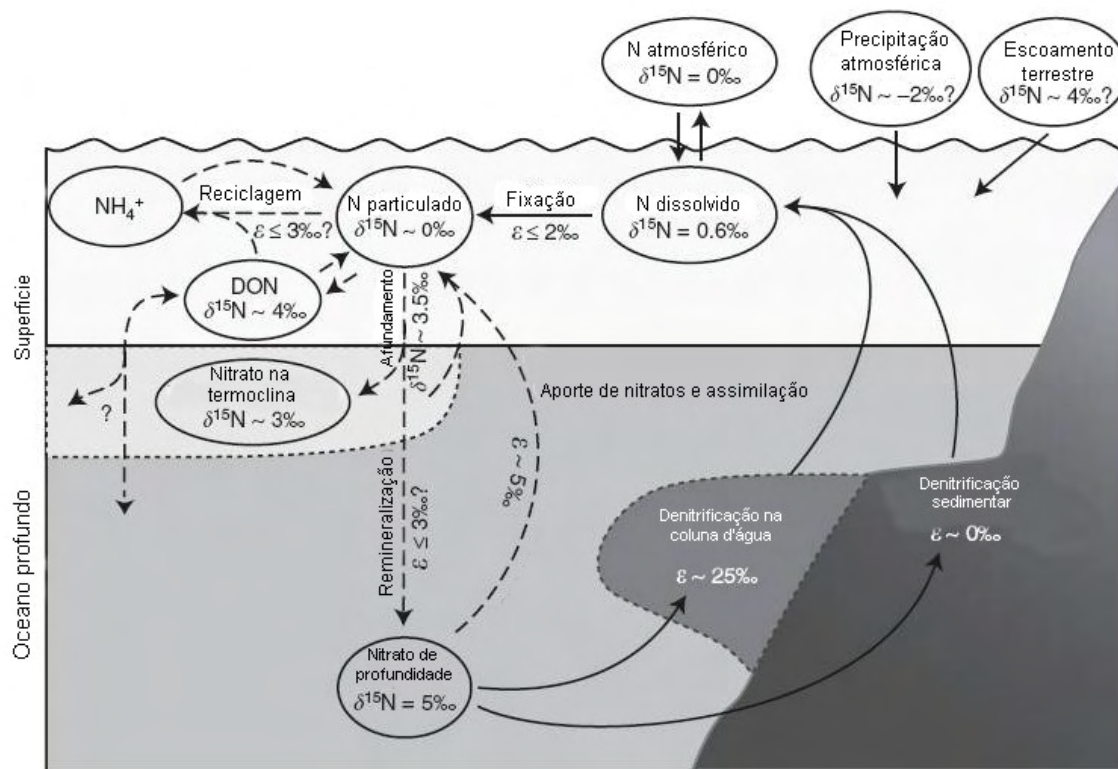


Figura 4.



Figura 5.

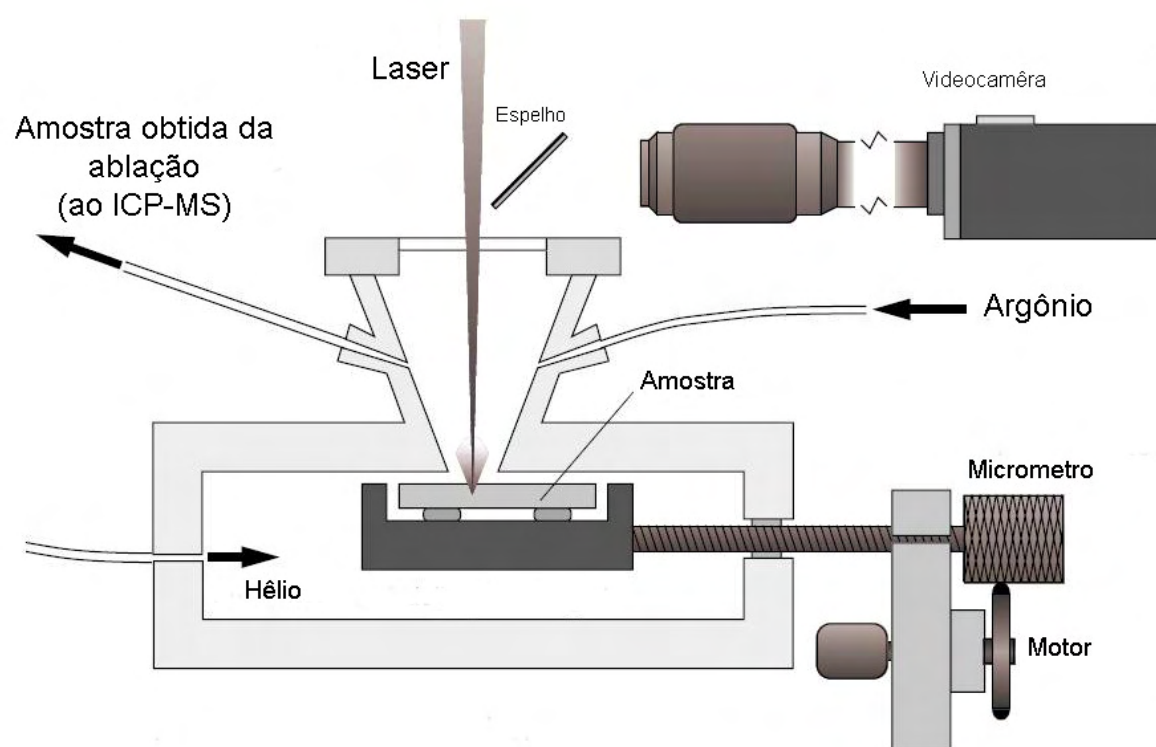
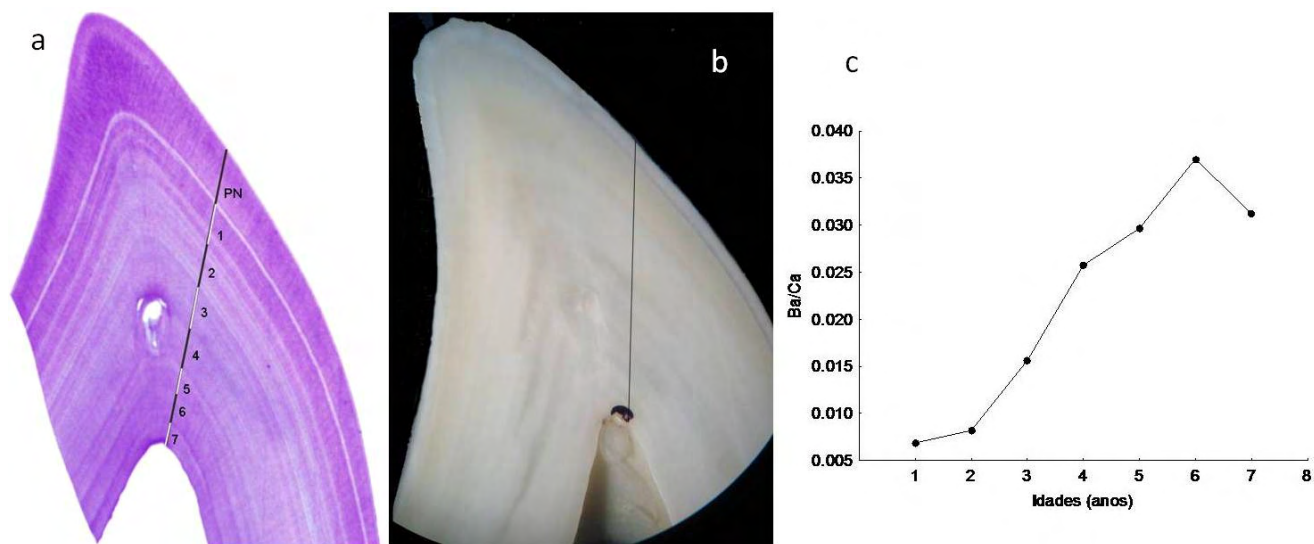


Figura 6.



ANEXO I

Ecological stocks of Guiana dolphins (*Sotalia guianensis*) along the Brazilian coast as inferred from stable isotopes

Silvina Botta, Aleta A. Hohn, Stephen A. Macko, Vera F. da Silva, Ana Carolina
Meirelles, Lupercio Barbosa, Ana Paula M. Di Benedetto, Renata Maria A. Ramos,
Marcos C. de O. Santos, Marta J. Cremer, Eduardo R. Secchi.

(A ser submetido a *Marine Ecology Progress Series*)

Ecological stocks of Guiana dolphins (*Sotalia guianensis*) along the Brazilian coast as inferred from stable isotopes

Silvina Botta^{1*}, Aleta A. Hohn², Stephen A. Macko³, Vera F. da Silva⁴, Ana Carolina Meirelles⁵, Lupercio Barbosa⁶, Ana Paula M. Di Benedetto⁷, Renata Maria A. Ramos⁸, Marcos C. de O. Santos⁹, Marta J. Cremer¹⁰, Eduardo R. Secchi¹.

(1) Instituto de Oceanografia-Universidade Federal do Rio Grande-FURG, Cx.P. 474, Rio Grande, RS 96500970, Brazil; (2) National Marine Fisheries Service, National Oceanic and Atmospheric Administration, Beaufort, NC, USA; (3) Department of Environmental Sciences, Clark Hall, University of Virginia, Charlottesville, VA 22903 USA; (4) Laboratório de Mamíferos Aquáticos, Instituto Nacional de Pesquisas da Amazônia, Manaus, Brazil; (5) Associação de Pesquisa e Preservação de Ecossistemas Aquáticos – AQUASIS, SESC, Caucaia, CE, Brazil; (6) Organização Consciência Ambiental—ORCA, Vila Velha, ES, Brazil; (7) Universidade Estadual do Norte Fluminense, CBB/LCA, Campos dos Goytacazes, RJ, Brazil (8) Everest Tecnologia em Serviços Ltda. Vitória, ES 29050-912 Brazil; (9) Laboratório de Biologia da Conservação de Mamíferos Aquáticos, Departamento de Oceanografia Biológica, Instituto Oceanográfico, Universidade de São Paulo, São Paulo, SP, Brazil (10) Departamento de Ciências Biológicas; Universidade da Região de Joinville; Joinville - SC - Brazil.

ABSTRACT

A dual isotope approach was used to identify stocks of the Guiana dolphin, *Sotalia guianensis*, along the coast of Brazil. Teeth were obtained from dolphins incidentally caught in fisheries or washed ashore. Sampling areas included the mouth and adjacent areas of the Amazon estuary (AmE, n=20), the marine coastal areas of Ceará (CE, n=20), Espírito Santo (ES, n=34), northern Rio de Janeiro (NRJ, n=46), southern São Paulo and northern Paraná (SP/PR, n=21) and the Babitonga Bay in northern Santa Catarina (SC, n=15). Acidified tooth powders were used for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ analyses. Mean $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ were not significantly different between males and females (t-tests, $p>0.05$), except for animals from AmE where males were significantly more enriched in

^{15}N than females. Four groups could be recognized based on a $\delta^{13}\text{C}$ vs. $\delta^{15}\text{N}$ values. The two northernmost regions, AmE and CE, were homogeneously clustered, with high values of $\delta^{13}\text{C}$ and the lowest $\delta^{15}\text{N}$ values (aprox. -12‰ and 11‰). ES is isolated with mean values of -12.4‰ for $\delta^{13}\text{C}$ and 13.2‰ for $\delta^{15}\text{N}$. NRJ had the most depleted $\delta^{13}\text{C}$ (-13.6‰) and high $\delta^{15}\text{N}$ (15.9‰). The fourth group was formed by individuals from the southernmost area, SC, with high values of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ (-11.7‰ and 16.0‰). Values from SP/PR overlapped mainly with values of NRJ and, to a lesser extent, with SC (mean $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ of -12.8‰ and 14.9‰, respectively). Results of ANOVAs confirmed that areas differed significantly with respect to $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ ($p < 0.001$). Geographical variations in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ proved to be a reliable tool for identifying ecological stocks of Guiana dolphins.

KEY WORDS: *Sotalia guianensis*, Guiana dolphin, stable isotopes, teeth, stock identification, Brazil.

INTRODUCTION

The Guiana dolphin, *Sotalia guianensis* (Van Bénédén, 1864), was recently recognized as a different species from the freshwater tucuxi dolphin, *Sotalia fluviatilis*, based on both morphological (Monteiro-Filho et al. 2002) and molecular (Cunha et al. 2005, Caballero et al. 2007) evidences. The newly recognized species occurs from Santa Catarina State, southern Brazil (Simões-Lopes 1988), northwards along the Atlantic coast of South America and the Caribbean coast of Central America up to Nicaragua (Borobia et al. 1991, Carr & Bonde 2000) and possibly Honduras (Flores & da Silva 2009), in a supposedly discontinuous distribution.

In Brazil, the species inhabits shallow waters, with long-term residence in bays and estuaries including the mouth of the Amazon River (Flores & da Silva 2009). Due to their coastal habitat Guiana dolphins are vulnerable to human threats such as incidental (Siciliano 1994, Di Benedetto 2003, Netto & Barbosa 2003, Monteiro-Neto et al. 2004, Netto & Di Benedetto 2008) and deliberate captures in fisheries (Secchi 2009), human-induced injuries (Azevedo et al. 2008), pollution and habitat loss (Yogui et al. 2003, Lailson-Brito et al. 2010). Abundance, mortality rates and other population parameters are either nonexistent or available for only a few areas (Santos et al. *in press*). As a consequence, no formal assessment of the population status have been performed (Secchi 2009) and the species is listed as Data Deficient in IUCN's Red List (IUCN 2010).

Population structure, investigated through molecular markers, suggested the existence of at least six Management Units (MUs, sensu Mortitz 1994) in Brazil: Pará, Ceará, Rio Grande do Norte, Bahia, Espírito Santo and the South-Southeastern area (from Rio de Janeiro to Santa Catarina states) (Cunha et al. 2010). Severe restrictions to gene flow among them indicate that those MUs are highly differentiated and the observed homogeneity in the South-Southeastern area was probably due to a recent colonization through a southwards range extension (Cunha et al. 2010).

Management Units (or stocks) are a less restrictive definition of stock than ESUs (Evolutionary Significant Unit or biological stock, Moritz 1994, Wang 2002), as the latter implies that gene flow between groups is absent while some level of genetic exchange must be present among MUs (Wang 2002). For defining stocks, differences in life-history parameters are important as they can reveal recently isolated stocks (Wang 2002). Since a large data set is needed in order to obtain accurate demographic

parameters, other ecological differences can provide alternative evidence that some degree of spatial structuring is occurring. Such differences can therefore be used to identify ecological stocks (Dizon et al. 1992, Wade & Angliss 1997, Wang 2002). For example, acoustic whistle parameters were different between northern and southern populations of Guiana dolphins along the Brazilian coast, while the magnitude of the differences between adjacent areas was comparatively smaller, suggesting less exchange at greater distances (Azevedo & Van Sluys 2005). Differences have also been found in contaminant loads among Guiana dolphins from different locations in South-southeastern Brazil (Lailson-Brito et al. 2010). In general, these and other chemical indicators such as elemental concentrations (Born et al. 2003), fatty acid profiles (Smith et al. 1996, Herman et al. 2005) and stable isotopes (Born et al. 2003, Herman et al. 2005) can also be used to show as evidences of species' ecological structuring..

Although stable isotope composition in animal tissues has more commonly been used for studying trophic ecology (e.g. Hobson et al. 1997, Hammill et al. 2005, Lusseau & Wing 2006), it has also been proposed as a tool for identifying population structuring in marine mammals (e.g. Abend & Smith 1995, Smith et al. 1996, Born et al. 2003, Herman et al. 2005, Borrell et al. 2006, Barros et al. 2010). Because stable isotopes are assimilated by predator from its prey, distinct dietary preferences and the geographical distribution of isotope values (e.g. isotopic maps or *isoscaples*, Graham et al. 2010) lead to differences in isotopic values in predator's tissues (Newsome et al. 2010) that can indicate discrete ecological niche and, thus, differentiate stocks.

Stable isotope ratios of nitrogen ($^{15}\text{N}/^{14}\text{N}$) and to a lesser extent carbon ($^{13}\text{C}/^{12}\text{C}$) show a stepwise enrichment with increasing trophic level in the marine environment (DeNiro & Epstein 1978, 1981). Indeed, nitrogen isotopes values change in a

predictable fashion between trophic levels (approximately 3.4‰ per trophic level, Post 2002), owing to the preferential excretion of the light isotope (Caut et al. 2009), and thus reflecting trophic position (De Niro & Epstein 1981, Cabana & Rasmussen 1996, McCutchan et al. 2003). An increase of only roughly 1‰ in the ^{13}C content is typically observed (De Niro & Epstein 1978, Peterson & Fry 1987). Therefore, predators' carbon isotope values are used as an indicator of the sources at the base of the food web where they feed (Hobson 1999, Graham et al. 2010).

The measured levels of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in an organism reflect the trophic dynamics noted above as well as the isotopic baseline of the habitat or ecosystem. Baseline carbon isotope values result from combination of producers with different photosynthetic pathways (Fry & Sherr 1984) and, in phytoplankton-based food webs, $\delta^{13}\text{C}$ values can vary in relation to cell physiology, morphology, growth and the source of inorganic carbon (Michener & Kaufman 2007). In general, major aquatic habitat types have distinct $\delta^{13}\text{C}$ values (seagrass habitat > kelp forest > nearshore marine > offshore marine > freshwater, Fry & Sherr 1988, Clementz & Koch 2001, Michener & Kaufman 2007). Furthermore, productivity, dissolved CO_2 concentration, and bicarbonate utilization could shape different carbon isotopic patterns in the ocean (Clementz & Koch 2001). Spatial patterns in coastal basal nitrogen isotopes values are more complex, mainly reflecting contributions of potential sources of nitrogen (deepwater nitrate, continental inputs, nitrogen fixation and anthropogenic inputs) and differences in nitrate utilization (reviewed by Montoya 2007).

The Brazilian coast includes a considerably wide range of marine coastal habitats, such as estuaries, bays, coastal areas under the influence of seasonal upwelling events, and areas with minimal freshwater discharge. Each area should differ not only in the

combination of producers but also in the diversity of consumers supported by them, thus resulting in differences in stable isotope compositions through the food webs. The Guiana dolphin, a small cetacean usually associated to relatively small home ranges (e.g. Flores & Bazzalo 2004, Hardt 2005, Batista 2008, Oshima et al. 2010), will reflect particular stable isotope compositions acquired from the food web and baseline isotopic values where it forages. By sampling a tissue with little or no turnover, it is possible to obtain a long-term isotopic composition. Teeth may be an ideal tissue because they form a permanent record and are composed of incremental layers that are deposited until death, thus, the diet over an individual's lifetime can be assessed (Walker et al. 1999, Walker & Macko 1999, Niño-Torres et al. 2006). Consequently, stable isotope compositions can be used as a proxy for average differences in diet and foraging areas among putative ecological stocks. In this study, we propose that geographical differences in baseline stable isotope compositions, combined with dietary preferences of Guiana dolphins from different areas along the Brazilian coast will be reflected in stable isotope compositions of their teeth and, therefore, be used as a tool for identifying discrete units for conservation purposes.

MATERIAL AND METHODS

Geographic distribution of samples

Teeth collected from 156 dolphins stranded or incidentally caught in fishing nets along the coast of Brazil were included in this study. Sampling areas included the mouth and adjacent areas of the Amazon estuary, (AmE, n=20), the marine coastal areas of Ceará (CE, n=20), Espírito Santo (ES, n=34), northern Rio de Janeiro (NRJ, n=46), southern São Paulo and northern Paraná (SP/PR, n=21) and the estuary of Babitonga Bay in northern Santa Catarina (SC, n=15) (Figure 1, Tables 1 and 2).

Age estimation

Evidence exists that lactation may influence the carbon and, especially, nitrogen stable isotope compositions (Walker & Macko 1999, Knoff et al. 2008). Additionally, variation in growth rates could lead to different trophic discrimination factors which, in turn, alter tissue isotope values, even without a modification in diet (Newsome et al. 2009). As a result, ages were estimated by counting dentinal incremental layers or GLGs (*Growth Layer Groups*, Perrin & Myrick 1980) in order to exclude possible lactating animals (<1yr of age, Rosas & Monteiro-Filho 2002, Di Benedetto & Ramos 2004) and to investigate age-related factors. Ages from AmE samples could not be estimated due to reduced number of teeth per individual.

Teeth were processed for age estimation following Hohn et al. (1989). The method included the use of a low speed saw with a diamond-embedded blade to cut a thick section (1-2mm) through the longitudinal axis in order to obtain a section from the center of the tooth that included all of the growth layers. These thick sections were then fixed in 10% formalin and decalcified with RDO[®] (a commercial mixture of acids). The decalcified thick sections were then thin-sectioned to 25µm using a freezing-stage microtome. The thin-sections were stained in Mayer's hematoxylin, "blued" in ammonia, dehydrated in glycerin, and mounted on a microscope slide in 100% glycerin. Age estimation was performed without reference to biological data, such as length or sex. Each GLG was considered to represent one year of age (Hohn et al. 1989).

Analysis of isotope compositions

Stable isotope analysis of teeth was performed following the protocol described in Walker & Macko (1999). Teeth were dried for 3–4 days in a 60°C oven and cleaned of

outer soft tissue with a carbide burr attached to a drill. A low speed saw with a diamond-embedded blade was used to cut through the center of the tooth in the longitudinal buccal-lingual axis in order to expose the GLGs. The exposed dentine was sampled with a small drill bit, ensuring that all GLGs were sampled uniformly so that the resulting powder would provide the averaged isotope composition of the entire life of the individual. The powder obtained was acidified with 30% hydrochloric acid to remove biogenic carbonates, which could alter the organic $\delta^{13}\text{C}$ measurements, and then dried again for 1 hr in a 60°C oven. The goal of preparation is the conversion of the organic samples into gases of suitable purity that can then be analyzed by the mass spectrometer. Samples of approximately 5 mg of residual acidified tooth were used for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ analysis using an elemental analyzer connected to a Micromass Optima Isotope Ratio Mass Spectrometer (GV Instruments, Manchester, UK). Natural abundance of stable isotope ratios ($^{13}\text{C} / ^{12}\text{C}$ and $^{15}\text{N} / ^{14}\text{N}$) are expressed in a delta notation (δ) as per mil enrichment (‰) when compared with international standards (e.g. Pee Dee Belemnite for carbon and atmospheric N_2 , for nitrogen). Results were expressed as:

$$\delta X = [(R_{\text{sample}}/R_{\text{standard}}) - 1] * 1000$$

where R_{sample} and R_{standard} are the $^{13}\text{C}/^{12}\text{C}$ or $^{15}\text{N}/^{14}\text{N}$ ratios of the sample and standard, respectively.

Data analysis

All results are presented as the mean $\pm$ SD. Data were tested for normality and homogeneity of the variances using Kolmogorov-Smirnov and Levene's test, respectively. The difference in the mean $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values between males and

females were tested using the Student-t test. Relationships between $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ and age, length or latitude were evaluated through non-parametric Spearman correlation analysis.

Differences in isotope ratios among areas were investigated using 1-way ANOVA followed by a Tukey's HSD test. Dual stable isotope plots of carbon versus nitrogen were used to visualize geographical variations on teeth isotopic values.

Classification

Classification tree analysis was used to explore the power of stable isotope compositions as predictor variables for determining origin of Guiana dolphins to areas where they were sampled. Classifications trees were constructed from hierarchical decisions, performing univariate splits into mutually exclusive groups via algorithms, following *StatSoft* procedures (2011).

Samples from each area were randomly separated in two subsamples in a 1.5:1.0 ratio of learning and test samples, respectively. Trees were built from the learning sample using areas as the dependent variable and $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ as independent variables. The optimal tree was selected as the smallest-sized (simplest tree) with the highest predictive accuracy (less *cost*, or proportion of misclassified samples when *a priori* probabilities are taken to be proportional to the class sizes). Once selected, the accuracy of the final model was assessed by the test sample cross-validation. A good cross-validation was met when the *cost* in the test sample did not exceed that of the learning sample.

RESULTS

Mean $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ were not significantly different between males and females in all areas (t-tests, $p>0.05$), except for animals from AmE where males were significantly more enriched in ^{15}N than females (t-test, $p=0.024$, $\delta^{15}\text{N}=12.0 \pm 0.3$, $n=10$ and $\delta^{15}\text{N}=10.6 \pm 0.4$, $n=10$, for males and females respectively). As this was the only exception, males and females were clumped together for comparison among areas.

The average $\delta^{13}\text{C}$ for all 156 dolphins was $-12.5 \pm 1.1\text{‰}$ and that for $\delta^{15}\text{N}$ was $14.0 \pm 2.1\text{‰}$. Age or length were not significantly correlated with $\delta^{13}\text{C}$ or $\delta^{15}\text{N}$ in almost all areas (Spearman's correlation tests, $p>0.05$). However, $\delta^{13}\text{C}$ correlation with age and length in dolphins from NRJ was weak though significant (Spearman's correlation coefficient, $\rho=0.4$ and 0.32 , respectively, $p<0.05$) and SC (Spearman's correlation coefficient, $\rho=0.62$ and 0.45 , respectively, $p<0.05$). Furthermore, $\delta^{15}\text{N}$ was negatively correlated with age and length in SC (Spearman's correlation coefficient, $\rho=-0.63$ and -0.60 , respectively, $p<0.05$) (Figure 2). Because these significant correlations were generally weak, nor age or length were considered in the subsequent analysis.

A weak but significant negative correlation with latitude was found for $\delta^{13}\text{C}$ (Spearman's correlation coefficient, $\rho=-0.34$, $p<0.05$). $\delta^{13}\text{C}$ values were higher in the northernmost and southernmost areas (AmE, CE and SC). Nitrogen isotope ratios, on the other hand, were clearly positively correlated with latitude (Spearman's correlation coefficient, $\rho=0.77$, $P<0.05$).

Mean $\delta^{13}\text{C}$ ranged from -13.6‰ in NRJ to -11.8‰ in AmE, while mean $\delta^{15}\text{N}$ increased from 11.3‰ in CE to 16.0‰ from SC samples (Table 3, Figure 3). Moreover, considering males and females from AmE separated due to their difference in $\delta^{15}\text{N}$ values, the latter presented the lowest mean values found in this study ($\delta^{15}\text{N}=10.6\text{‰}$).

Based on $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ scatter plot, four groups could be recognized (Figure 4). The two northernmost regions (AmE and CE) cluster together, with high $\delta^{13}\text{C}$ and the lowest $\delta^{15}\text{N}$ values. Specimens from ES form a separate cluster from those sampled in the southern adjacent area of NRJ. This latter has some overlap with SP/PR values, making difficult to recognized them as two distinct clusters. The fourth group is formed by individuals from the southernmost area of SC, with high values of both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$.

Areas differed significantly with respect to $\delta^{13}\text{C}$ (ANOVA, $F_{5,150}=39.13$, $p<0.001$) and $\delta^{15}\text{N}$ (ANOVA, $F_{5,150}=100.85$, $p<0.001$). $\delta^{13}\text{C}$ values from AmE and CE were not significantly different (Tukey's HSD test, $p=0.71$) neither between AmE and SC (Tukey's HSD test, $p=0.99$) or between CE and SC (Tukey's HSD test, $p=0.90$). Finally, values of carbon isotope ratios from ES and SP/PR were not significantly different (Tukey's HSD test, $p=0.20$). Similarly, differences in $\delta^{15}\text{N}$ between the two northernmost areas, AmE and CE, as well as between NRJ and SC were non-significant (Tukey's HSD test, $p=0.99$, for both comparisons) (Figures 3 and 4).

The classification tree model correctly predicted sampling area for 62% and 61% of the learning and test samples, respectively. The tree obtained had four terminal nodes, assigned to CE, ES, NRJ and SC. The model was not able to correctly predict sampling region for AmE samples, which were mostly assigned to CE and, to a lesser extent, to ES. All SP/PR samples were misclassified, with the majority of the samples assigned to NRJ, followed by ES en SC. The highest percentage of accurate classification was in NRJ, ES, CE and SC, both in the learning and the test sample (Table 4, Figure 5). Ranking of predictor variables in a 0-100 scale (less to most important) showed that

$\delta^{15}\text{N}$ was highly important with a value of 100 while $\delta^{13}\text{C}$ was also important, although with a value of 88.

DISCUSSION

Isotopic composition in teeth can be used to separate Guiana dolphins into regional ecological stocks. A northern group (AmE and CE) as well as ES Guiana dolphins presented low $\delta^{15}\text{N}$ values, while these two groups were clearly separated from the other groups. Individuals from NRJ and SC could be clearly identified based on their $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values, however SP/PR samples presented overlap mainly with NRJ, and to a lesser extent, with SC. Two main factors could explain the observed isotopic variation: geographical differences in Guiana dolphins' diet (trophic level, represented by $\delta^{15}\text{N}$ values) or the isotopic baseline values ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) which are reflected up in the marine trophic food web (Hobson 1999, Born et al. 2003, Michener & Kaufman 2007).

Additionally, non-dietary factors such as age (Zhao et al. 2004, Mendes et al. 2007), nutritional status, lactation (Hobson & Sease 1998, Knoff et al. 2008, Newsome et al. 2009) and growth rate (Newsome et al. 2009) can especially influence nitrogen, and to a lesser extent, carbon isotope values. However, samples from potentially unweaned animals were not included and the similar mean age of animals was similar among areas, composed mostly of sub-adult or adult animals, thus minimizing the influence of at least some of these factors.

Carbon and nitrogen isotope compositions were not different between sexes in any of the study areas with the exception of AmE. Nitrogen isotopes ratios in this area were significantly higher in males (12.0‰) than in females (10.6‰), indicating that a resource partitioning between sexes might take place in this area. Some dimorphic

species such as the long-finned pilot whale (*Globicephala melas*, Desportes & Mouritsen 1993), the Steller sea lion (*Eumetopias jubatus*, Hobson et al. 1997) and the beluga whale (*Delphinapterus leucas*, Lesage et al. 2001) exhibit some level of trophic niche segregation. Although no sexual dimorphism exist in Guiana dolphins (Flores & da Silva 2009)), more than 90% of the prey items are shared between sexes in AmE, some species, such as the cutlass fish, *Trichiurus lepturus*, and Ariidae fish, were more commonly found in males than in females (see Table 1, Beltrán-Pedrerros & Pantoja 2006). The lack of information on growth parameters and sexual dimorphism for this northern populations preclude further explanations for the observed difference. Nevertheless, both the cutlass fish and the Ariidae are relatively high trophic level predators (Martins et al. 2005, Giarizzo 2007), which might explain the higher $\delta^{15}\text{N}$ values in males.

The intrapopulation variance in $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$, interpreted as a consequence of a wider variety of prey species and/or foraging locations, could be used as a measure of niche width (Bearhop et al. 2004, reviewed by Newsome et al. 2007). Guiana dolphins from the northern group presented $\delta^{15}\text{N}$ values three times more variable than values found in the southern group suggesting that dolphins from these northern areas are preying upon a wider range of prey/trophic levels. The presence of other coastal species, such as the franciscana (*Pontoporia blainvillei*), rough-toothed dolphins (*Steno bredanensis*) and common bottlenose dolphins (*Tursiops truncatus*) in the southern areas might moderate the trophic niche width due to interspecific competition as observed in other animal groups (Begon et al. 2006). The decreasing diversity of prey species as latitude increases (e.g. Levinton 1995), might also affect the diet composition of this opportunist dolphin (Di Benedetto & Ramos 2004, Lopes 2010). Nevertheless,

these are only hypotheses yet to be tested as more information on Guiana dolphins feeding ecology become available.

Prey composition and, consequently, trophic level of Guiana dolphins in the food web can be considered a potential cause for the observed differences in nitrogen isotopes values among areas. The lower nitrogen isotopes values in Guiana dolphins from the northern group and ES could be interpreted as if they occupy lower trophic levels than dolphins from the remaining areas. Conversely, despite the fact that demersal fish, and, to a lesser extent, squids (e.g. *Loligo plei*) represent the main prey of Guiana dolphins along their distribution in Brazil (Table 5), only in ES and in the southern group that low trophic level clupeids and engraulids were described as important components of their diets (Table 5). Representatives of these two families (e.g. *Pellona harrowerii*, *Sardinella brasiliensis*, *Anchoa filifera* and *Cetengraulis edentulus*) are mainly pelagic zooplanktivores thus ^{15}N -depleted values are typical of this group, compared to that of demersal, higher trophic level fishes (Bugoni et al. 2010, Bissi et al. *submitted*). Nevertheless, due to the extended latitudinal range and hydrogeomorphological diversity covered by this study, geographic differences in basal isotope values, affecting both prey and predator, are expected. Therefore, the comparison of prey items in an effort to explain the observed variation might be misleading, unless if isotope compositions of potential prey in each area is known.

Basal carbon and nitrogen isotopes compositions must shape isotopically distinct habitats or “isoscapes” (Graham et al. 2010) along the Brazilian coast. Up to date no information on basal nitrogen and carbon isotope values is available for most areas analyzed in this study. However, alternative approaches can be used to interpret the isotopes patterns observed in Guiana dolphins’ teeth.

Nitrogen isotope compositions presented a clear latitudinal trend, with higher values found in NRJ, SP/PR and SC areas. The Coastal Water (CW) receiving low $\delta^{15}\text{N}$ nutrients from natural continental sources and the presence of the oligotrophic Tropical Water (TW), carried by the North Brazilian Current (NBC) and the Brazilian Current (BC) (Knoppers et al. 2009), might be the cause of the relatively low $\delta^{15}\text{N}$ values found in teeth of Guiana dolphins from the northern group and ES, respectively. N_2 -fixation by cyanobacteria and recycling N (low $\delta^{15}\text{N}$) are important in oligotrophic regions (Checkley Jr. and Miller 1989, Sigman et al. 2009, Landrum et al. *in press*). Indeed, $\delta^{15}\text{N}$ value from nitrogen fixed by diazotrophs ranges from -1 to -2‰ (McClelland et al. 2003, Montoya et al. 2002) as there is little isotopic fractionation during its uptake by phytoplankton (Minagawa & Wada 1984, Montoya 2007).

Dolphins from NRJ presented clearly higher $\delta^{15}\text{N}$ values than ES, despite their geographical proximity, which might be caused by a substantial change in nitrogen sources. High $\delta^{15}\text{N}$ values were also found in SP/PR and SC samples. South of the Vitória-Trindade Chain (VTC, 20°S), upwelling cells and plumes of nutrient-rich South Atlantic Central Waters (SACW T-S=5-20°C, 34-36, Stramma & England 1999) are frequent (Rodrigues & Lorenzzetti 2001). Although this oceanographic process is especially important around the Cabo Frio region (Rio de Janeiro State, Rodrigues & Lorenzzetti 2001), bottom intrusions of this cold and nutrient-rich water mass are also present in all areas from the Southern Brazilian ecosystem (Castro-Filho et al. 1987, Brandini et al. 2007). The SACW reaching the coastal euphotic zone mainly in summer time, carries deep water nitrate that is typically ^{15}N -enriched (~5‰, Sigman et al. 2009). This seasonal influence of high $\delta^{15}\text{N}$ nitrate changes the trophic structure due to different nutrient sources and species' succession (O'Reilly et al. 2002, Bode et al. 2003,

2004, Sigman et al. 2009). High $\delta^{15}\text{N}$ values due to the ^{15}N -enriched food webs resulted from the upwelling of denitrified intermediate and deep waters were also recorded in tissues from bowhead whales (*Balaena mysticetus*) feeding in coastal regions of the Beaufort Sea (Schell et al. 1989), California sea lions (*Zalophus californianus*) in the Gulf of California (Aurioles-Gamboa et al. 2009) or in consumers in general from the Galician upwelling ecosystem (Bode et al. 2006) and the Benguela system of southern Africa (Hill et al. 2006, Hill & McQuaid 2008). Bisi et al. (*submitted*) and Corbisier et al. (2006) also reported high $\delta^{15}\text{N}$ values in coastal food webs from southern Rio de Janeiro and northern São Paulo, respectively. In Babitonga Bay (SC), Barros et al. (2010) also found high $\delta^{15}\text{N}$ values in organic matter from this ecosystem, and attributed them to ocean sources and, in some sites inside the bay, to sewage. In this Bay, the Guiana dolphin has a preference for areas close to the entrance where they capture their main prey, including the mullet, *Mugil curema*, which seasonally migrates from the coast to estuarine areas (Cremer et al. 2009). As a consequence, inherited ^{15}N -enriched nitrate from deeper oceanic waters might be transferred to these top predators through the food web. In fact, Hardt (2008) found ^{15}N -enriched values found for the species' main prey sampled inside the bay and adjacent coastal waters.

Besides this seasonal input of SACW, coastal waters of SP/PR and SC also receive nutrient loads from the Subtropical Shelf Front, which carries shelf waters and nutrient-rich waters from the Rio de la Plata and Patos Lagoon estuaries (Piola et al. 2000, 2005), and local terrestrial runoff including dissolved inorganic nitrogen from sewage and farm/industrial wastes. Although terrestrial organic matter is often depleted in ^{15}N (Montoya 2007) anthropogenic sources are recognized to be source of high $\delta^{15}\text{N}$ to coastal waters worldwide (Kendall et al. 2007). Nevertheless, if this anthropogenic

input was the cause of the high nitrogen isotope found in these southern areas then other areas like CE or ES would also present high $\delta^{15}\text{N}$ values. Therefore, the diversity in basal nitrogen isotopes due to differences in hydrologic conditions might be the main cause for the latitudinal trend in nitrogen isotope compositions of Guiana dolphins.

As for $\delta^{13}\text{C}$, values among geographic areas were similar, and typical of carbon sources derived from phytoplankton-based coastal food webs (e.g. Burton & Koch 1999, Lesage et al. 2001). The only exception was NRJ, which presented the lowest $\delta^{13}\text{C}$ values. A more ^{13}C -depleted freshwater-influenced mean $\delta^{13}\text{C}$ value was expected for the AmE Guiana dolphins due to the strong influence of the Amazon River estuary (Table 1), which potentially drains its adjacent coastal area with river-transported ^{13}C depleted organic matter of terrestrial (Schell et al. 1998) or mangrove origin (Dittmar et al. 2001). However, due to the influence of the North Brazil Current, the main transport of the Amazon river is to the northwest (Lentz 1995, Santos et al. 2008). This might contribute to a differentiation in the coastal region adjacent to the mouth of this rivers, with a more similar to CE environmental conditions (e.g. less freshwater-influenced food webs) in the southwestern area. If AmE Guiana dolphins forage more frequently in the latter, their isotopes compositions would be more similar to that of CE. Stomach contents of Guiana dolphins incidentally killed in fishing nets in the AmE area revealed a diet based mainly on marine-estuarine fishes (e.g. Sciaenidae, Trichiuriidae, Ariidae), though some freshwater species were eventually ingested during periods with high freshwater discharge (Beltrán-Pedrerros & Pantoja 2006). Besides this, no information on the habitat use of Guiana dolphins is available from this area, nor is the exact procedence of the individuals used in the analysis, thus precluding further support to this hypothesis. Studies conducted in other regions (e.g. Kieckbusch et al. 2004),

including coastal areas of Brazil (Schwamborn et al. 2002, Corbisier et al. 2006, Giarrizo 2007), indicate that mangroves and other ^{13}C depleted primary organic matter sources were proved to have little or no contribution to coastal/estuarine food webs. This pattern might contribute to the ^{13}C -enriched values found in AmE dolphins.

The lowest $\delta^{13}\text{C}$ found in dolphins from NRJ could be due to the presence of a source of freshwater organic matter, which is usually ^{13}C depleted, entering the coastal food web. The Paraíba do Sul river drains a mean of $796 \text{ m}^3 \cdot \text{s}^{-1}$ to this region (Nicolite et al. 2009). However, similar freshwater inputs from the Doce river (Coelho 2006) seem not to be reflecting low $\delta^{13}\text{C}$ values in Guiana dolphins from ES. An alternative interpretation of the low mean carbon isotope values found in NRJ could be a change in feeding habit from a benthos linked to a ^{13}C -depleted offshore-pelagic food web (e.g. France 1995, Burton & Koch 1999, Clementz & Koch 2001, Pinela et al. 2010). Considering that an offshore feeding is unlikely for this coastal dolphin species, upwellings might be playing a role in labeling the food web it is part of, with more offshore-like carbon isotopes values. Deep water dissolved inorganic carbon (DIC) is depleted due to organic matter oxidation (which releases isotopically light CO_2 , Deuser & Hunt 1969, Swart et al. 1999). The upwelling of a ^{13}C -depleted deep water eventually alters basal $\delta^{13}\text{C}$ values, labeling the entire food web with lighter isotopes values (O'Reilly et al. 2002, Hill et al. 2006). Pierre et al. (1991) found lower ^{13}C contents in intermediate, deep and bottom waters, compared to that of upper water layers from the Central Brazil Basin. Although in a resident/small home range species, such as the Guiana dolphin, isotopic changes between upwelling and non-upwelling seasons would be averaged in a long-term and slow turnover tissue such as teeth, a distinct isotopic signal would still be possibly identified in NRJ individuals.

Babitonga bay samples (SC) also presented a mean $\delta^{13}\text{C}$ value typical of coastal phytoplankton-based food webs. Although this bay has several freshwater inputs, these are small (IBAMA 1998). Barros et al. (2010) demonstrated that $\delta^{13}\text{C}$ values in POC increased with increasing salinities from the rivers to the sites close to the bay entrance, up to values ~ -22 to -23‰ , considered typical of ocean samples (Schwamborn et al. 2002, Barros et al. 2010). Furthermore, Hardt (2005) and Cremer et al. (2009) reported that the central portion of the bay was the main concentration area for Guiana dolphins, thus explaining the marine-influenced carbon isotope values found. A previous work conducted at this bay analyzed some components of the diet of the species, measuring stable isotopes compositions in fish samples from inside and outside the bay (Hardt 2008) as well as muscle, liver and kidneys from ten Guiana dolphins. The author also found stable carbon isotopes compositions in dolphins and their prey with marine-influenced values ($\sim -17\text{‰}$). These values, after accounting for a combination of tissue-dependent $\Delta^{13}\text{C}$ collagen-muscle ($\sim 4\text{‰}$), plus a trophic discrimination factor, in the case of the prey (1‰) (Koch 2007), were not remarkably different to the values found in the present study.

Stable isotopes compositions as a tool for identifying ecological stocks

Based on $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ data, we could separate Guiana dolphins into four groups: AmE+CE, ES, NRJ and SC. Individuals from SP/PR could not be identified as a separate group, and were mainly misclassified as belonging to NRJ (Table 4). This division differs from the genetic population structure of Guiana dolphins from Brazil proposed by Cunha et al. (2010), which considered that animals from AmE and CE were part of separated stocks and included all animals from the southeastern/south region (from Rio de Janeiro to Santa Catarina states) as a single stock.

The division based on isotopes data was stronger between AmE, CE and ES and the southern areas (N RJ, SP/PR and SC) than within them. Studies based on whistles characteristics also showed a separation between northern areas (Pará -equivalent to AmE- and CE) and southern areas (Rio de Janeiro, SP/PR and SC) (Azevedo & Van Sluys 2005). Due to the proximity of ES to N RJ one would expect similar $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values between these adjacent areas. However, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ from ES were more similar to AmE or CE than to N RJ values. Morphometric data also showed a clear separation of ES samples from the southern/southeastern populations, suggesting that the probable hiatus in the distribution between Vitória (ES) and Atafona (N RJ) might be due to an ecological barrier that separates these two units, however reduced sampling effort could be the cause of the lack of species' records in this region (Ramos 2001). Another suggested hiatus in the distribution of Guiana dolphins is the Cabo Frio-Arraial do Cabo area (Ramos 2001). Although the seasonal presence of colder waters due to the frequent upwelling cells and plumes are characteristic of the region between Vitória and Cabo Frio, stronger events are recorded in the latter (Rodrigues & Lorenzzetti 2001). Indeed, morphometrics also showed that N RJ dolphins differed from individuals found in southern RJ (Ramos 2001). Therefore, both the change in hydrographic conditions south of the VTC and the colder waters around Cabo Frio could be acting as natural barriers for the NRJ Guiana dolphin population to disperse.

In northern Brazil, mtDNA control region data separated AmE and CE individuals in two stocks (Cunha et al. 2010). This was somewhat corroborated by morphometrics, as evidence exists that AmE individuals are smaller than coastal populations of CE and Santa Catarina (Fettuccia 2006). In the present work, Guiana dolphins from AmE and CE had statistically indistinguishable $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values, thus demonstrating that,

sometimes, similar $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values can be obtained from animals from different stocks, mainly in high trophic level predators, as their isotopes values are obtained from a combination of prey values which eventually can result in predators' similar values. In this case, without the strong genetic and morphometric evidences, the solely use of an ecological tracer, such as stable isotopes, would lead to the incorrect lumping of populations or "conservation error" (Taylor 2005). To avoid this, and in the lack of other evidence, the use of the stable isotopes of alternative elements (e.g. $\delta^{34}\text{S}$, Peterson & Fry 1987, Hoekstra et al. 2002, Connolly et al. 2004, Barros et al. 2010; or $\delta^{18}\text{O}$, Clementz & Koch 2001) can help to better define the ecological separation of the populations.

The southern/southeastern populations were considered by Cunha et al. (2010) as a single stock based on genetic data, as they observed a lack of variation in the control region among dolphins from these regions. Morphometrics, on the other hand, provide evidence for a separation of RJ from SP/PR, with smaller individuals found in the latter (Ramos 2001). In the present study mean stable isotope values differed between NRJ and SC areas, with overlapping values found in samples from SP/PR. Samples from the latter, were composed of dolphins incidentally killed in fishing nets operating in the adjacent coastal area and individuals found washed ashore along the coast or floating inside the estuary. Mean $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in dolphins from the estuary (n=5) were slightly lower than those of bycaught animals (n=7) ($\delta^{13}\text{C}$ =-13.3 and -12.6‰ and $\delta^{15}\text{N}$ =14.1 and 15.3‰, respectively). Differences were also observed in diet composition between these two groups, with larger prey recorded in coastal dolphins (Lopes 2010). Therefore, differences in habitat and prey might explain the broader isotope values SP/PR, thus further sampling of animals from each region should help clarify this. A certain

ecological structure among three estuarine complexes (Guanabara Bay in Rio de Janeiro, Sepetiba and Ilha Grande in southern Rio de Janeiro, and Paranaguá Bay in northern Paraná state) were also demonstrated through organochlorines (Lailson-Brito et al. 2010) and stable isotope compositions (Bisi et al. *submitted*). As for stable isotopes, diet is the main route for organochlorine uptake, thus demonstrating an ecological separation among the groups.

Regardless of the roles of dietary versus oceanographic differences as factors for explaining isotopic patterns described, this approach proved to be valuable for identifying MUs of Guiana dolphins in Brazil. Similarly to other such as organochlorine compounds in bottlenose dolphins (*Tursiops truncatus*) from the Iberian Peninsula (Borrell et al. 2006), heavy metals in narwhals (*Monodon monoceros*) from West Greenland (Dietz et al. 2004) and fatty acids in killer whales (*Orciuns orca*) from the North Pacific (Herman et al. 2005) local differences in stable isotopes in Guiana dolphins can be used as proxy for the identification of discrete ecological stocks. Additional sampling, especially in the region between CE and ES (Brazilian Northeastern region) and a further refinement in the Rio de Janeiro-Santa Catarina area is required to verify the existence of other ecological units of the species in Brazil. For example, two stocks between CE and ES composed of individuals from Rio Grande do Norte and Bahia states were described based on molecular data (Cunha et al. 2010).

Although the species has been experiencing incidental captures along all its distribution in Brazil (e.g. Siciliano 1994, Monteiro-Neto et al. 2004, Di Benedetto 2003, Netto & Barbosa 2003, Netto & Di Benedetto 2008), the real impact of this non-natural mortality on the populations remains unknown, mainly due to the lack of relevant parameters for a proper assessment along most of its distribution. Genetic,

morphometric, population parameters and ecological information, especially from the populations north of VTC, are still lacking. Efforts must be put in place in order to fill the essential gaps of knowledge needed for reliable identification of genetic or ecological stocks of Guiana dolphins which is crucial for assessment of the conservation status and proper management of these units.

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Figure 1. Study areas of Guiana dolphins, *Sotalia guianensis*, from Brazil.

Figure 2. Age and length in relation to $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values for teeth dentine of Guiana dolphins from Brazil: Amazon Estuary (AmE), Ceará (CE), Espírito Santo (ES), Northern Rio de Janeiro (N RJ), Southern São Paulo and Paraná (SP/PR) and Babitonga Bay, Santa Catarina (SC).

Figure 3. Mean (symbols) and $\pm\text{SD}$ (whiskers) $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values for teeth dentine of Guiana dolphins from Brazil: Amazon Estuary (AmE), Ceará (CE), Espírito Santo (ES), Northern Rio de Janeiro (N RJ), Southern São Paulo and Paraná (SP/PR) and Babitonga Bay, Santa Catarina (SC).

Figure 4. Teeth $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of Guiana dolphins from Brazil: Amazon Estuary (AmE), Ceará (CE), Espírito Santo (ES), Northern Rio de Janeiro (N RJ), Southern São Paulo and Paraná (SP/PR) and Babitonga Bay, Santa Catarina (SC).

Figure 5. Classification Tree for Guiana dolphins from Brazil: Amazon Estuary (AmE), Ceará (CE), Espírito Santo (ES), Northern Rio de Janeiro (N RJ), Southern São Paulo and Paraná (SP/PR) and Babitonga Bay, Santa Catarina (SC) using $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values in teeth as predictor variables. Split conditions for each node are indicated. The observed area N for each node is represented by a bar plot.

Table 1. Main characteristics of study areas: Amazon Estuary (AmE), Ceará (CE), Espírito Santo (ES), Northern Rio de Janeiro (N RJ), Southern São Paulo and Paraná (SP/PR) and Babitonga Bay, Santa Catarina (SC).

	AmE	CE	ES	N RJ	SP/PR	SC
Main Ecosystems	Mangroves Estuaries Mud plains	Mangroves Estuaries Reefs	Estuaries Reefs	Estuaries Coastal upwellings	Estuaries (bays and lagoons) Coastal upwellings	Estuaries (bays and lagoons)
Tides	Macro tides (>4m)	Mesotides (2-4m)	Mesotides (2-4m)	Mesotides (2-4m)	Micro tides (<2m)	Micro tides (<2m)
Freshwater discharge (m ³ s ⁻¹)	180,000 ^a	11 ^b	1139 ^c	796 ^d	435 ^e	<20 ^f
Main Ocean Currents	North Brazilian Current	North Brazilian Current	Brazilian Current	Brazilian Current	Brazilian Current	Brazilian Current

^aAmazon River (Geyer et al. 1996)

^bPacoti River, Fortaleza (Lacerda et al. 2007)

^cDoce River (Coelho 2006)

^dParaíba do Sul River (Nicolite et al. 2009)

^eCananéia Estuary (Schaeffer-Noveli et al. 1990)

^fBabitonga Bay (Barros et al. 2010)

Table 2. Total Length (TL, in cm), age (in yr), sample number (n) and sampling years (SY) of Guiana dolphins, *Sotalia guianensis*, per study area: Amazon Estuary (AmE), Ceará (CE), Espírito Santo (ES), Northern Rio de Janeiro (N RJ), Southern São Paulo and Paraná (SP/PR) and Babitonga Bay, Santa Catarina (SC).

AREA	Sex	TL Mean (min.-max.)	Age Mean (min.-max.)	n	SY
AmE	Females	144.1 (120.0-170.0)	-	10	1996-2001
	Males	135.6 (111.0-170.0)	-	10	
	Unknown	-	-	-	
	Total	139.8 (111.0-170.0)	-	20	
CE	Females	185.8 (160.0-208.0)	10.5 (5-21)	10	1993-2006
	Males	178.7 (153.0-192.0)	12 (5-23)	10	
	Unknown	-	-	-	
	Total	182.4 (153.0-208.0)	11.3 (3-23)	20	
ES	Females	186.7 (162.0-200.0)	12.9 (3-33)	10	1996-2007
	Males	177.5 (151.5-198.0)	10.2 (4-23)	16	
	Unknown	183.4 (156.0-193.0)	12.6 (7-17)	8	
	Total	181.8 (151.5-200.0)	11.3 (3-33)	34	
N RJ	Females	180.7 (164.0-198.0)	10.7 (5-30)	18	1989-2000
	Males	181.1 (164.0-200.0)	7.1 (5-18)	28	
	Unknown	-	-	-	
	Total	180.9 (164.0-200.0)	8.5 (5-30)	46	
SP/PR	Females	159.5 (130.0-200.0)	11.2 (3-29)	6	1996-2007
	Males	167.5 (115.0-200.0)	10.4 (3-28)	13	
	Unknown	-	29	2	
	Total	168.8 (115.0-200.0)	11.63 (2-29)	21	
SC	Females	171.5 (144.0-191.0)	7.2 (2-18)	6	2000-2006
	Males	174.2 (159.0-202.0)	10.8 (2-24)	7	
	Unknown	160.0	5-8	2	
	Total	171.8 (144.0-202.0)	8.9 (2-24)	15	

Table 3. One-way ANOVA and mean $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values for *Sotalia guianensis* teeth from six areas of Brazil: Amazon Estuary (AmE), Ceará (CE), Espírito Santo (ES), Northern Rio de Janeiro (N RJ), Southern São Paulo and Paraná (SP/PR) and Babitonga Bay, Santa Catarina (SC). Means followed by the same superscript letter are not significantly different ($p>0.05$, Tukey's HSD test).

Areas	n	Mean $\delta^{13}\text{C}$	SD	Mean $\delta^{15}\text{N}$	SD
Am E	20	-11.8 ^a	0.8	11.3 ^a	1.4
CE	20	-11.5 ^{a,b}	0.8	11.3 ^a	1.4
ES	34	-12.4 ^c	0.5	13.2	1.1
N RJ	46	-13.6	0.8	15.9 ^b	0.8
SP/PR	21	-12.8 ^c	0.7	14.9	0.7
SC	15	-11.7 ^{a,b}	0.5	16.0 ^b	0.8

Table 4. Classification tree analysis of Guiana dolphins from six areas of Brazil: Amazon Estuary (AmE), Ceará (CE), Espírito Santo (ES), Northern Rio de Janeiro (NRJ), Southern São Paulo and Paraná (SP/PR) and Babitonga Bay, Santa Catarina (SC) using $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values in teeth as predictor variables. Numbers in parenthesis are sample size for the learning and test samples. Number of samples assigned to each region are shown. Correct classifications are in **bold**.

<i>Learning sample</i>							
Predicted sampling area	Known sampling area						% correct
	AmE (12)	CE (12)	ES (20)	NRJ (28)	SP/PR (12)	SC (9)	
AmE	0	0	0	0	0	0	0
CE	7	9	3	0	0	0	75
ES	5	3	17	0	1	0	85
NRJ	0	0	0	26	8	3	93
SP/PR	0	0	0	0	0	0	0
SC	0	0	0	2	3	6	67
						Overall	62
						<i>Cost</i>	0.44

<i>Test sample</i>							
	AmE (8)	CE (8)	ES (14)	NRJ (18)	SP/PR (8)	SC (6)	% correct
AmE	0	0	0	0	0	0	0
CE	6	5	1	0	0	0	62
ES	2	3	11	0	2	0	79
NRJ	0	0	2	18	5	2	100
SP/PR	0	0	0	0	0	0	0
SC	0	0	0	0	1	4	67
						Overall	61
						<i>Cost</i>	0.39

Table 5. Main fish prey identified based on stomach content analysis for Guiana dolphins, *Sotalia guianensis*, from six areas of Brazil: Amazon Estuary (AmE), Ceará (CE), Espírito Santo (ES), Northern Rio de Janeiro (N RJ), Southern São Paulo and Paraná (SP/PR) and Babitonga Bay, Santa Catarina (SC). Main four fish species based on Index of Relative Importance (IRI) are listed.

Area	Main prey species	Prey habits	References
AmE*	<i>Trichiurus lepturus</i>	Demersal-pelagic	Beltrán-Pedrerros & Pantoja (2006)
	<i>Plagioscion squamosissimus</i>	Demersal	
	<i>Bagre bagre</i>	Demersal	
	<i>Macrodon ancylodon</i>	Demersal	
CE**	Gerreidae	Demersal	Gurjão et al. (2003)
	Mullidae	Demersal	
	Haemulidae	Demersal	
	Ophichthidae	Demersal	
ES	<i>Trichiurus lepturus</i>	Demersal-pelagic	Araujo et al. (2010)
	<i>Cynoscion jamaicensis</i>	Demersal	
	<i>Pellona harroweri</i>	Pelagic	
	<i>Stellifer sp.</i>	Demersal	
NRJ	<i>Trichiurus lepturus</i>	Demersal-pelagic	Di Benedetto & Ramos (2004)
	<i>Porichthys porosissimus</i>	Demersal	
	<i>Anchoa filifera</i>	Pelagic	
	<i>Paralonchurus brasiliensis</i>	Demersal	
	and <i>Ariosoma opisthoptalma</i>	Bathydemersal	
SP/PR	<i>Paralonchurus brasiliensis</i>	Demersal	Lopes 2010
	<i>Sardinella brasiliensis</i>	Pelagic	
	<i>Stellifer sp.</i>	Demersal	
	<i>Trichiurus lepturus</i>	Demersal-pelagic	
SC	<i>Mugil curema</i>	Demersal	Cremer (2007)
	<i>Micropogonias furnieri</i>	Demersal	
	<i>Diapterus rhombeus</i>	Demersal	
	<i>Stellifer brasiliensis</i>	Demersal	

* Values in parenthesis are FO (%)

** Prey listed as families, because no data on FO and/or IRI was available at the species level.

Figure 1

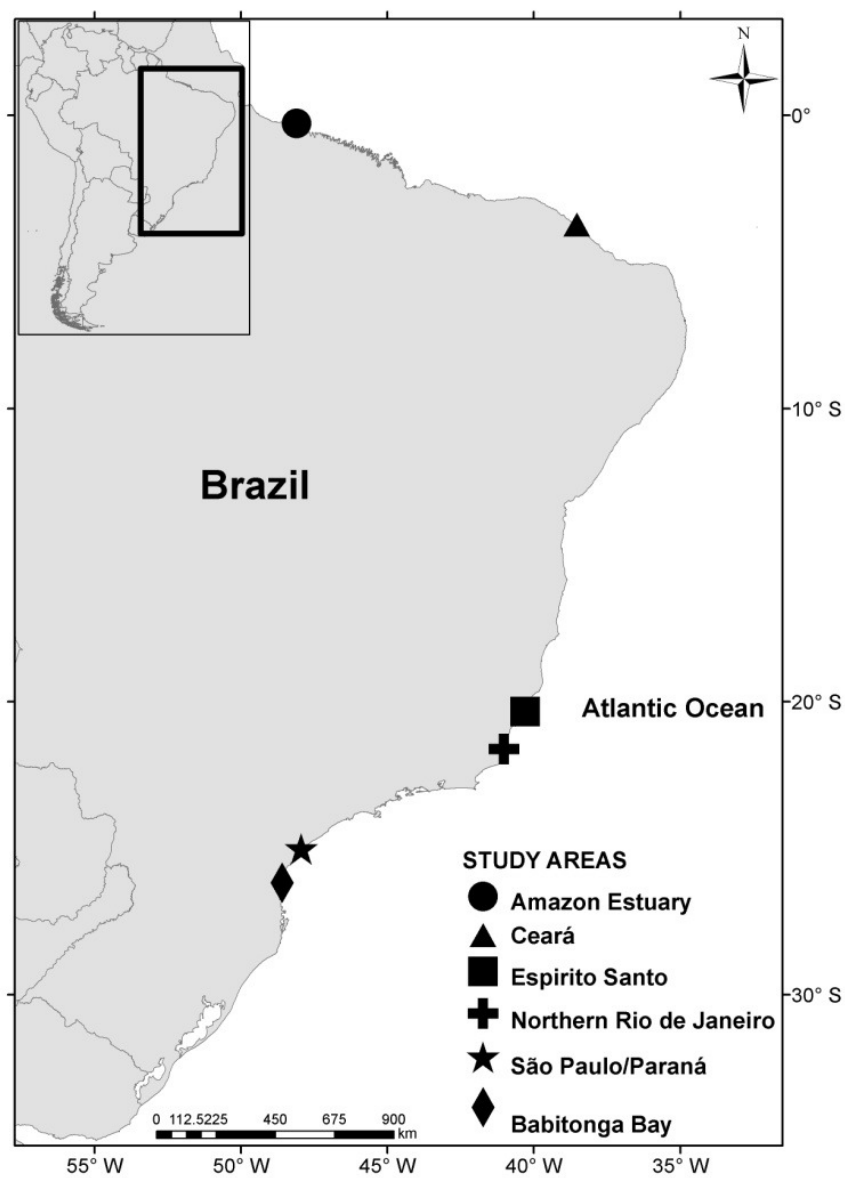


Figure 2

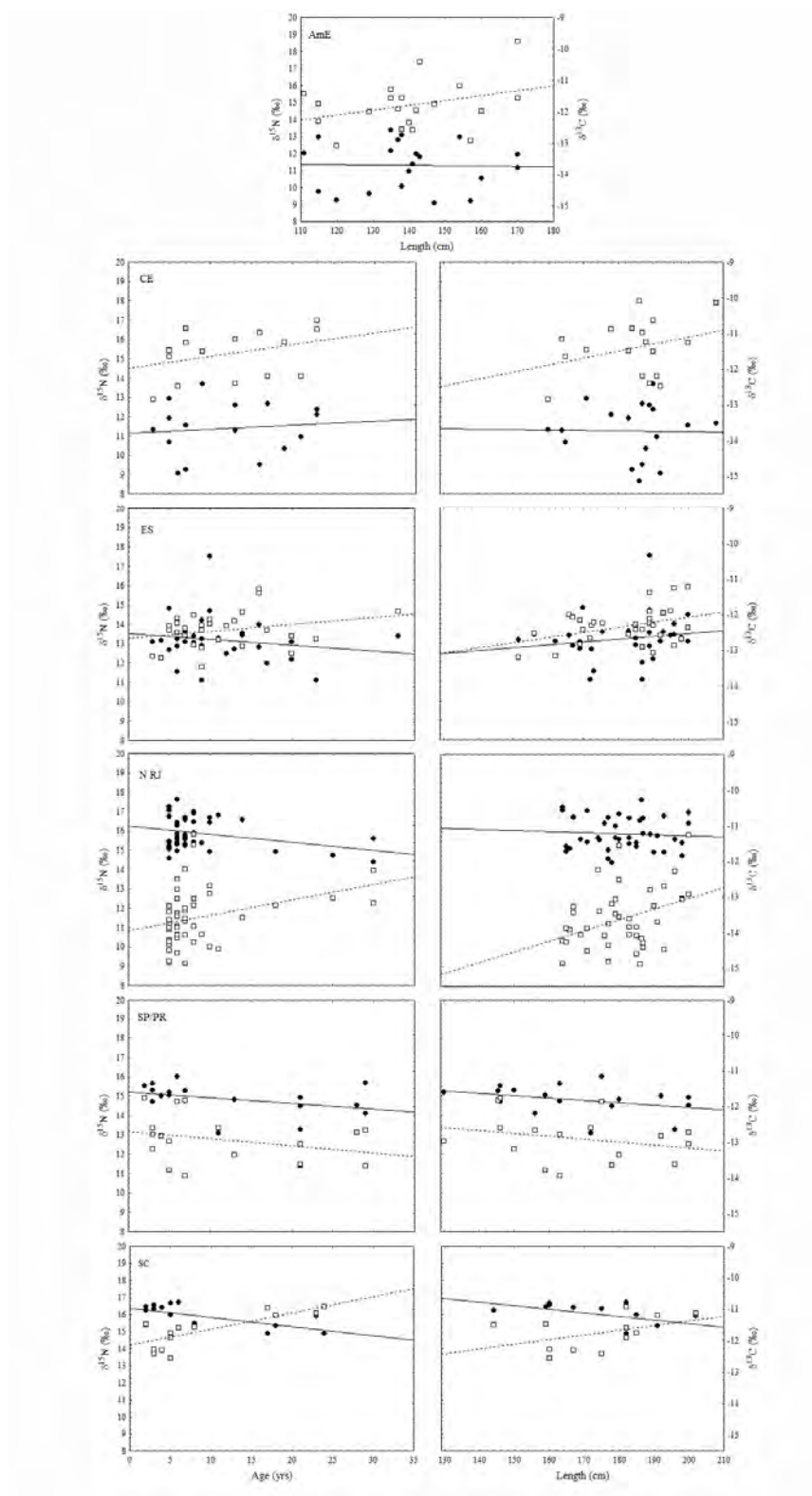


Figure 3

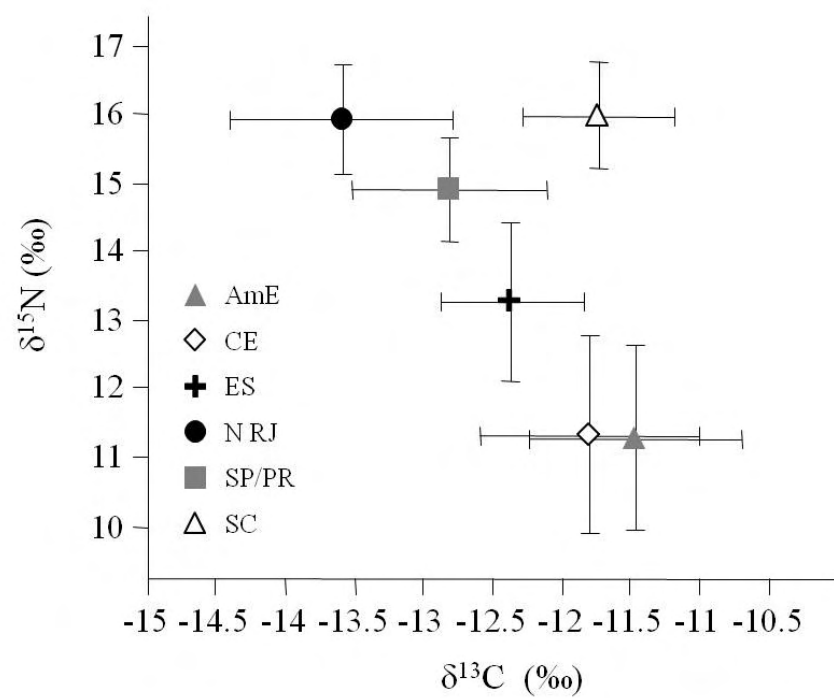


Figure 4

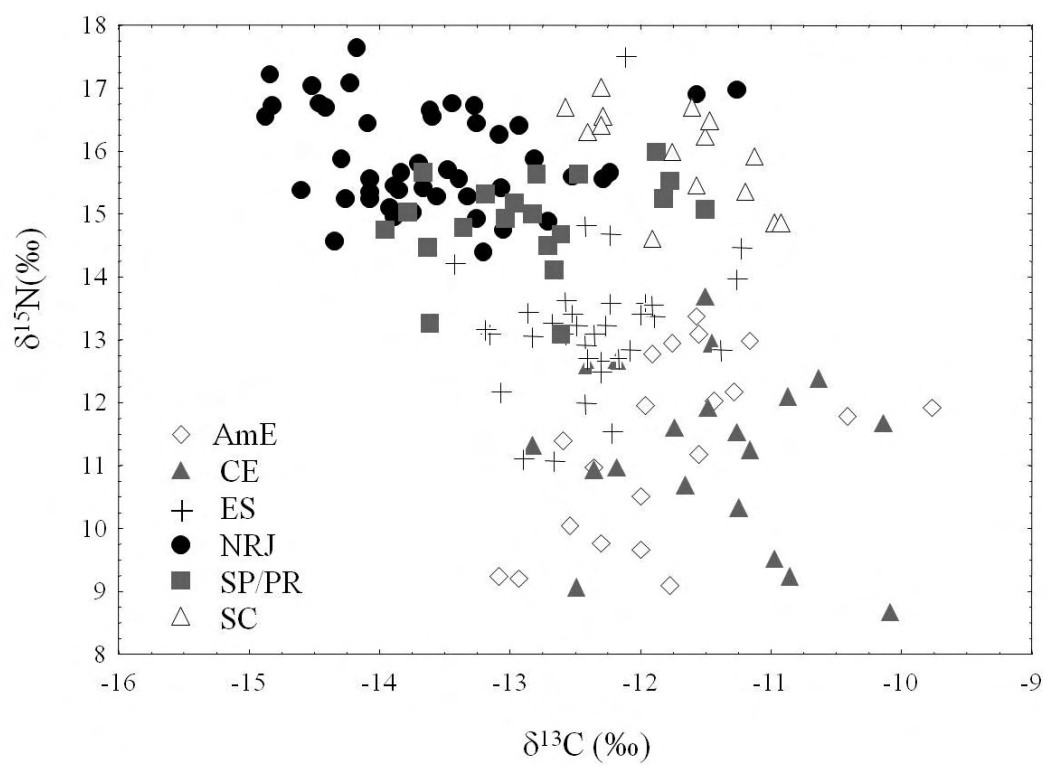
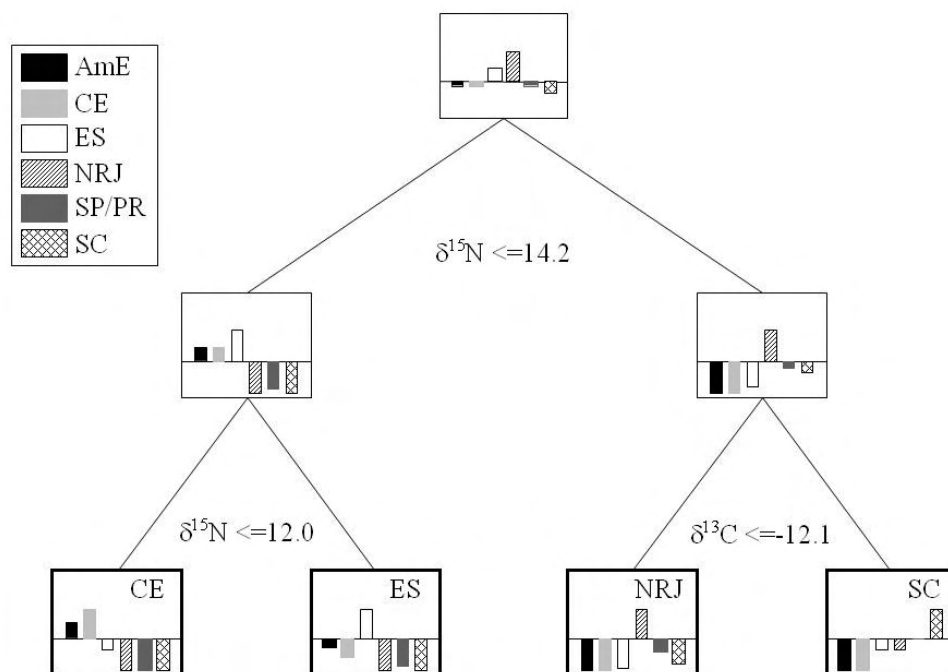


Figure 5



ANEXO II

Evidence of ecological separation in franciscana dolphins (*Pontoporia blainvillei*) based on stable isotopes

Botta, S.; Hohn, A.; Macko, S.; Ferreira, E.C.; Santos, M.C. de O.; Di Benedetto, A.P.
M.; Ramos, R.; Bertozzi, C.; Franco-Trecu, V.; Barbosa, L.; Cremer, M.; Cappozzo,
L.H, Failla, M.; Iñiguez, M.; Secchi, E.R.

(A ser submetido a *Marine Biology*)

**Evidence of ecological separation in franciscana dolphins (*Pontoporia blainvillei*)
based on stable isotopes**

Botta, S.¹; Hohn, A.²; Macko, S.³; Ferreira, E.C.¹; Santos, M.C. de O.⁴; Di Benedetto,
A.P. M.⁵; Ramos, R.⁶; Bertozzi, C.⁷; Franco-Trecu, V.⁸; Barbosa, L.⁹; Cremer, M.¹⁰;
Cappozzo, L.H.¹¹, Failla, M.¹²; Iñiguez, M.¹³; Secchi, E.R.¹

(1) Instituto de Oceanografia-Universidade Federal do Rio Grande-FURG, Cx.P. 474, Rio Grande, RS 96500970, Brazil.;(2) National Marine Fisheries Service, NOAA, Beaufort Laboratory, NC 28516, USA;(3) Department of Environmental Sciences, University of Virginia, Charlottesville, VA 22903 USA;(4) Laboratório de Biologia da Conservação de Mamíferos Aquáticos, Departamento de Oceanografia Biológica, Instituto Oceanográfico, Universidade de São Paulo, São Paulo, SP Brazil 05508-120;(5) Universidade Estadual do Norte Fluminense, CBB/LCA, Campos dos Goytacazes, RJ Brazil 28013-602.;(6) Everest Tecnologia em Serviços Ltda. Vitória, ES, 29050-912 Brazil.;(7) Projeto BioPesca, Ubatuba and Guarujá, São Paulo, Brazil;(8) Cetáceos Uruguay, Sección Etología-Facultad de Ciencias, Montevideo-Uruguay;(9) Organização Consciência Ambiental—ORCA, Vila Velha, ES, 29101-300, Brazil;(10) Departamento de Ciências Biológicas; Universidade da Região de Joinville; Joinville - SC - Brasil;(11) Museo Argentino de Ciencias Naturales C1405DJR Buenos Aires, Argentina;(12) Giani 367, Barrio El Cóndor, (8500) Río Negro, Argentina;(13) Fundación Cethus, Potosí 2087, (B1636BUA), Olivos, Argentina.

ABSTRACT

In this work stable isotope analysis was used to identify ecological stocks of franciscana dolphins (*Pontoporia blainvillei*). Teeth from 176 dolphins were obtained from incidentally caught or stranded specimens along the entire species distribution range: Espírito Santo (ES, n=10), northern Rio de Janeiro (NRJ, n=35), northern São Paulo (NSP, n=20), southern São Paulo/northern Paraná (SP/PR, n=19), Santa Catarina (SC, n=7) and Rio Grande do Sul (RS, n=51) in Brazil, and from Uruguay (URU, n=17) and Argentina (ARG, n=17). Teeth were used for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ analysis. The $\delta^{13}\text{C}$ and

$\delta^{15}\text{N}$ were different among areas. Differences in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ between NRJ and ES provide further evidence that these areas might be treated as separate management units. The central areas, NSP, SP/PR and SC presented common stable isotope compositions of C and N. Among the southern areas, data from RS and URU suggest that they share the same isotopic values. Mean $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of the southernmost area (ARG) indicated its separation from the RS/URU stock. Results of ANOVAs confirmed that dolphins from ES, NRJ, the central areas, RS/URU and ARG differed significantly with respect to $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ ($p < 0.001$). Geographical variations in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ proved to be a reliable tool for identifying ecological stocks of franciscana dolphins.

Keywords: *Pontoporia blainvillei*, stable isotopes, stock structure, cetacea.

INTRODUCTION

The negative impact of anthropogenic activities on marine mammals is of global concern. One of the main threats to coastal populations is the incidental mortality in fishing nets (Read et al. 2006). The level and characteristics of this bycatch probably differ along the distribution of the species being impacted. Therefore, for understanding the effects of this non-natural removal on the species' survival and for the implementation of conservation strategies on a local basis, the identification of discrete units is critical.

Choosing the stock concept that is going to be used depends on the purposes of the study. The stock concept as Evolutionary Significant Unit or ESU deals with structuration in an historical scale, and putative populations present low or no genetic exchange (Dizon et al. 1992, Moritz 1994). However, for short term conservation purposes of threatened populations, it is usually necessary to define management units

or "stocks" in a less strict sense, using other sources of information, besides genomics (Dizon et al. 1992, Wang 2002). Information such as distribution and movements, satellite telemetry data, parasite and contamination loads, and feeding habits can provide evidence of ecological partitioning (Wang 2002, Taylor 2005). Recently, chemical approaches, such as fatty acid profiles (Smith et al. 1996, Herman et al. 2005), elemental signatures (Born et al. 2003) and stable isotopes (Born et al. 2003, Herman et al. 2005), have become a common tool for stock identification in marine organisms, especially vertebrates. Isotope compositions have been proposed as a tool for population identification in cetaceans such as bottlenose dolphins (*Tursiops truncatus*, Borrell et al. 2006, Barros et al. 2010) and minke whales (*Balaenoptera acutorostrata*, Born et al. 2003).

Because stable isotopes assimilate from prey into predator, distinct dietary preferences and the geographical distribution of isotope values (e.g. isotopic maps or *isoscaples*, Graham et al. 2010) lead to differences in isotopic values in predator's tissues (Newsome et al. 2010) that can indicate niche separation and, thus, ecological stocks. The nitrogen and carbon stable isotope compositions in a consumer's tissues are typically more enriched in ^{13}C and, especially in ^{15}N with respect to their prey (Hobson 1999, Kelly 2000, Rubenstein & Hobson 2004). Typically, the ^{13}C enrichment per trophic level is 1‰ (De Niro & Epstein 1978, Peterson and Fry 1987) while an approximate ^{15}N enrichment of 3.4‰ is generally accepted (Post 2002). Due to this difference in fractionation, carbon isotopes are used as an indicator of the sources at the base of the food web where they feed (Hobson 1999, Graham et al. 2010) while nitrogen isotopes are mainly used for estimating the trophic position (De Niro & Epstein 1981, Cabana & Rasmussen 1996, McCutchan et al. 2003).

The measured levels of ^{13}C and ^{15}N in an organism reflect the trophic dynamics as well as the isotopic baseline of the habitat or ecosystem. Baseline carbon isotope values result from combination of producers with different photosynthetic pathways (Fry & Sherr 1984) and, in phytoplankton-based food webs, carbon isotopes values can vary in relation to cell physiology, morphology, growth and the source of inorganic carbon (Michener & Kaufman 2007). In general, major aquatic habitat types have distinct $\delta^{13}\text{C}$ values (seagrass habitat > kelp forest > nearshore marine > offshore marine > freshwater, Fry & Sherr 1988, Clementz & Koch 2001, Michener & Kaufman 2007). Furthermore, productivity, dissolved CO_2 concentration, and bicarbonate utilization could shape different carbon isotopic patterns in the ocean (Clementz & Koch 2001). Consequently, predators foraging within the same hydrological features would retain the same $\delta^{13}\text{C}$ values (Jaeger et al. 2010).

Spatial patterns in coastal basal nitrogen isotopes values are more complex, mainly reflecting contributions of potential sources of nitrogen (deepwater nitrate, nitrogen fixation, and natural and anthropogenic continental inputs) and differences in nitrate utilization (see review by Montoya 2007).

The franciscana (*Pontoporia blainvillei*) is a small dolphin endemic of coastal/estuarine waters of the western South Atlantic Ocean. Its occurrence is well documented throughout its range, which extends from Itaúnas in Espírito Santo, Brazil, to Golfo Nuevo in Chubut, Argentina (Crespo et al. 1998, Siciliano et al. 2002). Its distribution is not continuous, with two hiatuses being reported: one between Regência (19°40'S) and Barra de Itabapoaba (21°18'S) in Espírito Santo State, and the other between Macaé (22°25'S) and Ubatuba (23°20'S) (Siciliano et al. 2002), although records up to Ilha Grande (23°01'S) in Rio de Janeiro State were reported (Azevedo et al. 2002).

The species is especially vulnerable to incidental catch in gillnets (Corcuera 1994, Secchi et al. 1997, Di Benedetto & Ramos 2001, Bertozzi & Zerbini 2002, Rosas et al. 2002, Secchi et al. 2003b, Cappozzo et al. 2007, Franco-Trecu et al. 2009, Secchi 2010). As a result, the franciscana has been recently included in the “Vulnerable” category of the International Union for the Conservation of Nature and Natural Resources Red List (IUCN 2010).

In order to evaluate the potential of these threats on a fine scale, Secchi et al. (2003a) applied the phylogeographic concept (Dizon et al. 1992) for identifying franciscana stocks. By combining available information on distribution, population response, genetics and morphometrics, four Franciscana Management Areas (FMA) were provisionally proposed. FMA I comprises Rio de Janeiro and Espírito Santo States, Brazil; FMA II ranges from São Paulo to Santa Catarina States, Brazil; FMA III includes Rio Grande do Sul State, Brazil and Uruguay; and FMA IV covers the species range in Argentine waters (Figure 1). However, refinement is needed to determine the boundaries or the existence of further structuring, especially in FMA IV where data are scarce. Furthermore, the confirmation of the northern hiatus in the species distribution with increased survey effort combined with biological differences between the Rio de Janeiro and Espírito Santo states might point to the splitting of FMA I into two management areas.

The small home range and resident pattern of franciscanas were documented in only a few areas throughout its distribution (Babitonga Bay, Santa Catarina, Cremer and Simões-Lopes 2005, Buenos Aires, Bordino et al. 2008). As demonstrated by Bassoi (2005), who conducted a comprehensive analysis of the feeding habits of the species in southern Brazil, this resident pattern, small home range and opportunist feeding habit is reflected in different prey composition among its populations, even in contiguous areas.

Moreover, the geographic distribution of the species covers a wide range of oceanographic conditions, food webs and dominant prey. This variation should reflect in unique stable isotope compositions among franciscanas from different areas along the distribution range. By sampling a tissue with little or no turnover, it is possible to obtain a long-term isotopic composition. Teeth may be an ideal tissue because they form a permanent record and are composed of incremental layers that are deposited until death; thus, the diet over an individual's lifetime can be assessed (Walker et al. 1999, Walker & Macko 1999, Niño-Torres et al. 2006). In this paper, we hypothesize that geographical differences in baseline stable isotope compositions, combined with the diet preferences of franciscanas from different areas the species distribution range will be reflected in differing stable isotope compositions in teeth which in turn would represent ecological stocks.

MATERIAL AND METHODS

Study area and samples

Teeth for isotopic analysis were collected from 176 franciscanas washed ashore or incidentally captured in fishing nets along the coasts of Brazil, Uruguay and Argentina, covering the entire distributional range of the species. Areas sampled included the marine coasts of Espírito Santo (ES, n=10), northern Rio de Janeiro (NRJ, n=35), northern São Paulo (NSP, n=20) and southern São Paulo/Northern Paraná (SP/PR, n=19), the Babitonga Bay, northern Santa Catarina (SC, n=7), the adjacent coastal areas of the Patos Lagoon estuary, southern Rio Grande do Sul (RS, n=51), the La Plata River estuary and adjacent coastal waters, Uruguay (URU, n=17) and coastal waters of southern Buenos Aires, including three samples from northern Rio Negro, Argentina (ARG, n=17) (Figure 1, Table 1).

Age estimation

Ontogenetic diet changes (Zhao et al. 2004, Mendes et al. 2007), different growth rates (Newsome et al. 2009) and/or lactation (Walker & Macko 1999, Auriolles et al. 2006, Knoff et al. 2008, Newsome et al. 2009) may influence stable isotopes compositions. Therefore, in order to exclude possible lactating individuals (<9 months of age, Brownell 1989, Rodriguez et al. 2002) and to analyze the relationship between age and isotopes values, ages were estimated through the number of growth layer groups (GLGs, Perrin & Myrick 1980) in the dentin and cementum. Teeth were processed following established procedures (Pinedo & Hohn 2000). One clean tooth of each individual was fixed in 10% formalin, decalcified with RDO® (a commercial mixture of acids), sectioned to 25µm using a freezing microtome, stained in Mayer's hematoxylin, "blued" in ammonia, dehydrated in glycerin and mounted on a microscope slide in 100% glycerin. Age estimation was performed without reference to biological data, such as length or sex. Each GLG was considered to represent one year of age (Pinedo & Hohn 2000).

Sample preparation and stable isotope analyses

One cleaned tooth of each animal was crushed to a thin powder, acidified with 30% HCl and dried. Approximately 5 mg of residual acidified tooth powder were used for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ analyses using an elemental analyzer (EA) connected to a Isotope Ratio Mass Spectrometer. Natural abundance of stable isotope ratios ($^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$) are expressed in a delta notation (δ) as per mil enrichment (‰) when compared with international standards (e.g. Pee Dee Belemnite for carbon and atmospheric N_2 for nitrogen). Results were expressed as:

$$\delta X = [(R_{\text{sample}}/R_{\text{standard}}) - 1] * 1000$$

where R_{sample} and R_{standard} are the $^{13}\text{C}/^{12}\text{C}$ or $^{15}\text{N}/^{14}\text{N}$ ratios of the sample and standard, respectively.

Statistical analysis

All results are presented as the mean $\pm$ SD. Data were tested for normality and homogeneity of the variances using Kolmogorov-Smirnov and Levene's test, respectively. The difference in the mean $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values between males and females were tested using the Student-t test. Relationships between $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ and age or latitude were evaluated through non-parametric Spearman regression analysis. Differences in isotopes ratios among areas were investigated using 1-way ANOVA followed by a Tukey's HSD test. Null hypothesis was rejected if $p < 0.05$. Dual stable isotope plots of carbon versus nitrogen were used to visualize geographical variations on teeth isotopic values.

Isomaps

To allow a visualization of the latitudinal trends in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ within the distributional range of the franciscanas *isomaps* were constructed using the geographic information system software ArcMAPTM 9.3.1 (ESRI). The sampling locations/sites were geo-referenced and interpolations were conducted by ordinary kriging methods with spherical models, utilizing the Spatial Analyst. Due to the costal habitat of the franciscana (Danilewicz et al. 2009), the interpolation was delimited by an area of 100 km from the coast.

RESULTS

No significant differences were found between sexes for $\delta^{13}\text{C}$ or $\delta^{15}\text{N}$ (Student's t-tests, $p > 0.05$), with the exception of the higher $\delta^{15}\text{N}$ found in males ($n=10$; 17.4‰) than

in females (n=9; 16.5‰) from SP/PR (Student's t-test, $p<0.05$). Since this was the only difference found, tests among areas were performed with sexes combined.

Age was not correlated with $\delta^{13}\text{C}$ or $\delta^{15}\text{N}$ values in most areas (Spearman's correlation tests, $p<0.05$). However, $\delta^{13}\text{C}$ was positively correlated with age in N SP (Spearman's correlation coefficient, $\rho = 0.6$, $p<0.05$), SP/PR (Spearman's correlation coefficient, $\rho = 0.9$, $p<0.05$) and ARG (Spearman's correlation coefficient, $\rho = 0.5$, $p<0.05$). On the other hand, $\delta^{15}\text{N}$ was negatively correlated with age in ES (Spearman's correlation coefficient, $\rho = -0.8$, $p<0.05$) and ARG (Spearman's correlation coefficient, $\rho = -0.5$, $p<0.05$) (Figure 2).

Correlation between $\delta^{13}\text{C}$ and latitude, was positive and significant (Spearman's correlation coefficient, $\rho = 0.63$, $p<0.05$). However, minimum and maximum values were found in intermediate areas (minimum of $-13.9 \pm 0.7\text{‰}$ in N RJ, maximum of $-10.7 \pm 0.57\text{‰}$ in RS, Table 2, Figure 3). The lowest ($14.4 \pm 0.66\text{‰}$) and the highest ($20.6 \pm 1.24\text{‰}$) mean $\delta^{15}\text{N}$ values were found the northernmost and the southernmost areas, respectively (Table 2). Indeed, $\delta^{15}\text{N}$ was highly and linearly correlated with latitude (Spearman's correlation coefficient, $\rho = 0.86$, $p<0.05$) (Figure 3). These latitudinal gradients in isotope values could be clearly seen in isoscape maps drawn from interpolated $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values (Figure 4)

Based on $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ scatter plot, five groups could be recognized (Figures 5 and 6). Individuals from ES clustered together and highly separated from the southern adjacent area of NRJ. Franciscanas from this latter region were, in turn, separated from the cluster formed by individuals from NSP, SP/PR and SC. Dolphins from RS and URU clustered together with the highest carbon isotopes values while ARG samples formed a separate group with the highest nitrogen isotopes values. Areas differed

significantly with respect to $\delta^{13}\text{C}$ (ANOVA, $F_{7,168}=91.30$, $p<0.001$) and $\delta^{15}\text{N}$ (ANOVA, $F_{7,168}=87.34$, $p<0.001$) (Tables 2 and 3).

DISCUSSION

Stable isotope compositions revealed distinct location-specific values that might represent discrete ecological groups of franciscanas. Similarities in teeth $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values clustered individuals into five groups: ES, NRJ, a group composed of individuals from NSP, SP/PR and SC, another group with RS and URU samples and finally, ARG specimens clustered together in a single group. These differences in stable isotope compositions may be significantly influenced by a variation in the stable isotope values at the base of the food web, as well as differences in the specie's prey preferences among areas. Additionally, non-dietary factors such as age (Zhao et al. 2004, Mendes et al. 2007), nutritional status and growth rate (Newsome et al. 2009) can especially influence nitrogen and, to a lesser extent, carbon isotope values. Nitrogen isotopes were negatively correlated with age in ES and ARG. Higher $\delta^{15}\text{N}$ relative to the mother's signal is expected for suckling calves because mothers are catabolizing their own tissues for producing milk, which leads to a higher "trophic level" of their offspring (Walker & Macko 1999, Knoff et al. 2008, Newsome et al. 2009). As franciscanas are believed to be weaned before the first year (Brownell 1989, Rodriguez et al. 2002), by sampling the whole tooth of a 1 yr-old dolphin, a considerable proportion of dentine formed during lactation is included, thus a high $\delta^{15}\text{N}$ is expected. Samples from ES and ARG were composed by a high percentage of 1yr-old individuals (30 and 50%, respectively, Table 1) which could explain the observed significant correlation with age. As for carbon isotopes, a positive correlation with age was found in NSP, SP/PR and ARG. As in the case of nitrogen, lactating calves usually present lower $\delta^{13}\text{C}$ value than their mothers which is probably influenced by the high lipid content and consequent ^{13}C depleted milk

(Hobson & Sease 1998, Newsome et al. 2006, Newsome et al. 2009). This could be a possible cause for the correlation found in ARG, but not for NSP and SP/PR samples, as they were composed by >1yr old animals. Nevertheless, excluding 1 yr-old samples from ES and ARG did not change their mean $\delta^{15}\text{N}$ values (Students' t-tests, $p=0.08$ and $p=0.7$, respectively), nor $\delta^{13}\text{C}$ values in ARG (Students' t-test, $p=0.14$). As for NSP and SP/PR, an ontogenetic change in diet composition with age and/or foraging areas might explain the age-related increase in $\delta^{13}\text{C}$ values, though more data are needed to elucidate this issue.

In SP/PR male franciscanas presented $\delta^{15}\text{N}$ values 1‰ higher than females, indicating that a resource partitioning between sexes might take place in this area. Information on franciscana diet from this region is scarce, however, a study based on contents of 29 stomachs indicate that 2-5yr-old females consume more cephalopod prey than males of the same age (Oliveira 2003) which might be the cause for the higher nitrogen values found in the latter.

Geographical variations in $\delta^{13}\text{C}$

The isomap created from interpolated $\delta^{13}\text{C}$ values found in franciscanas teeth suggests a clear influence of different hydrographical features in the distribution of carbon values. Indeed, the most depleted mean $\delta^{13}\text{C}$ value was found in NRJ and the highest in the two main estuarine areas, RS and URU. Dolphins from ES, NSP, SP/PR, SC and ARG shared similar carbon values ($\sim 12\text{‰}$), suggesting carbon sources derived from phytoplankton-based coastal food webs (Burton & Koch 1999, Lesage et al. 2001).

The lowest $\delta^{13}\text{C}$ found in dolphins from NRJ could be due to the presence of a source of freshwater organic matter, which are usually ^{13}C depleted, entering the coastal food web. The Paraíba do Sul river drains a mean of $796 \text{ m}^3 \cdot \text{s}^{-1}$ to this region (Nicolite

et al. 2009). However, similar freshwater inputs from the Doce River (Coelho 2006) seem not to be reflecting in low $\delta^{13}\text{C}$ values in franciscanas from ES. An alternative hypothesis would be that franciscanas from NRJ had a different feeding habitat as depleted $\delta^{13}\text{C}$ values are expected for an offshore feeding (Botta et al. *in press*). Nearshore, benthos-linked food webs are more ^{13}C enriched compared to more offshore, pelagic food webs (Burton & Koch 1999, Clementz & Koch 2001). Although franciscanas prey mostly upon bottom-dwelling juvenile teleosts, in NRJ two pelagic fish species are the second and third most important prey in their diet (Table 4). The ingestion of these pelagic prey and the relatively high importance of pelagic squids in their diet could be the cause of the lower $\delta^{13}\text{C}$ values found in this area. Even though geographically close, franciscanas from ES have a slightly different diet from NRJ (Table 4). In ES, the pelagic zooplanktivore *Pellona harroweri* is the most important prey species, followed by demersal fishes and a demersal-pelagic fish, *Trichiurus lepturus* (Araujo et al. 2010). Furthermore, despite the reduced sample size no squids were reported as franciscana prey in this area (Araujo et al. 2010). In spite of this variation in prey preferences, oceanographic habitat differences are the most likely explanation for the difference found in isotopic values between ES and NRJ. Both areas are separated by the Vitória-Trindade Chain (VTC, 20°S), south of which upwelling cells and plumes of nutrient-rich South Atlantic Central Waters (SACW T-S=5-20°C, 34-36, Stramma and England 1999) are frequent (Rodrigues & Lorenzzetti 2001, Calado et al. 2010). Therefore, the boundaries of these two areas coincide with a transition of environmental conditions that change from oligotrophic tropical waters found in ES to naturally eutrophized waters due to upwelling processes in NRJ (Rodrigues & Lorenzzetti 2001, Calado et al. 2010). Isotopic values at the base of food webs in upwelling ecosystems vary according to a cyclic change in available nutrients

and carbon sources influencing the overall values in upper trophic levels (O'Reilly et al. 2002). In the case of carbon, the upwelling of a ^{13}C -depleted deep water (Pierre et al. 1991) eventually alter basal $\delta^{13}\text{C}$ values, labeling the entire food web with lighter isotope values (O'Reilly et al. 2002, Hill et al. 2006). Although in a resident species, such as the franciscana, isotopic changes between upwelling and non-upwelling seasons would be integrated over the entire life of the individual due to the slow turnover of a hard tissue such as teeth, a distinct isotopic signal could still be identified in NRJ individuals. Whichever the causes of the low- $\delta^{13}\text{C}$ are, isotope values were very similar to those found in Guiana dolphins incidentally killed in NRJ area (this thesis, Annex I), despite main prey differ between these dolphin species (Di Benedetto & Ramos 2004), which indicate a strong influence of hydrological regimes in stable isotope values of high trophic level predators.

In areas further south (NSP, SP/PR and SC) and in ARG stable carbon isotope compositions ($\sim -12\text{‰}$) are similar to ES and to those of other coastal cetaceans elsewhere (e.g. *Tursiops truncatus*, Díaz-Gamboa 2003, and *Delphinus capensis*, Niño-Torres et al. 2006, from the Gulf of California, *Tursiops truncatus* from Florida, Barros et al. 2010). Indeed, all samples from NSP and SP/PR came from animals incidentally captured in the coastal waters of São Paulo and Paraná. However, franciscanas from SC were found stranded in the Babitonga Bay area, thus a more freshwater influenced carbon signal was expected for these samples as river-transported organic matter of terrestrial origin is typically ^{13}C depleted (Schell et al. 1998). Franciscanas have a marked preference for the innermost portions of the bay (Cremer and Simões-Lopes 2005). In these inner areas, Barros et al. (2010) found high salinity values (32-34) and $\delta^{13}\text{C}$ POC values typical of oceanic samples (~ -22 to -23‰), which possibly explain the high $\delta^{13}\text{C}$ values found in franciscanas.

High values of carbon isotopes were found in the two main estuarine areas (RS and URU). These estuarine regions are characterized by extensive salt-marshes composed of ^{13}C -enriched C_4 producers such as *Spartina* and *Ruppia* (Isacch et al. 2006). In RS, the important contribution of these marsh plants to the food web of the Patos Lagoon estuary was stressed by Abreu et al. (2006) and Garcia et al. (2007). Although franciscanas do not enter this estuary, sympatric bottlenose dolphins (*Tursiops* sp.) that do feed inside the estuary presented identical carbon values (Botta et al. *in press*). Estuarine ^{13}C -enriched food web could influence the isotopic composition of adjacent waters due to the interaction between those habitats through processes of production, transport of matter and organisms and migrations (Seeliger 2001). However, no data on stable isotopes of the food chain of the coastal waters adjacent to the estuary are available, thus precluding further interpretations. On the other hand, franciscanas in Uruguay inhabit the La Plata river estuary and adjacent coastal marine areas and their carbon isotope values reflect the influence of the high $\delta^{13}\text{C}$ inherited from salt-marshes from this estuary and other minor coastal creeks (Isacch et al. 2006). In agreement to this, Franco-Trecu (2010) estimated a mean $\delta^{13}\text{C} = -16\text{‰}$ in Uruguayan coastal fish. After accounting for a tissue-dependent $\Delta^{13}\text{C}$ collagen-muscle ($\sim 4\text{‰}$) (Koch 2007) this is similar to the mean value found for franciscanas (-11‰).

Geographical variations in $\delta^{15}\text{N}$

$\delta^{15}\text{N}$ values were lowest in ES (14‰), increasing with latitude up to a highest mean value of 21‰ found in ARG. This pattern could be interpreted as a variation in prey composition and, consequently, trophic level of franciscana dolphins or a different contribution of pelagic vs. benthic prey. In this context, dolphins from ES would be consuming lower trophic level/more pelagic prey than dolphins from the other areas. Conversely, despite the pelagic fish *Pellona harroweri*, the remaining main prey are all

demersal fish and no cephalopod prey have so far been reported for this area. Franciscanas from NRJ prey considerably upon pelagic fish and cephalopods, however, their nitrogen values do not significantly differ from NSP, SP/PR or SC dolphins, which prey mostly on demersal fish and cephalopods. Highest $\delta^{15}\text{N}$ values were found in the southernmost areas (RS, URU and ARG) despite the contribution of small pelagic prey such as *Anchoa mardinii* and *Engraulis anchoita*, and the cephalopod *Loligo sanpaulensis*. Although differences in prey composition should be accounting for some of the observed variation, isotopically distinct local food webs must be the most probable explanation.

The Coastal Water (CW) receiving low $\delta^{15}\text{N}$ nutrients from continental sources and the presence of the oligotrophic Tropical Water (TW), carried by Brazilian Current (BC) (Knoppers et al. 2009), might be the cause of the relatively low $\delta^{15}\text{N}$ values found in teeth of franciscanas from ES. N_2 -fixation by cyanobacteria and recycling N (low $\delta^{15}\text{N}$) are important in oligotrophic regions (Checkley Jr & Miller 1989, Sigman et al. 2009, Landrum et al. 2011). Indeed, $\delta^{15}\text{N}$ value from nitrogen fixed by diazotrophs ranges from -1 to -2‰ (McClelland et al. 2003, Montoya et al. 2002) as there is little isotopic fractionation during its uptake by phytoplankton (Minagawa & Wada 1984, Montoya 2007).

Franciscanas from NRJ, NSP, SP/PR and SC presented intermediate nitrogen isotopes values (~16-17‰). The region covered by these areas is frequently fertilized by the SACW reaching the coastal euphotic zone, especially in the NRJ area (Castro-Filho et al. 1987, Gaeta & Brandini 2006, Brandini et al. 2007). Deep waters like SACW carry nitrate that is typically ^{15}N -enriched (~5‰, Sigman et al. 2009). This seasonal influence of high $\delta^{15}\text{N}$ nitrate changes the trophic structure due to different nutrient

sources and species` succession (O'Reilly et al. 2002, Bode et al. 2003, 2004, Sigman et al. 2009), thus labeling consumers with high $\delta^{15}\text{N}$ values.

Highest nitrogen isotope values were found in the southern areas (RS, URU and ARG). RS and URU are seasonally influenced by an important freshwater discharge from the La Plata River and, to a lesser extent, the Patos-Mirim lagoon complex. This coastal zone is also influenced by the convergence of Subantarctic Shelf Waters (SASW), transported by the Malvinas current, and Tropical and Subtropical (Central) Waters (STCW), from the Brazil current, which determine nutrients concentration and biological productivity (Ciotti et al. 1995, Seeliger et al. 1996). High nitrogen isotope values were also found in bottlenose dolphins (*Tursiops* sp.) from this region (Botta et al. *in press*) thus special oceanographic conditions must be sources of high $\delta^{15}\text{N}$ nutrients fertilizing coastal food webs.

ARG samples collected in southern Buenos Aires province, presented the highest $\delta^{15}\text{N}$ values. This region is under influence of the El Rincón estuarine complex (Acha et al. 2004) and continental shelf waters (Lucas et al. 2005), the latter resulting from modified nutrient-rich subantarctic waters (Guerrero & Piola 1997, Lucas et al. 2005). Lara et al. (2010) characterizing carbon and nitrogen isotope compositions of POM along a transect from southern Buenos Aires to Antarctica, found the highest values in the former. This high N isotopic signature found was attributed to a combination of different sources of N supporting primary production (^{15}N -enriched nitrate pool and low $\delta^{15}\text{N}$ ammonium excreted by consumers). The uptake of nitrate is complete in regions where its concentration is low, thus increasing the $\delta^{15}\text{N}$ value of the phytoplankton, approaching the value of the nitrate pool (Graham et al. 2010).

Finally, inputs of high $\delta^{15}\text{N}$ to coastal waters worldwide derive from anthropogenic sources like domestic and industrial sewage (Kendall et al. 2007). Nitrogen isotope content of wastewater has high $\delta^{15}\text{N}$ values due to ammonium volatilization and denitrification processes during sewage treatment that removes the lighter ^{14}N faster than the ^{15}N (Macko & Ostrom 1994, McClelland et al. 1997). In addition, stormwater may also be considered as an enrichment factor, due to the thermodynamically favored volatilization of its isotopically depleted $^{14}\text{NH}_3$ as it flows across warm surfaces (Dillon & Chanton 2007). Bisi et al. (*submitted*), Corbisier et al. (2006), Barros et al. (2010) and Abreu et al. (2006) already attributed high $\delta^{15}\text{N}$ values in coastal and estuarine food webs along the Brazilian coast (Rio de Janeiro, São Paulo, Babitonga Bay in Santa Catarina and the Patos Lagoon estuary in Rio Grande do Sul, respectively) to anthropogenic inputs. Indeed, this input of high $\delta^{15}\text{N}$ must certainly contribute to the nitrogen isotope values found, however, highest ^{15}N -enriched values do not correspond to highest values of pollution reported. For example, the Baixada Santista estuarine complex in NSP area, is the most polluted region along the Brazilian Atlantic coast, mainly due to domestic and industrial wastes. However, $\delta^{15}\text{N}$ values found in this region were not the highest. Therefore, although anthropogenic sources might be playing a role in elevating the natural $\delta^{15}\text{N}$ values of local food webs, differences in hydrogeomorphologic patterns might be the major functional reasons for the latitudinal variation observed in nitrogen isotopes values.

Stock identification

The separation of franciscanas into five groups based on stable carbon and nitrogen isotope compositions is consistent with the Franciscana Management Areas proposed by Secchi et al. (2003a). The exception was the FMA I which can be split into two areas (ES and NRJ) according to their highly differing isotope compositions. This was

somewhat expected due to the existence of a gap between these areas (Siciliano et al. 2002) and the decision of including these two areas into one single stock was only because there were not sufficient biological data for ES (Secchi et al. 2003a). Genetic (Cunha et al. *in prep.*) and morphometric (Ramos et al. 2002) evidences strongly support splitting these two areas.

The phylogeographic approach considers the three central areas (NSP, SSP and SC) as one single stock (FMA II). Franciscanas from these areas are the smallest found along the species distribution (Ramos et al. 2002, Barreto & Rosas 2006) and genetic data demonstrate that animals from these locations are highly different from the adjacent FMAI and FMAIII (Valsecchi & Zanelatto 2003, Ott 2002, Cunha et al. *in prep.*). Our stable isotope analyzes corroborate those evidences as detected carbon and nitrogen isotope values were more similar among areas within FMAII than between any of the regions in this area and adjacent regions.

Samples from RS and URU were undistinguishable in terms of their isotope compositions, but were different from ARG values (Figures 5 and 6). Franciscanas from ARG were considered as a separate Management Area by Secchi et al. (2003a). Despite some ecological differences such as feeding and reproductive ecology (Rodriguez et al. 2002, Danilewicz et al. 2002), their separation was considered weak, and the splitting was mostly due to a pragmatic political separation in order to apply management action on a local basis. Although control region genetic data analyzed by Ott (2002) and Lazaro et al. (2004) suggested that all putative populations between Argentina and southern Brazil belong to a single genetic population, studies taken place in a finer scale inside this region found strong evidence of population structuring. Costa et al. (2010) and Mendez et al. (2010) found a significant genetic structuring among individuals from Uruguayan and Argentinean Rio de La Plata estuary in regard to contiguous Atlantic

Ocean. Both studies postulated that environmental factors and niche specialization are probable causes for the observed structuring. In agreement to this finding, Barbato et al. (*in press*) reported that animals from the Rio de La Plata estuarine region in Argentina were more similar in external morphology to NRJ (FMAI) than to neighboring RS (FMAIII) dolphins. Samples from Uruguay analyzed in the present study were obtained from animals incidentally killed or washed ashore in both the La Plata river estuary and the coastal marine areas. However, limited samples from of the estuarine specimens precludes us to further verification if this difference is also detectable in stable isotopes. Stable isotope values analyses of genetically identified individuals from both populations (environments) are recommended.

This study demonstrate further evidence that carbon and nitrogen stable isotope compositions measured in a slow turnover-tissue such as teeth is a valuable tool in identifying ecological stocks of resident/small home range cetaceans as long as the distributional range of the species includes marine environments with differing conditions and/or food web structures (trophic dynamics).

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Figure 1. Study areas of franciscana dolphins, *Pontoporia blainvillei*, from Brazil, Uruguay and Argentina.

Figure 2. Age in relation to $\delta^{13}\text{C}$ (open squares) and $\delta^{15}\text{N}$ (closed circles) values for teeth dentine of franciscanas (*Pontoporia blainvillei*) from Espirito Santo (ES), northern Rio de Janeiro (N RJ), northern São Paulo (N SP), southern São Paulo/northern Paraná (SP/PR), Babitonga Bay, northern Santa Catarina (SC), southern Rio Grande do Sul (RS), Uruguay (URU) and Argentina (ARG).

Figure 3. Mean (symbols) and $\pm\text{SD}$ (whiskers) $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values for teeth dentine of franciscana dolphins, *Pontoporia blainvillei*, in relation to latitude: Espirito Santo (ES), northern Rio de Janeiro (N RJ), northern São Paulo (N SP), southern São Paulo/northern Paraná (SP/PR), Babitonga Bay, northern Santa Catarina (SC), southern Rio Grande do Sul (RS), Uruguay (URU) and Argentina (ARG).

Figure 4. Calculated $\delta^{13}\text{C}$ (a) and $\delta^{15}\text{N}$ (b) isoscapes after interpolation from the isotopic values of franciscana (*Pontoporia blainvillei*) teeth. Espirito Santo (ES), northern Rio de Janeiro (N RJ), northern São Paulo (N SP), southern São Paulo/northern Paraná (SP/PR), Babitonga Bay, northern Santa Catarina (SC), southern Rio Grande do Sul (RS), Uruguay (URU) and Argentina (ARG).

Figure 5. Mean (symbols) and $\pm\text{SD}$ (whiskers) $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values for teeth dentine of franciscanas (*Pontoporia blainvillei*) from Espirito Santo (ES), northern Rio de Janeiro (N RJ), northern São Paulo (N SP), southern São Paulo/northern Paraná (SP/PR), Babitonga Bay, northern Santa Catarina (SC), southern Rio Grande do Sul (RS), Uruguay (URU) and Argentina (ARG).

Figure 6. Teeth $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of franciscanas (*Pontoporia blainvillei*) from Espirito Santo (ES), northern Rio de Janeiro (N RJ), northern São Paulo (N SP),

southern São Paulo/northern Paraná (SP/PR), Babitonga Bay, northern Santa Catarina (SC), southern Rio Grande do Sul (RS), Uruguay (URU) and Argentina (ARG).

Table 1. Main characteristics of study areas: Espírito Santo (ES), northern Rio de Janeiro (N RJ), northern São Paulo (N SP), southern São Paulo/northern Paraná (SP/PR), Babitonga Bay, northern Santa Catarina (SC), southern Rio Grande do Sul (RS), Uruguay (URU) and Argentina (ARG).

	Ecosystems	Freshwater discharge (m³ s⁻¹)	Main Ocean Currents
ES	Estuaries	1139 ^a	Brazilian Current
	Reefs		
NRJ	Estuaries	796 ^b	Brazilian Current
	Coastal upwellings		
NSP	Estuaries	24-127 ^c	Brazilian Current
	Coastal upwellings		
SP/PR	Estuaries (bays and lagoons)	435 ^d	Brazilian Current
	Coastal upwellings		
SC	Estuaries (bays and lagoons)	<20 ^e	Brazilian Current
	Coastal upwellings		
RS	Estuaries	2000 ^f	Brazilian and Malvinas currents
	Salt marshes		
URU	Estuaries	23000 ^g	Malvinas current
	Salt marshes		
ARG	Estuaries	960 ^h	Malvinas current
	Salt marshes		

^aDoce River (Coelho 2006)

^bParaíba do Sul River (Nicolite et al. 2009)

^cBaixada Santista Estuary (Moser et al. 2005)

^dCananéia Estuary (Schaeffer-Noveli et al. 1990)

^eBabitonga Bay (Barros et al. 2010)

^fPatos Lagoon Estuary (Calliari et al. 2009)

^gLa Plata River Estuary (Piola & Romero 2004)

^hEl Rincón estuarine system (Acha et al. 2004)

Table 2. Mean $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values (‰) for teeth dentine of franciscanas (*Pontoporia blainvillei*) from Espirito Santo (ES), northern Rio de Janeiro (N RJ), northern São Paulo (N SP), southern São Paulo/northern Paraná (SP/PR), Babitonga Bay, northern Santa Catarina (SC), southern Rio Grande do Sul (RS), Uruguay (URU) and Argentina (ARG). Range of sample ages is expressed in years.

AREA	n	Ages	$\delta^{13}\text{C}$	SD	Min/Max	$\delta^{15}\text{N}$	SD	Min/Max
ES	10	1-7	-12.6	0.48	-13.3/-11.6	14.4	0.66	13.3/15.5
NRJ	35	3-9	-13.9	0.70	-14.8/-11.8	16.1	0.65	14.7/17.5
NSP	20	2-14	-11.9	0.53	-12.9/-10.7	16.1	0.99	14.2/17.8
SP/PR	19	2-18	-12.0	0.49	-13.2/-11.3	17.0	0.86	15.0/18.6
SC	7	1-10	-12.3	0.43	-13.2/-11.9	16.4	0.54	15.8/17.2
RS	51	4-12	-10.7	0.57	-12.3/-9.6	18.3	0.81	16.4/20.6
URU	17	1-7	-11.1	0.64	-12.1/-10.1	18.7	0.7	17.6/20.7
ARG	17	1-8	-12.0	0.60	-13.0/-10.7	20.6	1.24	18.8/23.3

Table 3. Tukey`s HSD test p values of multiple comparisons of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values for teeth dentine of franciscanas (*Pontoporia blainvillei*) from Espírito Santo (ES), northern Rio de Janeiro (NRJ), northern São Paulo (NSP), southern São Paulo/northern Paraná (SP/PR), Babitonga Bay, northern Santa Catarina (SC), southern Rio Grande do Sul (RS), Uruguay (URU) and Argentina (ARG). Up-right values from $\delta^{13}\text{C}$ comparisons and down-left values from $\delta^{15}\text{N}$ comparisons.

AREA	ES	NRJ	NSP	SP/PR	SC	RS	URU	ARG
ES		**	*	0.08	0.97	**	**	0.15
NRJ	**		**	**	**	**	**	**
NSP	**	1.00		1.00	0.71	**	**	1.00
SP/PR	**	*	*		0.87	**	**	1.00
SC	**	0.99	0.97	0.87		**	**	0.94
RS	**	**	**	**	**		0.39	**
URU	**	**	**	**	**	0.51		**
ARG	**	**	**	**	**	**	**	

* p<0.05

** p<0.001

Table 4. Main fish prey identified based on stomach content analysis for franciscanas (*Pontoporia blainvillei*): Espírito Santo (ES), northern Rio de Janeiro (NRJ), northern São Paulo (NSP), southern São Paulo/northern Paraná (SP/PR), Babitonga Bay, northern Santa Catarina (SC), southern Rio Grande do Sul (RS), Uruguay (URU) and Argentina (ARG). Main four fish species based on Index of Relative Importance (IRI) are listed.

Area		Main prey species	Prey habits	References
ES	Fish	<i>Pellona harroweri</i>	Pelagic	Araujo et al. 2010
		<i>Stellifer</i> sp.	Demersal	
		<i>Trichiurus lepturus</i>	Demersal- pelagic	
		<i>Stellifer brasiliensis</i>	Demersal	
NRJ	Squids	-		Bittar & Di Beneditto 2009
	Fish	<i>Stellifer</i> sp.	Demersal	
		<i>Anchoa filifera</i>	Pelagic	
		<i>Pellona harroweri</i>	Pelagic	
		<i>Isopisthus parvipinnis</i>	Demersal	
	Squids	<i>Loligo plei</i>		
<i>Loligo sanpaulensis</i>				
NSP		No information available		
SP/PR	Fish	<i>Pellona harroweri</i>	Demersal	Oliveira 2003
		<i>Isopisthus parvipinnis</i>	Demersal	
		<i>Stellifer</i> sp.	Demersal	
	Squids	<i>Lolliguncula brevis</i>		
		<i>Loligo plei</i>		
SC	Fish	<i>Stellifer rastrifer</i>	Demersal	Cremer 2007
		<i>Cetengraulis edentulus</i>	Pelagic	
		<i>Stellifer brasiliensis</i>	Demersal	
		<i>Lolliguncula brevis</i>		
RS	Squids	<i>Lolliguncula brevis</i>		Basso 2005
	Fish	<i>Cynoscion guatucupa</i>	Demersal	
		<i>Trichiurus lepturus</i>	Demersal- pelagic	
		<i>Paralonchurus brasiliensis</i>	Demersal	
		<i>Anchoa marinii</i>	Pelagic	
		<i>Loligo sanpaulensis</i>		
URU*	Squids	<i>Loligo sanpaulensis</i>		Görke et al. 2010
	Fish	<i>Anchoa marinii</i>	Pelagic	
		<i>Macrodon anticauda</i>	Demersal	
		<i>Porychthys porosissimus</i>	Demersal	
		<i>Urophycis brasiliensis</i>	Demersal	
ARG	Squids	<i>Loligo sanpaulensis</i>		Paso-Viola et al. 2010
	Fish	<i>Cynoscion guatucupa</i>	Demersal	
		<i>Trachurus lathami</i>	Demersal- pelagic	
		<i>Engraulis anchoita</i>	Pelagic	
	Squids	<i>Loligo sanpaulensis</i>		

*IRI not reported, thus prey are arbitrary ordered.

Figure 1

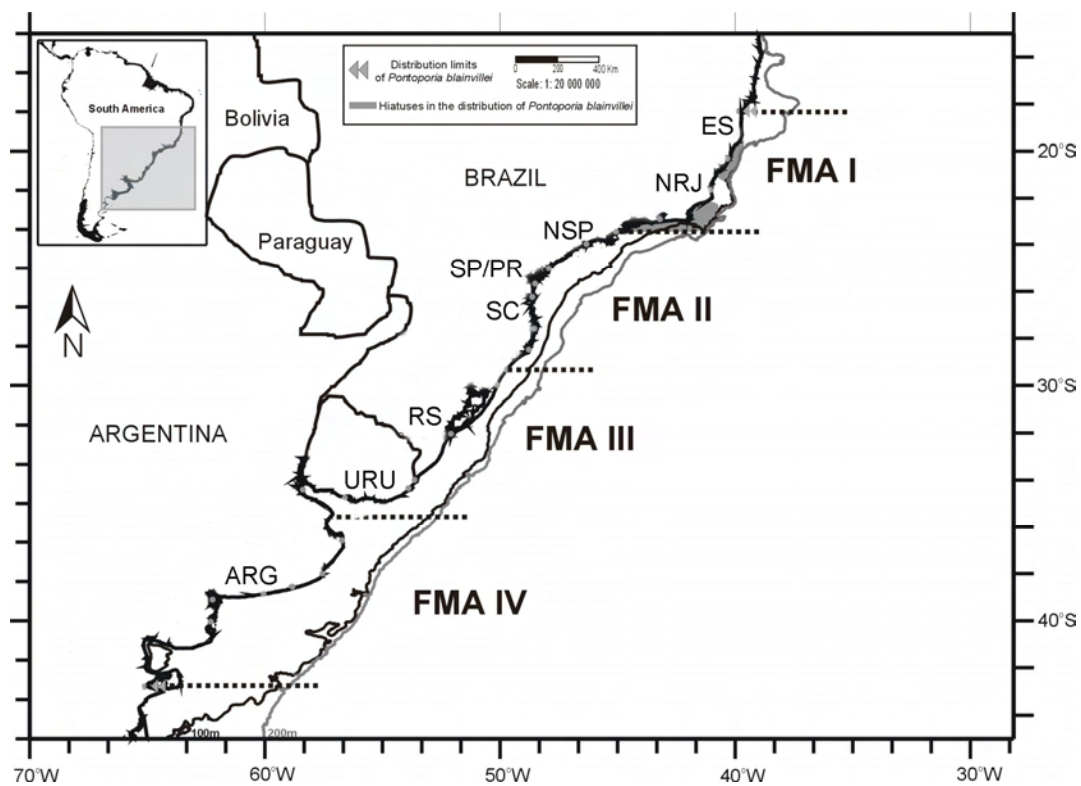


Figure 2.

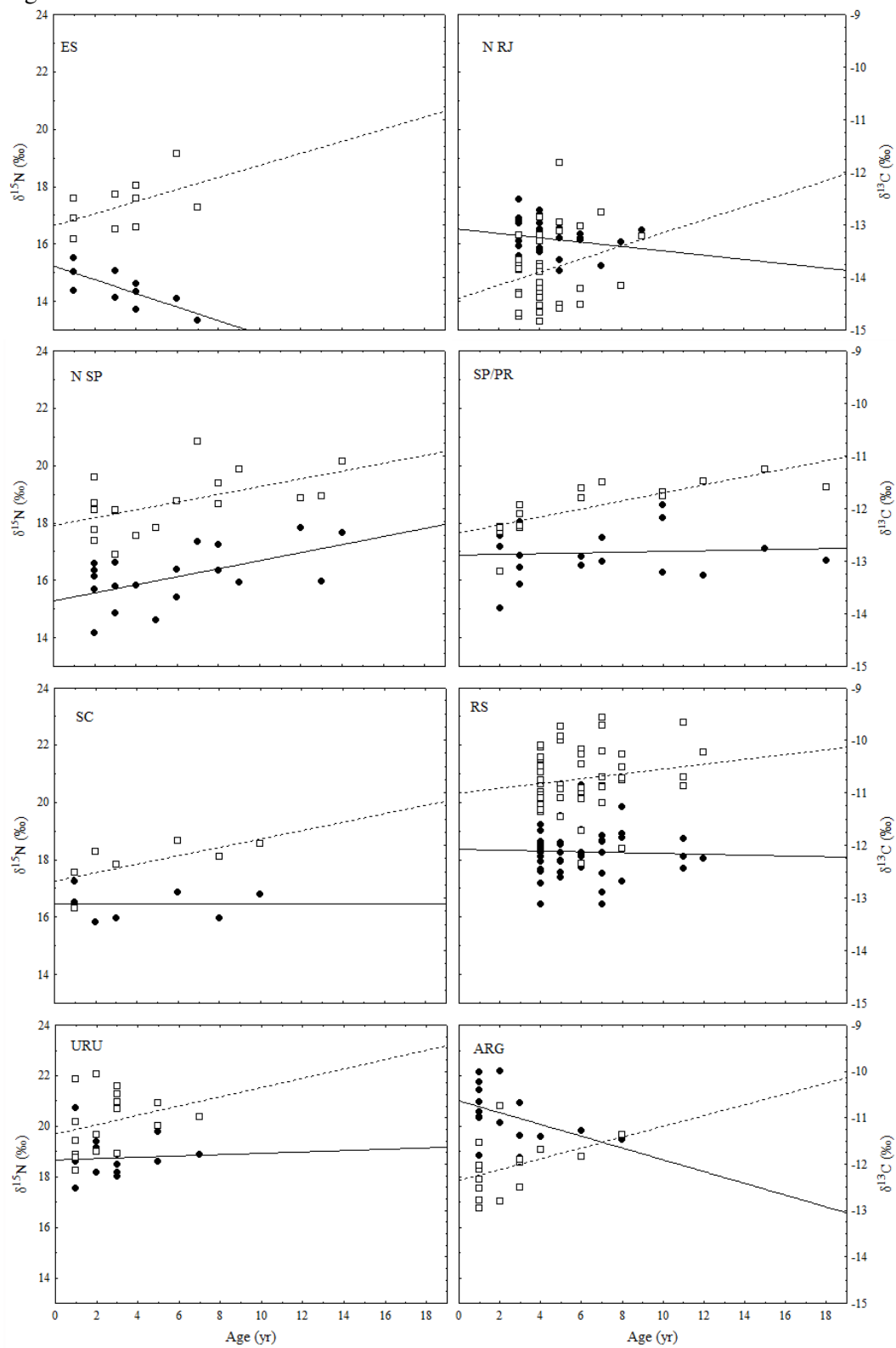


Figure 3.

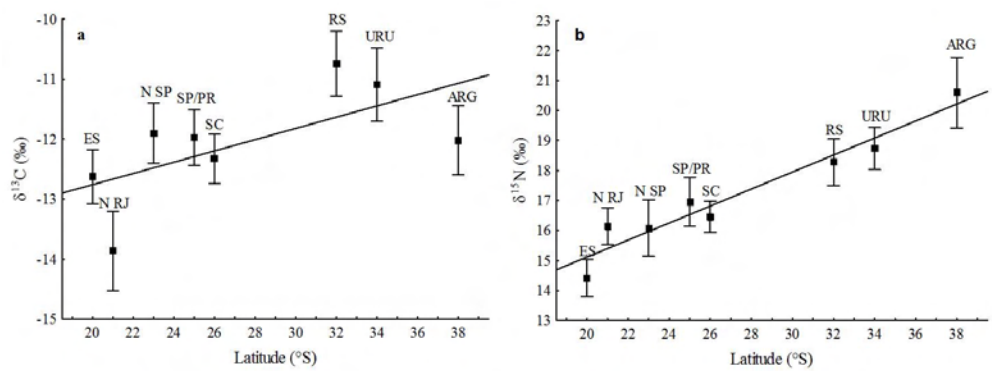
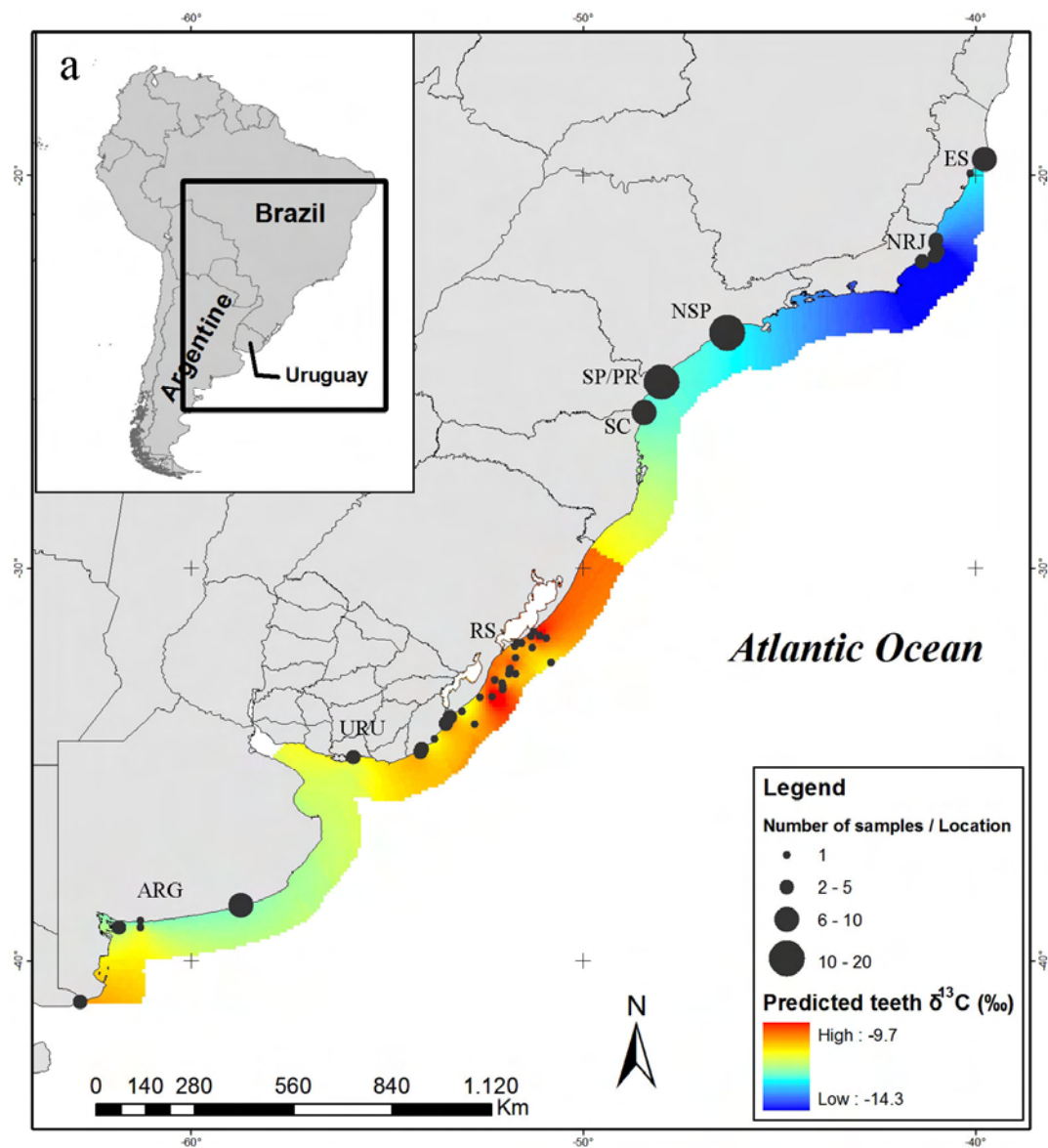


Figure 4.



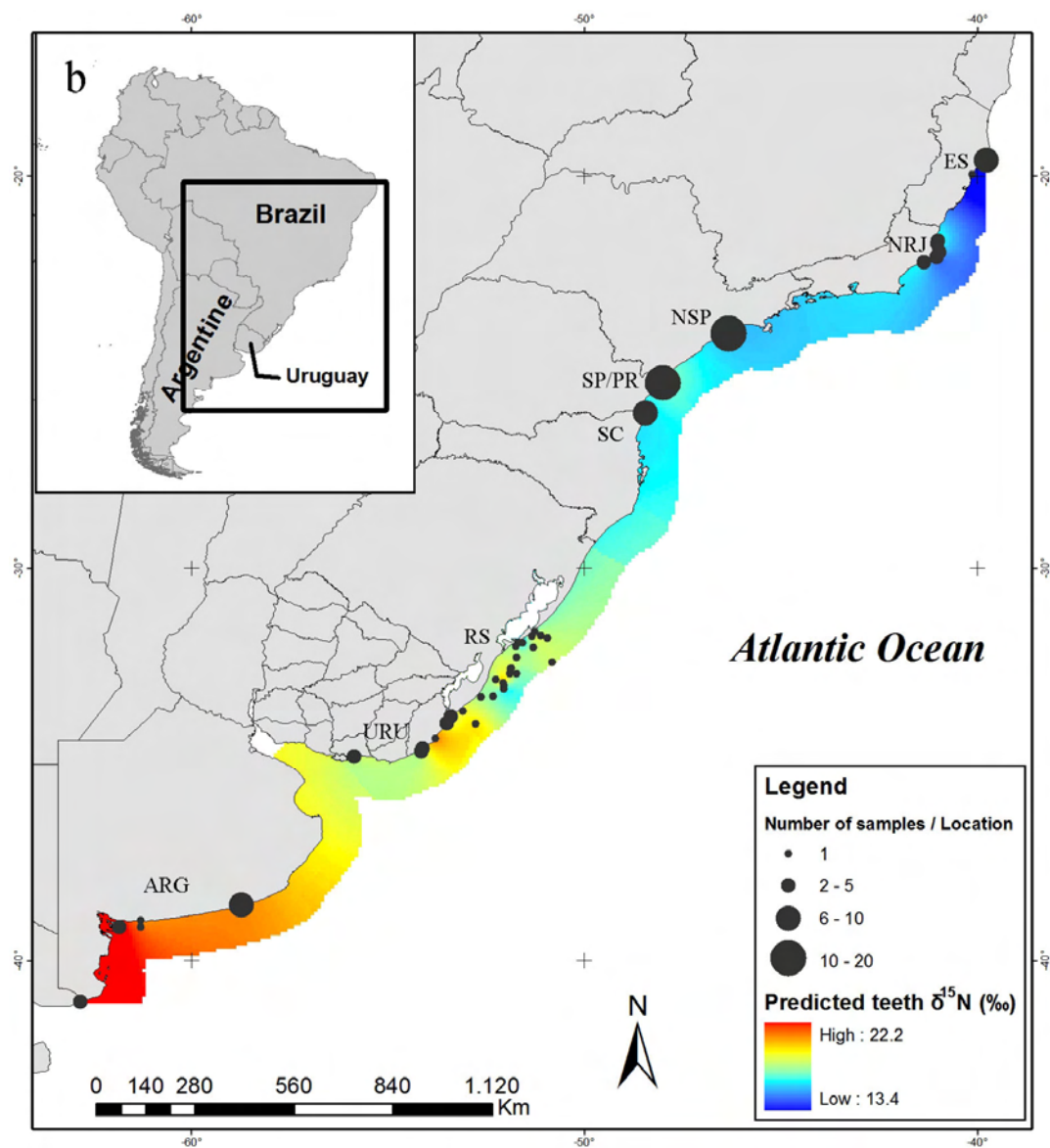


Figure 5.

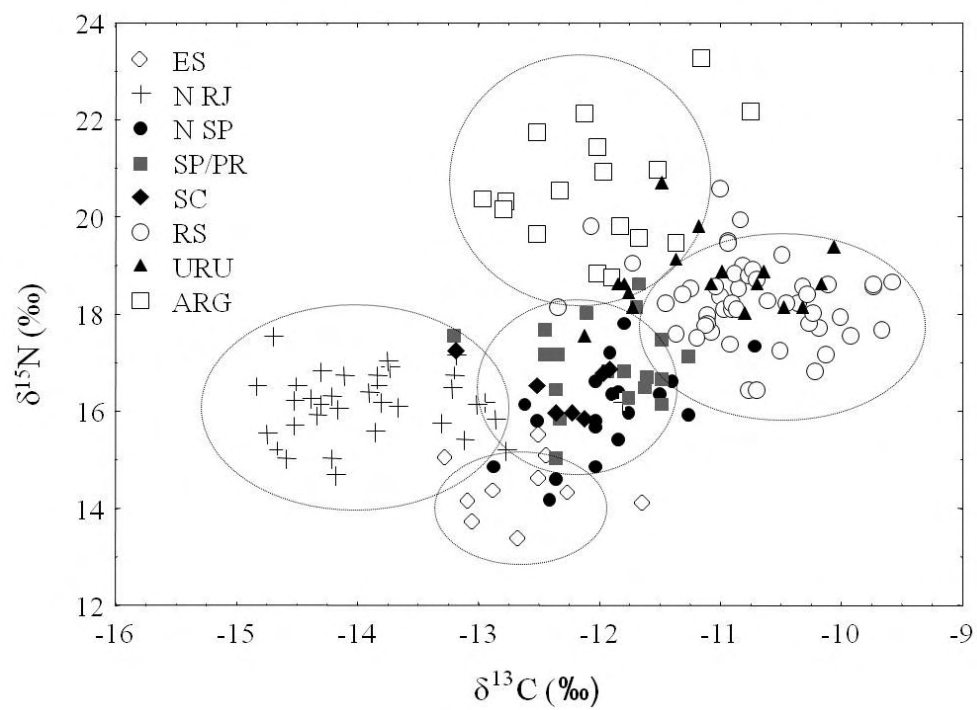
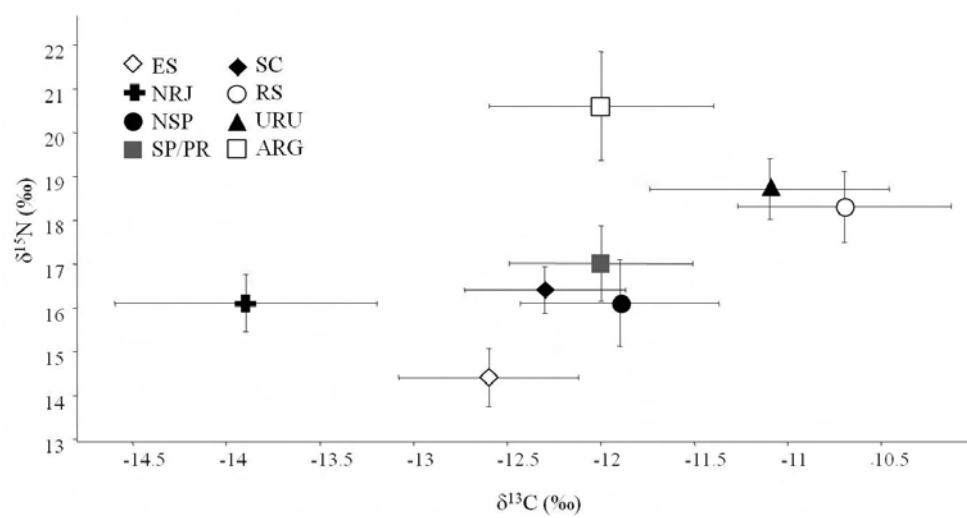


Figure 6.



ANEXO III

Isotopic variation in delphinids from the subtropical western South Atlantic

S. Botta, A.A. Hohn, S.A. Macko, E.R. Secchi

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Isotopic variation in delphinids from the subtropical western South Atlantic

BOTTA, SILVINA¹, HOHN, ALETA A.², MACKO, STEPHEN A.³, SECCHI, EDUARDO R.¹

¹Laboratório de Tartarugas e Mamíferos Marinhos, Instituto de Oceanografia, Universidade Federal do Rio Grande-FURG, Cx.P. 474, Rio Grande, RS 96500970, Brasil, ²National Marine Fisheries Service, National Oceanic and Atmospheric Administration Beaufort Laboratory, 101 Pivers Island Road, Beaufort, North Carolina 28516, US, ³Department of Environmental Sciences, Clark Hall, University of Virginia, Charlottesville, VA 22903 USA.

Abstract

A dual stable isotope approach ($\delta^{13}C$ and $\delta^{15}N$) was used to investigate inter- and intra-specific variations in feeding ecology and habitat use of 7 delphinids from coastal/estuarine, continental shelf and offshore marine environments from southern Brazil: Tursiops sp., Orcinus orca, Stenella frontalis, Steno bredanensis, Delphinus delphis, Pseudorca crassidens and Lagenodelphis hosei. Teeth from 50 specimens acquired from stranded animals were analyzed in this study. Tursiops sp. and O. orca are the most coastal species, and had the highest $\delta^{13}C$ values followed by the continental shelf species S. frontalis, S. bredanensis, D. delphis. Lagenodelphis hosei showed the lowest $\delta^{13}C$ value, demonstrating its typical offshore habitat. One group of P. crassidens had the lowest $\delta^{15}N$ values, indicating their low trophic level feeding habit while two specimens of the same species showed the highest mean nitrogen isotope value. This first study on stable isotope values of delphinids from southern Brazil provides substantial new information about the trophic ecology, habitat use and feeding environments of these animals.

Keywords: Delphinids, stable isotopes, trophic level, habitat use, western South Atlantic.

INTRODUCTION

Delphinidae is the most successful and diverse among all Cetartiodactyla families which occupy a wide variety of ecosystems (LeDuc, 2002), including freshwater (e.g. tucuxi, *Sotalia fluviatilis*), shallow coastal (e.g. short-beaked common dolphin, *Delphinus delphis*), and deep pelagic waters (e.g. Fraser's dolphin, *Lagenodelphis hosei*) in tropical and subpolar environments. Some species have restricted distribution while others are broadly distributed. Others have evolved by adapting to specific niches, generating different ecotypes such as the coastal and offshore bottlenose dolphin (*Tursiops truncatus*) (Segura *et al.*, 2006) and killer whales (*Orcinus orca*) (Ford, 2002).

A rich diversity in delphinids can be found in waters off the coast of Brazil (Bastida *et al.*, 2007). The continental shelf and slope waters in southern Brazil are influenced by the Subtropical Convergence and represent a biogeographic transition zone between Patagonian temperate and Brazilian tropical waters. In the neritic zone, cold and warm water circulation and upwelling processes (Castello *et al.*, 1997; Garcia, 1998, Piola *et al.*, 2000; Muelbert *et al.*, 2008) influence productivity that can be considered moderate to high (Odebrecht & Garcia, 1997).

Trophic relationships and habitat use are of key importance to the understanding, management and conservation of cetacean populations and their position in complex marine food webs. In southern Brazil, studies on the feeding ecology and habitat use of delphinids have typically focused on two coastal species: the Guiana dolphin (*Sotalia guianensis*) (e.g. Flores & Bazzalo, 2004; Oshima *et al.*, 2010) and the bottlenose dolphin (e.g. Simões-Lopes & Fabian, 1999; Fruet *et al.*, 2011). Information on the trophic ecology of the remaining delphinid species that inhabit the continental shelf or offshore waters is scarce or nonexistent, mostly due to their often difficult to work in habitats.

Carcasses washed ashore provide valuable biological material from species from those habitats. Stomach content analysis, for example, can yield direct information on the diet and indirect insight on the feeding environment (Barros & Clarke, 2009). However, this information is often biased owing to different digestion rates of prey, overestimating, for example, the importance of prey with chitinous structures (e.g. cephalopod beaks and crustaceans) relative to fishes, whose otoliths are rapidly digested by gastric acids (Jobling & Breiby, 1986; Santos *et al.*, 2001). Other limitations of analyses of stomach contents are due to the secondary ingestion of prey, which is prey found that was in the digestive tract of the predator's prey, as well as the short feeding time interval integrated by this kind of study, giving only information on recent feeding (Hobson *et al.*, 1994; Dehn *et al.*, 2006). These latter limitations can be reduced by increasing sample sizes and ensuring samples represent the appropriate temporal and spatial scales.

Fortunately, other complementary methods, such as naturally occurring stable isotopes of key elements and fatty acids (e.g. Herman *et al.*, 2005; Krahn *et al.*, 2007), are now available for studies on feeding ecology and habitat preference in aquatic vertebrates. Stable isotope values in animal tissues reflect those in the food webs where they feed (Rubenstein & Hobson, 2004; Graham *et al.*, 2010) and are useful particularly for determining trophic level, identifying major food sources, and assessing foraging habitats (e.g. Das *et al.*, 2003; Krahn *et al.*, 2007; Pinela *et al.*, 2010; Ricchialdelli *et al.*, 2010). Stable isotope ratios of nitrogen ($^{15}\text{N}/^{14}\text{N}$) and to a lesser extent carbon ($^{13}\text{C}/^{12}\text{C}$) show a stepwise enrichment with increasing trophic level in the marine environment (DeNiro & Epstein, 1978; 1981). Indeed, nitrogen isotopes values change in a predictable fashion between trophic levels, owing to the preferential excretion of the light isotope (Caut *et al.*, 2009), and so reflect trophic position (De Niro & Epstein, 1981; Cabana & Rasmussen, 1996; McCutchan *et al.*, 2003). Some studies with captive (Hilderbrand *et al.*, 1996; Hobson *et al.*, 1996; Lesage *et al.*, 2002) and

free-ranging (Newsome *et al.*, 2010) mammals showed that this increment varies among tissues, species, developmental stage and/or body condition. However, an approximate enrichment of 3.4‰ per trophic level is generally accepted (Post, 2002). Similar to ^{15}N , ^{13}C content also increases up trophic levels, although an increase of only roughly 1‰ is typically observed (De Niro & Epstein, 1978; Peterson & Fry, 1987). Therefore, predators' carbon isotope values are used as an indicator of the sources at the base of the food web where they feed (Hobson, 1999; Graham *et al.*, 2010).

An important aspect to be considered when using stable isotopes is that the turnover rate within a tissue is based on its metabolic rate. Therefore, diet information may be determined over a time frame that varies from a few days (e.g. blood plasma, liver), months (e.g. red blood cells or muscle) to years (e.g. whale baleen or teeth) (Walker & Macko, 1999; Kelly, 2000). Teeth are a particularly informative tissue for tracking the diet of delphinids over their lifetimes because they provide a permanent dietary record for an individual, as, under normal conditions, growth layers in teeth do not resorb or modify (Walker *et al.*, 1999; Walker & Macko, 1999; Niño-Torres *et al.*, 2006).

In this study a first estimation of carbon and nitrogen isotopes values in teeth of delphinids from southern Brazil, in subtropical Western South Atlantic, is presented. These stable isotope profiles were used to investigate inter and intra-specific variations in feeding ecology and habitat use of seven species from coastal/estuarine, continental shelf and offshore marine environments.

MATERIAL AND METHODS

Study site and sampling

Fifty specimens of seven species (Table 1) found washed ashore during systematic beach surveys conducted along the southern coast of Rio Grande do Sul State (RS), Brazil, (1993 to

2009) were used for this study (Figure 1). Teeth were extracted from the middle upper or lower jaw, cleaned and stored dry in the *Laboratório de Tartarugas e Mamíferos Marinhos* (LTMM-IO-FURG) collection.

Analysis of isotope compositions

Stable isotope analysis of teeth was performed following the protocol described in Walker and Macko (1999). Teeth were dried for 3–4 days in a 60°C oven and cleaned of outer soft tissue with a carbide burr attached to a drill. A low speed saw with a diamond-embedded blade was used to cut through the center of the tooth in the longitudinal buccal-lingual axis in order to expose the GLGs (*Growth Layers Groups*, Perrin & Myrick, 1980). Exposed dentine was sampled with a small drill bit, taking care that all GLGs were sampled so that the resulting powder would represent the entire life of the individual. The powder obtained was acidified with 30% hydrochloric acid (HCl) to remove biogenic carbonates, which could alter the organic $\delta^{13}\text{C}$ measurements, and then dried again for 1 hr in a 60°C oven. The goal of preparation is the conversion of the organic samples into gases of suitable purity that can then be analyzed by the mass spectrometer. Samples of approximately 5 mg of residual acidified tooth were used for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ analysis using an elemental analyzer (EA) connected to a Micromass Optima Isotope Ratio Mass Spectrometer (IRMS; GV Instruments, Manchester, UK). Natural abundance of stable isotope ratios ($^{13}\text{C} / ^{12}\text{C}$ and $^{15}\text{N} / ^{14}\text{N}$) are expressed in a delta notation (δ) as per mil variations (‰) when compared with international standards (e.g. Pee Dee Belemnite, PDB, for carbon and atmospheric N_2 , for nitrogen). Results were expressed as:

$$\delta X = [(R_{\text{sample}}/R_{\text{standard}}) - 1] * 1000$$

where R_{sample} and R_{standard} are the $^{13}\text{C}/^{12}\text{C}$ or $^{15}\text{N}/^{14}\text{N}$ ratios of the sample and standard, respectively.

Data analysis

All results are presented as the mean $\pm$ SD. Data were tested for normality and homogeneity of the variances using Kolmogorov-Smirnov and Levene's test, respectively. Comparison of isotopes values among species were done using 1-way ANOVA techniques followed by a Tukey's HSD test, when a significant difference was found. The null hypothesis of no differences was rejected if $p < 0.05$. In addition, a cluster analysis (Euclidean distances, complete linkage method) based on $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ mean values of each species was used for the detection of isotope groupings.

RESULTS

Carbon and nitrogen isotope compositions differed significantly among species (ANOVA, $F_{6,41}=10.78$; $p < 0.001$ and $F_{6,41}=49.99$; $p < 0.001$, for carbon and nitrogen isotopes, respectively) (Tables 1 and 2, Figure 3). Data from the Fraser's dolphin ($n=1$) could not be statistically tested.

One killer whale had the lowest $\delta^{13}\text{C}$ found in our samples (-20‰) and also a low $\delta^{15}\text{N}$ value (10.5‰) (Figure 2). This 396cm-long specimen was a very emaciated young female found stranded alive with the stomach full of oceanic salps (*Iasis zonaria*) and other unidentified planktonic invertebrates. This was considered atypical, thus, this animal was excluded from statistical analyses.

Some species showed considerable intraspecific variation, either in $\delta^{13}\text{C}$ or $\delta^{15}\text{N}$, however, in those animals presenting extreme values nothing atypical was detected when found washed ashore, as it was the case of the killer whale mentioned above. Therefore, their values were considered in the range of the normal distribution of isotopes values for the species. Carbon isotopes values for rough-toothed dolphins ranged from -14.3‰ to -11.5‰

and those for false killer whales were between -13.1‰ to -10.8‰ (Table 1, Figure 2 and 3). Furthermore, values of $\delta^{15}\text{N}$ for this last species revealed a bimodal distribution, with two specimens with high values (19.4‰ and 19.0‰) and the remainder with a mean $\delta^{15}\text{N}$ of 12.2‰. The latter included animals from a single mass stranding, while the ^{15}N -enriched individuals were found washed ashore alone. There is no stock information available for this species in the region thus we cannot infer if they belong to different ecotypes/stocks. However we treated them as two different groups (A and B), as their significantly different $\delta^{15}\text{N}$ values (Student's t-test, $p < 0.001$) suggest different feeding habits. The ^{15}N -enriched group (False killer whale A) was significantly different from the other species (Tukey's HSD test, $p < 0.01$), with the exception of the bottlenose dolphin (Tukey's HSD test, $p = 0.14$) and the killer whale (Tukey's HSD test, $p = 0.54$). $\delta^{15}\text{N}$ values of the second group (False killer whale B) were significantly different from all dolphin species (Tukey's HSD test, $p < 0.001$). As for $\delta^{13}\text{C}$, group A did not significantly differ from the remainder species (Tukey's HSD test, $p > 0.05$), while group B was significantly different from bottlenose dolphins and killer whales (Tukey's HSD test, $p < 0.01$).

Six bottlenose dolphins were more enriched in ^{15}N (4 males, one female, and one individual of unknown sex) (Figure 2). The mean $\delta^{15}\text{N}$ of these animals was 18.9‰, being 1.6‰ more enriched than the lower $\delta^{15}\text{N}$ group (17.2‰). Their $\delta^{15}\text{N}$ values were statistically different (t-test, $p < 0.0001$) but their carbon stable isotopes values were not different (t-test, $p = 0.51$), averaging -10.5‰ for the group with enriched nitrogen signatures, and -10.6‰ for the rest of the dolphins. Finally, one bottlenose dolphin showed an extremely different and depleted carbon and nitrogen signals ($\delta^{13}\text{C} = -12.0‰$ and $\delta^{15}\text{N} = 15.5‰$).

Cluster analysis of stable isotope values for carbon and nitrogen defined one group with high trophic level predators leaving the group B of false killer whales (low trophic level

predator) on a separate branch. Within the cluster of high trophic level predators, the oceanic Fraser's dolphin was separated from the continental shelf and coastal grouping, with the latter clustered together at a lower distance (Figure 4).

DISCUSSION

Carbon and nitrogen stable isotope values in teeth dentine of delphinids from southern Brazil reflected different trophic levels and/or feeding environments respectively. An offshore-inshore trend of increasing $\delta^{13}\text{C}$ values was observed, which is in agreement with the preferred habitat of the species analyzed here. Indeed, carbon isotope values varied from highly ^{13}C enriched values found in coastal species, such as bottlenose dolphins ($\delta^{13}\text{C} = -10.6\text{‰}$) to more depleted signals such as the carbon isotope ratio of the Fraser's dolphin ($\delta^{13}\text{C} = -12.8\text{‰}$), a typical species from deep pelagic environments. Similarly, previous studies revealed a longitudinal trend in marine environments, where nearshore, benthos linked food webs are more ^{13}C enriched compared to more offshore, pelagic food webs (France, 1995; Burton & Koch, 1999; Takai *et al.*, 2000; Clementz & Koch, 2001; Lesage *et al.*, 2001; Barros *et al.*, 2010; Pinela *et al.*, 2010; Riccialdelli *et al.*, 2010) which is probably a reflection of a gradient of decreasing macrophyte influence (Hill *et al.*, 2006). Indeed, phytoplankton have lower $\delta^{13}\text{C}$ values than many inshore plants (e.g. seagrasses, kelp forests, marsh plants), making inshore carbon sources able to be distinguished from more pelagic sources (Fry & Sherr, 1984; Hobson *et al.*, 1994; Clementz & Koch, 2001).

Bottlenose dolphins and killer whales had the highest $\delta^{13}\text{C}$ values, reflecting their coastal feeding habits (Table 1). Furthermore, their $\delta^{15}\text{N}$ values also overlap, suggesting a similar trophic level for these two coastal predators. In southern Brazil, coastal bottlenose dolphins form small resident populations usually associated with estuaries and river mouths (Castello & Pinedo, 1977; Simões-Lopes & Fabian, 1999). A resident population of 84-86 animals

(Dalla Rosa, 1999; Fruet *et al.*, 2011) inhabits the Patos Lagoon estuary and its adjacent coastal areas. Studies of the diet of bottlenose dolphins from RS based on stomach content analysis confirmed a coastal feeding habit with the white croaker (*Micropogonias furnieri*), the cutlass fish (*Trichiurus lepturus*) and the drum (*Paralichthys brasiliensis*) being the most important prey (Pinedo, 1982; Mehnen *et al.*, 2005). Nitrogen isotope values were also high. As noted above, due to the trophic enrichment in ^{15}N through the food chain, a high $\delta^{15}\text{N}$ is expected for this high-trophic-level predator. Two groups of bottlenose dolphins differing in their $\delta^{15}\text{N}$ values were identified (Figure 2), which can be interpreted as a resource partitioning that may be occurring among bottlenose dolphins from southern Brazil. The observed mean values of the ^{15}N -enriched group ($\delta^{15}\text{N} = 18.9\text{‰}$) and the ^{15}N -depleted group (17.2‰) are higher and similar, respectively, than values found for teeth of bottlenose dolphins from the western North Atlantic coast ($\delta^{15}\text{N} = 16.8\text{‰}$, Walker *et al.*, 1999; $\delta^{15}\text{N} = 17.6$ and 16.8‰ for the inner and outer part of the tooth dentine, Knoff *et al.*, 2008) and $\sim 5\text{--}7\text{‰}$ higher than values found by Barros *et al.* (2010) in Sarasota Bay ($\delta^{15}\text{N} = 11.9\text{‰}$) and the Gulf of Mexico ($\delta^{15}\text{N} = 12.7\text{‰}$). The latter used a different methodology for preparing the samples, centrifuging the dentine powder after demineralization, to separate collagen from noncollagenous proteins, and performing a lipid extraction. However, lipid extraction has a small influence in $\delta^{15}\text{N}$ by introducing an average fractionation of about 0.25‰ (Post *et al.*, 2007). Therefore, regional variations in diet/food web structure and/or nitrogen isotopes at the base of the food webs are likely to be the cause of the observed differences. Moreover, Abreu *et al.* (2006) reported high values of $\delta^{15}\text{N}$ inside the Patos Lagoon estuary and argued that this is probably an effect of nutrient input from domestic and industrial sewage. Nitrogen isotope content of wastewater has higher values due to ammonium volatilization and denitrification processes during sewage treatment that removes the lighter ^{14}N faster than the

^{15}N (Macko & Ostrom, 1994; McClelland *et al.*, 1997). In addition, stormwater may also be considered as an enrichment factor, due to the thermodynamically favored volatilization of isotopically depleted $^{14}\text{NH}_3$ from stormwater as it flows across hot surfaces (Dillon & Chanton, 2007). Estuarine ^{15}N -enriched waters could influence the isotopic composition of adjacent waters, however, no data on stable isotopes of the food chain of the coastal adjacent waters of the estuary are available at this time, thus precluding further interpretations.

Additionally, a bottlenose dolphin with a clearly different isotope signal was identified, which suggests that it may have fed in another region (Figure 2). In a preliminary isotope analysis comparing bottlenose dolphins from two areas, this animal was clustered together with a northern group of dolphins found stranded along the São Paulo state, Brazil (25°00'S, 47°50'W) (Botta *et al.*, 2010a). Three hypotheses exist for the origin of this animal: (a) this individual could be part of the northern form, as proposed by Barreto (2000); (b) it could be a disperser from a southern population of bottlenose dolphins from Uruguay, which are known to move to coastal adjacent waters of the Patos Lagoon estuary (Laporta *et al.*, 2008) and (c) it could belong to an offshore ecotype. However, based on the cranial characters proposed by Barreto (2000) to distinguish northern from southern forms (e.g. shape of the pterigoyds and their separation), we could infer that this animal was a northern form individual, which could explain its distinct isotope compositions. As stated above, carbon and nitrogen signals of the southern São Paulo population were similar to the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values found for this animal (Botta *et al.*, 2010a). Nevertheless, the possibility of this animal belonging to an offshore group cannot be discarded, as no information on cranial morphometry/shape and/or isotopic signatures for this group are available so far. Isotope compositions in teeth of offshore bottlenose dolphins from other areas are similar for nitrogen, but lower for carbon to those presented by this animal (e.g. $\delta^{15}\text{N}=14.8\text{‰}$ and $\delta^{13}\text{C}=-13.9\text{‰}$, western North Atlantic,

Walker & Macko, 1999). Indeed, the carbon isotope value for this bottlenose dolphin (-12‰) was similar to those of continental shelf species (e.g. Atlantic spotted dolphin) and higher than that presented by the offshore Fraser's dolphin.

The presence of killer whales in coastal waters of southern Brazil is seasonal, with records from winter and spring months being more common (Dalla Rosa *et al.*, 2005). The weakfish (*Cynoscion guatucupa*), the eagle stingray (*Myliobatis* sp.) and cephalopods have been recorded as prey for killer whales in Brazilian waters (Dalla Rosa, 1995; Dalla Rosa *et al.*, 2005). Although no remains of cetaceans were found in the stomach of the killer whales analyzed, a franciscana dolphin (*Pontoporia blainvillei*) was reported in the stomach content of a killer whale stranded in this region (Ott & Danilewicz, 1997). Owing to differences in the period of time integrated by stomach contents and stable isotopes, these two animals may have been also eating small cetaceans, as denoted by their high $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$, which are similar to values found in franciscana teeth from this area (Botta *et al.*, 2010b).

The killer whale with the lowest $\delta^{13}\text{C}$ found in our samples (-20‰) and also with a low $\delta^{15}\text{N}$ value (10.5‰) was considered atypical. The stomach content of this individual (oceanic salps (*Iasis zonaria*) and other unidentified planktonic invertebrates) could explain the low observed stable carbon and nitrogen isotope values found. Indeed, filter-feeder salps occupy low trophic levels in the oceanic environments (Madin, 1974). Elsewhere, salps and other components of the zooplankton have $\delta^{13}\text{C}$ values around -20‰ (Hatase *et al.*, 2002; Bode *et al.*, 2004).

In the western South Atlantic, short-beaked common dolphins and Atlantic spotted dolphins inhabit shallow waters over the continental shelf and upper slope (Zerbini *et al.* 2004; Moreno *et al.*, 2005; Tavares *et al.*, 2010), where they feed on small meso/epipelagic fishes and squids (Santos & Haimovici, 2002; Melo *et al.*, 2010; E.R. Secchi pers. obs.). The

intermediate $\delta^{13}\text{C}$ values found in teeth of these species' individuals are presumably reflecting this pelagic phytoplankton-dependent food web. The $\delta^{13}\text{C}$ value found for common dolphins is consistent with values found in other studies ($\sim -16\text{‰}$ in muscle, Das *et al.*, 2003; $\sim -12\text{‰}$ in bone, Pinela *et al.*, 2010).

The rough-toothed dolphin is generally found in deep-offshore waters (Miyazaki & Perrin, 1994; Jefferson, 2002), however coastal sightings of this species are relatively common along Brazilian waters, mainly for the southeastern coast (Lodi, 1992; Ott & Danilewicz, 1996; Flores & Ximenes, 1997; Lodi & Hetzel, 1998). The carbon isotope signal found for the species was similar to that of continental shelf species, indicating that a shallower water habitat is also used in the subtropical Western South Atlantic. Diet reported for this species included fish and squid (Miyazaki & Perrin, 1994). Nitrogen stable isotopes for this species did not differ from the rest of the continental shelf species, thus suggesting feeding at similar trophic positions (Figure 4). Finally, a rough-toothed dolphin with a ^{13}C depleted value was identified (Figure 2). This animal had $\delta^{13}\text{C}$ even lower than the oceanic Fraser's dolphin which can reflect a more oceanic feeding habitat used by this specimen, which could belong to an offshore group of rough-toothed dolphins.

False killer whale habitats are primarily oceanic and their main preys are deep sea cephalopods and fishes (Odell & McClune, 1999; Baird, 2002). Isotopically, two groups of divergent trophic level and/or habitats could be identified. One group of 6 false killer whales presented low carbon and nitrogen isotopes signals (False killer whale B, Figures 2 and 3, Table 1). These animals were part of a mass stranding of 14 individuals occurred in winter of 1995. The stomach contents of four of these animals (the remainder were empty) revealed only cephalopod prey, mainly *Ommastrephes bartramii* (Andrade *et al.*, 2001). This squid is a member of the oceanic Ommastrephidae family and together with *Illex argentinus* are

common prey found in mass stranded false killer whales from Argentina too (Koen-Alonso *et al.*, 1999) and are also important in the diet of other upper slope and oceanic adjacent water predators (Santos & Haimovici, 2001; 2002). The only isotope information published for these cephalopods off the RS area, revealed low carbon and nitrogen stable isotope content ($\delta^{13}\text{C} = -16.7\text{‰}$ and $\delta^{15}\text{N} = 9.3\text{‰}$, for a combined sample of *I. argentinus* and *O. bartramii*, Bugoni *et al.*, 2010). After accounting for a combination of tissue-dependent $\Delta^{13}\text{C}$ collagen-muscle ($\sim 4\text{‰}$) and a trophic discrimination factor (1‰) by subtracting a total of 5‰ from false killer whales' dentine $\delta^{13}\text{C}$ value (Koch, 2007), comparison with Omastrephids data confirmed the teuthophagic feeding habit of this group of false killer whales. On the other hand, two individuals presented different isotopic values from this mass stranded group (false killer whale A, Figures 2 and 3). One of these specimens, a 333cm-long false killer whale washed ashore in 2004, had a high ^{13}C content (-10.8‰), indicating a coastal habitat, and ^{15}N enriched dentine (19‰), indicating it was feeding at a high trophic level. Besides cephalopod prey, Sciaenidae and Serranidae fishes were reported in the diet of this species in southern Brazil (Pinedo & Rosas, 1989). Other authors indicate that this species could also prey upon small cetaceans (Odell & McClune, 1999; Baird, 2002). Nevertheless, the stable isotope proxy, together with previously-reported stomach content information suggesting that at least some false killer whales in southern Brazil have a more coastal piscivorous feeding habit. Indeed, this species has been observed in coastal areas off southern Brazil (LTMM-IO-FURG, unpublished data). Finally, one young specimen (total length=165cm) had a low $\delta^{13}\text{C}$ (-13.1‰) but a high $\delta^{15}\text{N}$ (19.4‰). This animal was found stranded alone in 2006. Higher $\delta^{15}\text{N}$ relative to the mother's signal is expected for lactate-feeding calves because mothers are catabolizing their own tissues for producing milk, which leads to a higher "trophic level" of their offspring (Hobson & Sease, 1998; Walker & Macko, 1999; Newsome *et al.*, 2006;

Knoff *et al.*, 2008; Newsome *et al.*, 2009). The calf's lower $\delta^{13}\text{C}$ value is probably influenced by the high lipid content in the ^{13}C depleted milk (Hobson & Sease, 1998; Newsome *et al.*, 2006; Newsome *et al.*, 2009). Based on its total length, this animal was probably still nursing, as false killer whales usually lactate for 18-24 months (Odell & McClune, 1999), which could be a plausible explanation for the isotopes signals found.

The carbon isotope content for the Fraser's dolphin was lower than that of typical continental shelf delphinids, which closely represents its oceanic, deep water habitat. This is a tropical species, but with unusual strandings reported for subtropical areas (e.g. Praderi *et al.*, 1992; Pinedo *et al.*, 2001; Laporta *et al.*, 2002; Moreno *et al.*, 2003). This individual was a male, with a total length of 236 cm. Length at sexual maturity is about 220-230 cm (Dolar, 2002) indicating that this animal was probably sexually mature. Mesopelagic fishes, crustaceans and cephalopods are among the preferential prey for this species, which are captured mainly from near surface to at least 600m (Robison & Craddock, 1983). However, the diet reported for individuals stranded in southern Brazil included demersal fishes, the cutlass fish (*Trichiurus lepturus*), the epipelagic squid *Loligo sanpaulensis* and the demersal/pelagic cephalopod, *Argonauta nodosa*, besides some penneidae shrimp (Pinedo *et al.*, 2001; Santos & Haimovici, 2001; Moreno *et al.*, 2003; Melo *et al.*, 2010). This coastal feeding habit can be considered as unusual and likely occurred because the animals were outside their home range (Moreno *et al.*, 2003). Therefore, stomach contents may occasionally represent unusual local feeding while stable isotope values of teeth reflect the typical long-term feeding habits for this individual.

This first study on stable isotope values of delphinids from southern Brazil some important information about trophic ecology and feeding environments of delphinids inhabiting coastal, shelf and oceanic environments in the subtropical western South Atlantic

was revealed. Substantial information about the trophic ecology and feeding environment can be derived from biochemical analyses in teeth. Furthermore, its combination with traditional methods, such as stomach content analysis, direct observation and telemetry, and biochemical methods could be a powerful tool to investigate the natural history of marine mammals (Pauly *et al.*, 1998). However, the almost nonexistent environmental isotope data for this southern region calls for additional sampling efforts and analyses involving the main components of these marine food webs which include these high trophic level predators.

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Figure captions

Fig. 1. Study area, western South Atlantic, southern Brazil. Northern and southern limits of the region surveyed for the collection of stranded delphinids is shown (Mostardas and Chui).

Fig. 2. Teeth $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values in teeth of delphinids found stranded along the southern coast of Brazil. Dashed line circle indicate ^{15}N -enriched bottlenose dolphins, *Tursiops sp.*

Fig. 3. Mean ($\pm\text{SD}$) $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values for teeth dentine of delphinids from southern Brazil.

Fig. 4. Tree diagram of delphinids from southern Brazil resulted from cluster analysis of stable isotope ratios of carbon and nitrogen in teeth (see Table 1 for codes).

Table 1. $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values $\pm$ SD (‰) of teeth of delphinids found washed ashore along the southern coast of Brazil.

Species	$\delta^{13}\text{C}$	Min Max	$\delta^{15}\text{N}$	Min Max	n
Bottlenose dolphin (BD)	-10.6	-12.0 -9.5	17.6	15.5 19.3	24
<i>Tursiops sp.</i>	(± 0.51)		(± 0.93)		
Killer whale (KW)	-10.5	-10.6 -10.3	17.7	17.6 17.7	2
<i>Orcinus orca</i>	(± 0.23)		(± 0.05)		
Short-beaked common dolphin (SBCD)	-11.7	-12.1 -11.4	16.6	15.7 17.1	4
<i>Delphinus delphis</i>	(± 0.32)		(± 0.59)		
Rough-toothed dolphin (RTD)	-12.3	-14.3 -11.5	16.7	15.6 17.8	5
<i>Steno bredanensis</i>	(± 1.18)		(± 0.82)		
Atlantic spotted dolphin (ASD)	-11.9	-12.4 -11.5	16.6	15.6 17.9	5
<i>Stenella frontalis</i>	(± 0.36)		(± 0.91)		
False killer whale A (FKW A)	-12.0	-13.1 -10.8	19.2	19.0 19.4	2
<i>Pseudorca crassidens</i>	(± 1.65)		(± 0.25)		
False killer whale B (FKW B)	-12.2	-12.5 -11.9	11.2	10.6 12.3	6
<i>Pseudorca crassidens</i>	(± 0.20)		(± 0.62)		
Fraser's dolphin (FD)	-12.8	-	14.9	-	1
<i>Lagenodelphis hosei</i>					

Table 2. Results of the Tukey's HSD post hoc test for multiple comparisons of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values from teeth of delphinids found washed ashore along the southern coast of Brazil (see Table 1 for codes). Carbon isotope ($\delta^{13}\text{C}$) p-values are reported below the diagonal and those for nitrogen isotopes ($\delta^{15}\text{N}$) are reported above the diagonal. Significant p-values (<0.05) are highlighted in **bold**.

	KW	BD	FKW A	SBCD	ASD	RTD	FKW B
KW		1.00	0.54	0.71	0.72	0.83	0.00
BD	1.00		0.14	0.29	0.24	0.41	0.00
FKW A	0.22	0.07		0.01	0.01	0.02	0.00
SBCD	0.23	0.03	1.00		1.00	1.00	0.00
ASD	0.09	0.00	1.00	1.00		1.00	0.00
RTD	0.02	0.00	1.00	0.88	0.98		0.00
FKW B	0.03	0.00	1.00	0.94	1.00	1.00	

Figure 1

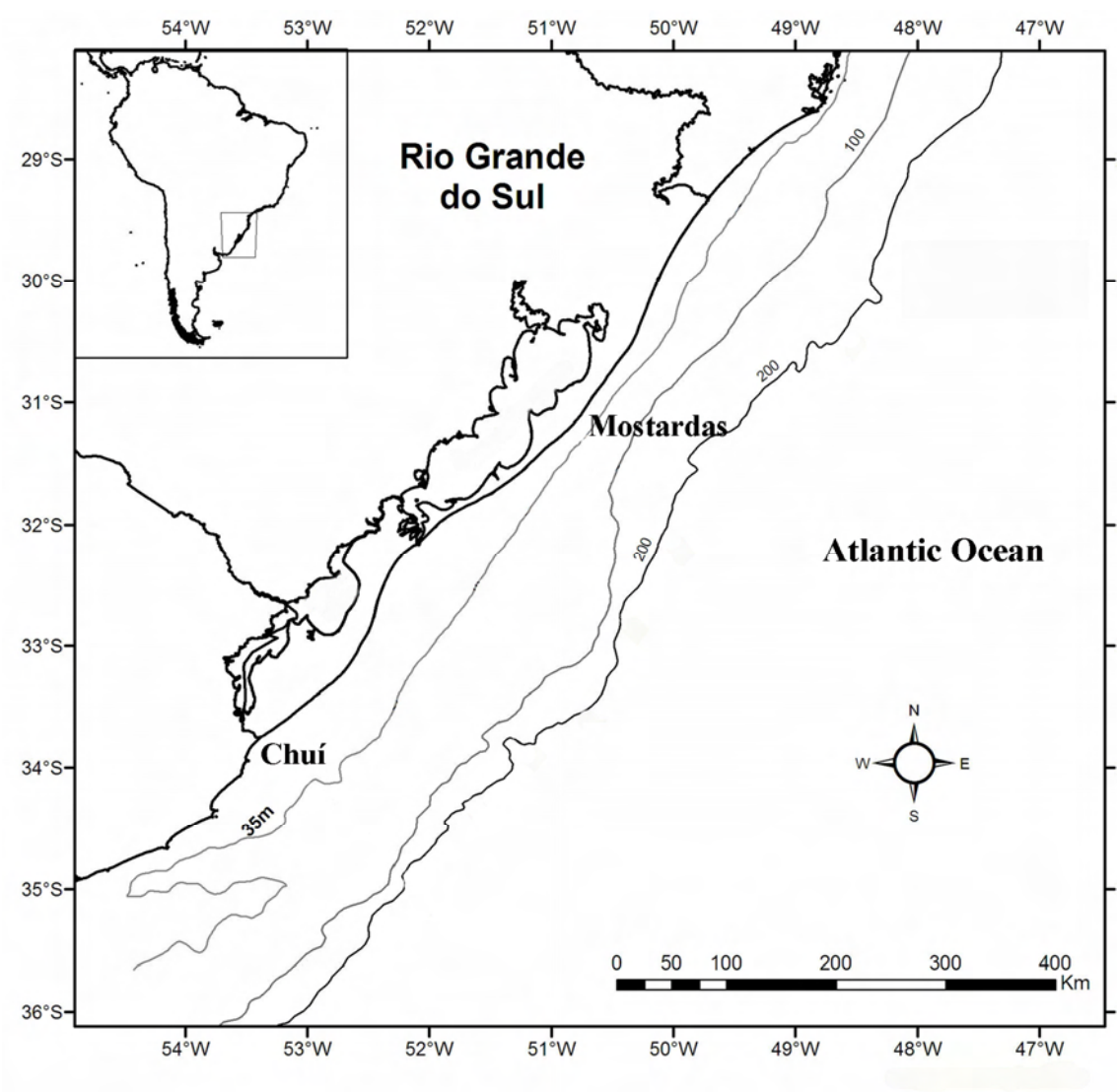


Figure 2

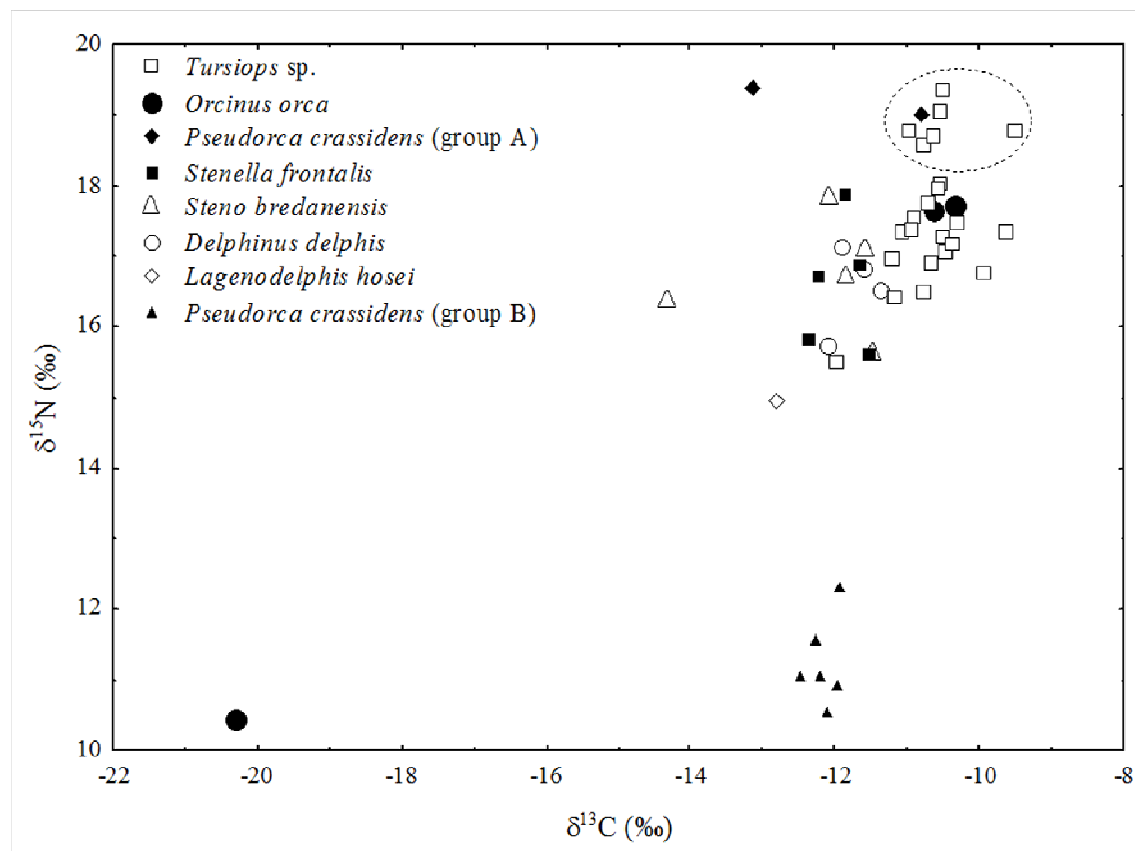


Figure 3

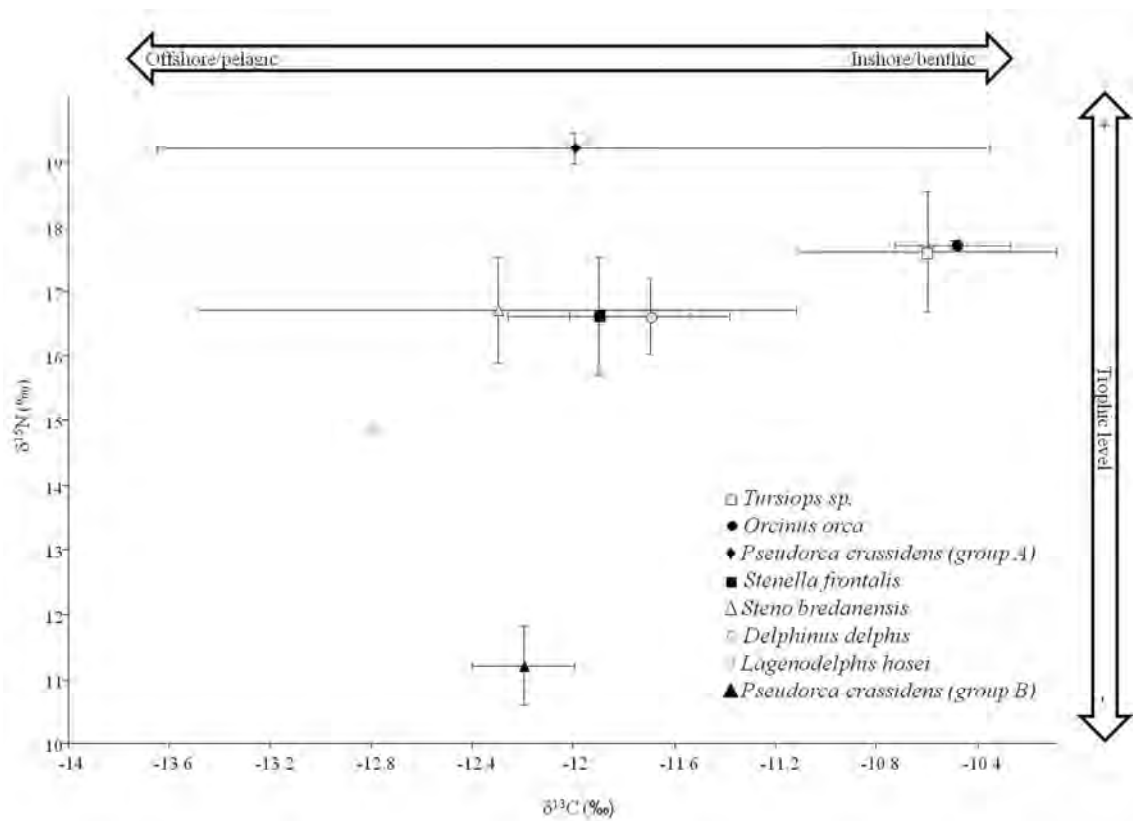
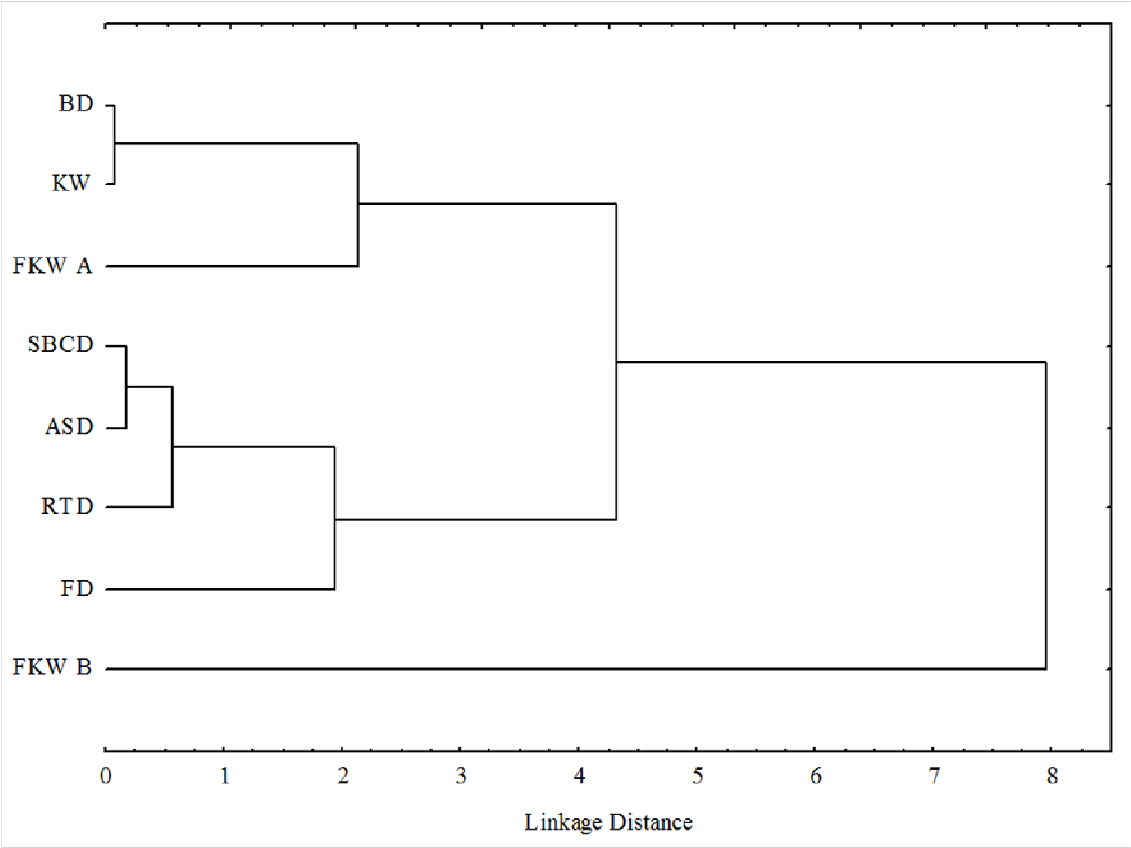


Figure 4



ANEXO IV

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

S. Botta, E. Secchi, C. Albuquerque, A.A. Hohn, V.M.F. da Silva, M.C.O. Santos, C. Meirelles,
L. Barbosa, A.P.M. Di Benedetto, R.M.A. Ramos, C. Bertozzi, M.J. Cremer, V. Franco-Trecu, N.
Miekeley

(A ser submetido a *Analytical and Bioanalytical Chemistry*)

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

S. Botta¹, E. Secchi¹, 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. Miekeley¹³

(1) Instituto de Oceanografia-Universidade Federal do Rio Grande-FURG, Cx.P. 474, Rio Grande, RS 96500970, Brazil; (2) Departamento de Ecologia e Oceanografia, Universidade Federal do Espírito Santo, ES, Brazil; (3) National Marine Fisheries Service, National Oceanic and Atmospheric Administration, Beaufort, NC, USA; (4) Laboratório de Mamíferos Aquáticos, Instituto Nacional de Pesquisas da Amazônia, Manaus, Brazil; (5) Laboratório de Biologia da Conservação de Mamíferos Aquáticos, Departamento de Oceanografia Biológica, Instituto Oceanográfico, Universidade de São Paulo, São Paulo, SP, Brazil (6) Associação de Pesquisa e Preservação de Ecossistemas Aquáticos – AQUASIS, SESC, Caucaia, CE, Brazil; (7) Organização Consciência Ambiental—ORCA, Vila Velha, ES, Brazil; (8) Universidade Estadual do Norte Fluminense, CBB/LCA, Campos dos Goytacazes, RJ, Brazil (9) Everest Tecnologia em Serviços Ltda. Vitória, ES 29050-912 Brazil; (10)) Projeto BioPesca, Ubatuba and Guarujá, São Paulo, Brazil; (11) Departamento de Ciências Biológicas; Universidade da Região de Joinville; Joinville - SC - Brazil; (12) Cetáceos Uruguay, Sección Etología-Facultad de Ciencias, Montevideo, Uruguay; (13) Departamento de Química, Pontifícia Universidade Católica do Rio de Janeiro, RJ, Brazil.

ABSTRACT

Bones, teeth and otoliths are metabolically inert structures and due to a cyclical deposition of material they preserve a chronology of chemical variations that may be related to the environmental histories of the organisms. Because of the natural increase of barium and decrease of strontium bioavailability in water with decreasing salinity, these elements were shown to be especially useful within this context. Therefore, we analyzed the Ba/Ca, Sr/Ca and Ba/Sr ratios in dolphins' teeth that represent a salinity gradient from freshwater to coastal open waters. The main aim was to determine whether these elements can be used as a natural tag for different aquatic environments and for the reconstruction of the environmental behavior of aquatic

mammals, so far not studied under this aspect. Teeth from two freshwater dolphins (*Inia geoffrensis* and *Sotalia fluviatilis*) and three marine species (*S. guianensis*, *Pontoporia blainvillei* and *Tursiops* sp.) from Brazil and Uruguay were analyzed using Laser Ablation ICP-MS. Intensity ratios (IR) of $^{138}\text{Ba}/^{43}\text{Ca}$, $^{86}\text{Sr}/^{43}\text{Ca}$ and $^{138}\text{Ba}/^{86}\text{Sr}$ were measured along a line that covered all growth increments in the dentin. Teeth from the freshwater species had mean Ba/Ca and Ba/Sr values tenfold higher than marine dolphins confirming the inverse relationship between salinity (and thus ambient Ba/Ca) and teeth IR. No significant differences were found for Sr/Ca values. Furthermore, Ba/Ca and Ba/Sr ratios could also differentiate dolphins from main estuarine areas from that of areas with minimal freshwater discharge. Ba/Ca and Ba/Sr age-related profiles identified three patterns among *Tursiops* sp.: low values with minimal ontogenetic variation, intermediate values with peaks and troughs and high values. Results presented encouraging indications for the application of this technique as a potential new tool for studying habitat use in aquatic mammals.

Keywords: barium, strontium, laser ablation ICP-MS, teeth, habitat use.

INTRODUCTION

Odontocete cetaceans occupy a variety of aquatic habitats from freshwater to marine environments such as coastal (estuarine or near shore), neritic (on the continental shelf) or oceanic (open seas or oceans) waters. Their distributions are a response to demographic, evolutionary, ecological, habitat-related, and anthropogenic factors (Forcada 2002). Prey abundance and distribution are accepted as the main factors affecting odontocete distribution, as they are usually found in waters with high densities of their main prey species (Hastie et al.

2004). Estuaries are highly productive coastal habitats (Lalli & Parsons 1997), supplying food and protection for many dolphin populations, usually showing high degree of residency and site fidelity to them. Furthermore, many cetacean populations inhabiting estuaries around the world were proved to be genetically isolated from adjacent populations, for example, Indo-Pacific bottlenose dolphins, *Tursiops aduncus*, from Port Stephens, Australia (Möller et al. 2007), bottlenose dolphins, *T. truncatus*, from estuarine regions along the western North Atlantic, USA (Rosel et al. 2009) and franciscanas, *Pontoporia blainvillei*, from Samborombón Bay, Argentina (Mendez et al. 2010).

Guiana dolphins (*Sotalia guianensis*), franciscanas (*Pontoporia blainvillei*) and bottlenose dolphins (*Tursiops* sp.) occupy several estuarine waters along the western south Atlantic (Bastida et al. 2007). In Brazil, based on long-term photo-ID studies, some populations of Guiana dolphins were considered resident to specific estuaries, such as the Cananéia estuary (São Paulo state, Oshima et al. 2010) and the Babitonga Bay (Santa Catarina state, Hardt 2005). Several resident populations of bottlenose dolphins can be also found along the southern Brazilian coast, including the estuaries of the Patos Lagoon (Dalla Rosa 1999, Fruet et al. 2011), Tramandaí river and Laguna (Simões-Lopes and Fabian 1999). Conversely, no studies on photo-ID in franciscana dolphins were conducted along its distributional range, probably due to its small size, shy behavior and boat avoidance (Bordino et al. 1999, Cremer & Simões-Lopes 2005) which makes the task extremely difficult. However, the species is known to occupy several estuaries such as the Paranaguá estuarine complex, northern Paraná state (Santos et al. 2009), Babitonga Bay, Santa Catarina state (Cremer & Simões-Lopes 2005) and the La Plata river estuary (Praderi 1986).

Photo-ID studies conducted in some of these areas (e.g Cananéia estuary, Babitonga Bay, Patos Lagoon estuary) revealed that part of the identified dolphins can be found year-round while other were never seen again after being first photographed. This could be interpreted as an indication of some structuring might be happening in this estuaries too, as not all identified individuals were considered resident to them. Therefore, while a portion of the individuals found in the area uses the estuarine waters on a regular basis some animals would eventually or never use it, remaining in adjacent coastal or distant areas. Understanding this patterns would require long-term studies based on remote-sensing or photo-ID techniques, covering both estuarine and adjacent coastal waters. Studies based on identified individuals require a long-term effort, which can sometimes be challenging due to logistical and/or financial constraints. Remote sensing techniques, on the other hand, it is still expensive and gives information on a few animals over a restricted time duration (Acevedo-Gutierrez 2009).

Fortunately, complementary approaches based on naturally occurring stable isotopes of key elements (e.g. stable isotopes and elemental concentrations) are now available for studies on habitat use in aquatic organisms such as fish (e.g. McCulloch et al. 2005, Hamer et al. 2006), sea turtles (e.g. Arthur et al. 2008), sea birds (e.g. Jaeger et al. 2010) and aquatic mammals (e.g. Fontaine et al. 2007, Botta et al. *in press*). Although these tracers are indirect evidences of habitat use, they offer advantages such as increased sample sizes and the possibility of developing short-term studies. On the other hand, depending on the chosen tissue, long-time integrated results can be achieved.

In particular, the determination of the elemental concentration in fish otoliths is a well developed technique applied for the reconstruction of their migration patterns between

freshwater/estuarine and open coastal areas. Elements such as barium and strontium are deposited chronologically in the calcareous matrix of otoliths in a direct relationship with the chemical and physical characteristics of the aquatic environment, thus tracking the different environments experienced by the fish throughout their lives (Elsdon & Gillanders 2002, Fowler et al. 2005). The availability of these two elements in aquatic systems depends mostly on salinity (Surge & Lohmann 2002, Jacquet et al. 2005). The high sulphate content of seawater results in its combination with Ba, forming stable solid barium sulphate (barite) that is precipitated, removing Ba^{2+} ions from the water column. Although Sr behaves in a similar way to Ba, it forms an unstable structure thus a greater proportion of Sr dissolves back in the water (Kitano 1975 *apud* Gilbert et al. 1994, Jacquet et al. 2005). Consequently, high Sr and low Ba bioavailabilities are found in the seawater in comparison to freshwater (Burton & Price 1990). Thus, the positive and negative relationship of Sr and Ba with water salinity can be detected in hard tissues such as fish otoliths (Elsdon & Gillanders 2006, Albuquerque et al. 2010).

In mammals, Sr and Ba have been extensively used in the bioarchaeological field, specifically in paleodietary human studies (Gilbert et al. 1994, Sponheimer et al. 2005, Szostek et al. 2009). The main route of their acquisition is through water and food being then incorporated into the hydroxyapatite $[Ca_{10}(PO_4)_6(OH)_2]$ structure of bone and teeth as a replacement of calcium (Burton et al. 2003). These two elements are not essential, thus are not subject to homeostatic control, but they suffer certain discrimination against Ca (Szostek et al. 2009). Consequently, Sr/Ca and Ba/Ca ratios are used as a proxy for trophic position due to their biopurification along the food chain (Burton & Price 1990), however they are more specifically used to differentiate herbivores from carnivores (Burton et al. 2003). Low Ba/Ca and Ba/Sr ratios, however, were proved to be valuable indicators of diet enrichment with products of

marine origin (Burton & Price 1990, Gilbert et al. 1994, Szostek et al. 2009). Furthermore, due to the fact that elements accumulated in dentine are not subject to strong remodeling and turnover, ontogenetic changes in Ba/Ca and/or Ba/Sr ratios should be analyzed by sampling dentine mineralized at different life stages. In this context, cetacean teeth offer an additional advantage over human teeth, due to their characteristic monofiodoncy and chronological deposition of discrete layers of dentine until death. Consequently, long time-integrated Ba/Ca and Sr/Ca values can be obtained by sampling the whole tooth. Furthermore, Ba/Ca and/or Ba/Sr profiles along dentine layers should also be a valuable proxy for ontogenetic and other temporal patterns of occupation of the habitat with different freshwater influence. Sampling a chronological series of hard tissue layers, like cetacean dentine GLGs (Growth Layers Groups, Perin & Myrrick 1980), is a difficult task, especially for small odontocetes. However, the relatively new technique of laser ablation sampling, has been increasingly used for the analysis of solid samples due to the achievable fine spatial resolution (1-50 μ m), the minimal sample preparation, contamination and amount of sample required for analysis (Evans & Outridge 1994). Preliminary applications of a laser ablation device coupled to an inductively coupled plasma mass spectrometer (LA-ICP-MS) in marine mammal teeth demonstrated its great potential for answering questions about temporal changes in elemental acquisition (Evans & Outridge 1994, Outridge & Evans 1995). However, besides its increasing application in humans and other primates teeth (e.g. Kang et al. 2004, Dolphin et al. 2005, Humphrey et al. 2008), to our knowledge, no work has been done in marine mammals since then.

Assessing the potential application of these tracers for studying habitat use will initially require the demonstration that the amount of Ba and Sr in dolphin teeth can differentiate aquatic environments along a salinity gradient. Indeed, resident/small home range dolphin populations

from habitats with variable salinity (e.g. freshwater, estuaries and coastal waters with minimal continental influence) would be evidenced by characteristic Ba/Ca, Sr/Ca and Ba/Sr ratios in teeth. Furthermore, with the possibility of a chronological sampling of teeth layers by LA-ICP-MS, temporal changes in the environment and/or ontogenetic movements among different habitat would be potentially visualized. Therefore, in this paper, we analyzed the Ba/Ca, Sr/Ca and Ba/Sr ratios in dolphins' teeth that represent a salinity gradient from freshwater to coastal open waters. The main aim was to determine whether these elements can be used as a natural tag for tracking habitat characteristics and habitat utilization patterns of aquatic mammals.

MATERIAL AND METHODS

Samples and study area

Teeth from two freshwater (n=41) and three marine dolphin species (n=243) were obtained from incidentally captured dolphins or washed ashore carcasses. The study area included rivers (e.g. Amazon River and tributaries) and coastal regions with diverse freshwater discharge from estuaries (e.g. La Plata River estuary) to regions with minimal freshwater influence (e.g. Ceará) (Figure 1, Table 1).

Age estimation

In order to evaluate potential influence of age on the elemental variables and to produce age-related profiles of elemental intensities, ages were estimated by counting dentinal and or cemental incremental layers or GLGs (*Growth Layer Groups*, Perrin and Myrick, 1980). Teeth were processed for age estimation following Hohn *et al.* (1989) for bottlenose and Guiana dolphins and Pinedo & Hohn (2000) for franciscanas. For Guiana and bottlenose dolphins a thick section (1-2 mm) of the center of the tooth was cut through the longitudinal axis with a low

speed saw with a diamond-embedded blade. These thick sections and the whole small sized franciscanas' teeth were then fixed in 10% formalin and decalcified with RDO[®] (a commercial mixture of acids). The decalcified teeth sections were then thin-sectioned to 25µm using a freezing microtome, stained in Mayer's hematoxylin, "blued" in ammonia, dehydrated in glycerin, and mounted on a microscope slide in 100% glycerin. GLGs counting performed without reference to biological data, such as length or sex. Each GLG was considered to represent one year of age (Hohn et al. 1989, Pinedo & Hohn 2000). Amazon dolphins and tucuxis were not aged due to the lack of enough teeth samples.

Chemical analyses

All samples were analyzed through LA-ICP-MS. However, due to the difficulty in finding solid standard reference materials for matrix-matched calibration purposes, data obtained by this method were of semi-quantitative nature instead of quantifiable trace-element concentrations, where calcium was used as an internal standard to control for variation in ablation yield. Therefore, in order to confirm the valid use of the semi-quantitative method, a subsample of 17 teeth (5 Amazon dolphins, 6 Guiana dolphins from AmE and 6 from CE) were analyzed by dissolved-solution methods (see below).

Although previous studies in humans demonstrated that both Sr/Ca and Ba/Ca are reduced at higher trophic levels (Elias et al. 1982, Burton & Price 1990) it was proved mainly for differences among herbivores and carnivores. As dolphins are 100% carnivores there was a concern about this trophic level effect would also be evidenced among carnivores occupying different trophic levels. Therefore, using stable nitrogen isotopes as a proxy for trophic position (Post 2002, see method below) we investigated its relationship with Ba/Ca and Sr/Ca values,

expecting to find no significant correlations among them, thus excluding trophic level as a confounding factor in results.

LA-ICP-MS

Teeth were cleaned of outer soft tissue with a carbide burr attached to a drill, when necessary. Teeth from the larger species were sectioned using a low speed saw with a diamond-embedded blade through the longitudinal axis in order to obtain a 2mm 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 a fast drying glue. Teeth from franciscanas were first glued in a glass slide and then polished by hand with a lapping paper until the entire pulp cavity and all the GLGs were visible. Teeth surfaces were then polished with a silicon carbide paper (n°8000), washed with deionized (DI) water (Mili-Q, Bedford, USA), sonicated for 3 minutes and rinsed three times with DI water. The slides were allowed to dry in a horizontal flow cabinet before analysis.

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). The laser was configured for Q-switched mode operation and worked under a pulse frequency of 20 Hz. The laser moved across the tooth at $40 \mu\text{m s}^{-1}$, with a mean beam energy of 1260mJ.

Data was acquired for the isotopes ^{43}Ca , ^{138}Ba , ^{208}Pb , ^{87}Sr , ^{86}Sr , ^{66}Zn , ^{25}Mg and ^{55}Mn , however only ^{138}Ba , ^{43}Ca and ^{86}Sr data are presented in this work. Each tooth were scanned from the pulp cavity to the edge (dentin-enamel limit) covering all pre- and post-natal dentin. Elemental intensities were corrected for argon background before calculating Ba/Ca, Sr/Ca and Ba/Sr ratios.

Dissolved-solution method

Teeth powder obtained from a cleaned teeth was obtained by drilling the dentine layers. The powder was then oven-dried to constant weight, weighed to an accuracy of 0.1mg and digested in a capped polypropylene tube placed in a heating block at about 90°C using a mixture of concentrated HNO₃ and H₂O₂.

Determinations of trace elements in samples of digested teeth powder were performed by conventional solution nebulization ICP-MS under standard conditions.

Nitrogen stable isotopes analysis

A sample from the teeth that comprises the whole dentine was used for determining isotopes concentrations with an elemental analyzer (EA) connected to a Micromass Optima Isotope Ratio Mass Spectrometer. In the case of the franciscana, the whole tooth was crushed so that a homogeneous sample is produced before processing for mass spectrometer analysis. Teeth from the remainder dolphin species were cut longitudinally in half, and a sample from the center that comprised the whole dentine/cement was drilled.

Sample preparation for mass spectrometer analysis was performed following standard procedures (Walker & Macko 1999) in order to convert the sample into gases (N₂ and CO₂) for analysis in mass spectrometer. Results will be expressed as:

$$\delta X = [(R_{\text{sample}}/R_{\text{standard}}) - 1] * 1000$$

where R_{sample} and R_{standard} are the ¹³C/¹²C or ¹⁵N/¹⁴N ratios of the sample and standard, respectively. Units are expressed as parts per thousand or per mil (‰). The standard for carbon is Pee Dee Belemnite (PDB) and the standard for nitrogen is atmospheric nitrogen.

Chronological variation in Ba/Ca and Ba/Sr in teeth

Ba/Ca and Ba/Sr profiles of nine individuals of the larger species (*Tursiops truncatus*) were used to study individual environmental histories. The tooth of a rough-toothed dolphin (*Steno bredanensis*), a more offshore continental shelf species, was also analyzed for comparison. Due to the difficult visualization of incremental lines in dolphin teeth without any treatment, stained teeth slides used for age estimation were used in order to measure the GLGs and match the elemental profiles to dolphin age. Digital images of the scanned slides and the stained sections were compared in order to measure GLGs along the same position of the scan (Figure 2). Measurements were taken with *Image J 1.42q* (National Institutes of Health, USA) software. By considering the ICP-MS sampling time of 0.516 seconds and a laser scanning speed of 40 $\mu\text{m/s}$, counts per second were assigned to specific ages, thus obtaining mean Ba/Ca and Ba/Sr ratios for each age.

Data analysis

Data were tested for normality and homogeneity of the variances using Kolmogorov-Smirnov and Levene's test, respectively. Variables were log-transformed to meet statistical assumptions of normality. The relationship between Ba/Ca, Ba/Sr and Sr/Ca values obtained through solution-based ICP-MS and LA-ICP-MS values were verified through regression and correlation analyses. Correlation analyses were performed between ages (or total length, whenever ages were not available) and $\log(\text{Ba/Ca})$, $\log(\text{Ba/Sr})$ and $\log(\text{Sr/Ca})$ to verify the existence of an age-related variation in the those ratios. Similarly, correlation between $\delta^{15}\text{N}$ values and $\log(\text{Ba/Ca})$ and $\log(\text{Sr/Ca})$ was assessed. Student's t-tests were used for comparing elemental mean values found for freshwater versus marine samples. Whenever significantly

differences were found, a 1-way ANOVA followed by Tukey's HSD tests were performed to test for differences among marine areas/species. Cluster analyses (Euclidean distances, complete linkage method) were used in order to detect groupings among areas/species based on mean elemental values.

RESULTS

LA-ICP-MS versus solution-based ICP-MS

Ba/Ca, Ba/Sr and Sr/Ca ratios calculated through semi -quantitative and quantitative values of Ba, Sr and Ca obtained by LA-ICP-MS and ICP-MS (solution-based), respectively were highly and linearly correlated in the case of Ba/Ca ($r^2=0.99$, $p<0.0001$) and Ba/Sr ($r^2= 0.98$, $p<0.0001$). On the other hand, values for Sr/Ca were significantly but not strongly correlated ($r^2=0.64$, $p<0.001$) as values obtained by LA-ICP-MS were slightly lower than by conventional ICP-MS(Figure 3).

Age, length and trophic level effects

Age or length were not significantly correlated with Ba/Ca or Sr/Ca in all areas/species (Spearman's correlation tests, $p>0.05$). Nitrogen stable isotope compositions were not significantly correlated with Ba/Ca or Sr/Ca in almost all areas/species (Spearman's correlation tests, $p>0.05$). Ba/Ca was positively correlated with $\delta^{15}\text{N}$ in Guiana dolphins from AmE (Spearman's correlation test, $r^2=0.20$, $p<0.001$, $n=16$) and franciscanas from ES (Spearman's correlation test, $r^2=0.96$, $p<0.001$, $n=5$). Because these significant correlations were weak (in the case of AmE), or probably due to a small sample size (in the case of ES) a potential trophic level effect was considered negligible in the subsequent analysis.

Teeth Ba/Ca, Ba/Sr and Sr/Ba differences among aquatic habitats

Mean $\log(\text{Ba}/\text{Ca})$ and $\log(\text{Ba}/\text{Sr})$ for freshwater dolphins were significantly higher than the marine dolphin samples (Student's t-test, $p < 0.001$). Conversely, $\log(\text{Sr}/\text{Ca})$ values were not significantly different among freshwater and marine dolphins (Student's t-test, $p = 0.12$) (Table 2, Figure 4). Therefore, $\log(\text{Sr}/\text{Ca})$ was not included in subsequent analyses.

Significant differences were found among marine regions for $\log(\text{Ba}/\text{Ca})$ and $\log(\text{Ba}/\text{Sr})$ (ANOVAs, $F_{(8,229)} = 19.19$, $p < 0.001$ and $F_{(8,229)} = 18.$, $p < 0.001$, respectively) (Figure 5). When species from each area were compared for $\log(\text{Ba}/\text{Ca})$ and $\log(\text{Ba}/\text{Sr})$, differences between species from the same area and from different species among areas were also observed ($F_{(13,224)} = 13.08$, $p < 0.001$ and $F_{(13,224)} = 13.11$, $p < 0.001$). A posterior comparison indicated that the main estuarine sites (AmE, URU and RS) differed significantly from CE and NSP (Table 3). This pattern was also observed when different species within areas were compared, however in the SP/PR area, franciscanas differed from Guiana dolphins both in Ba/Ca and Ba/Sr (Table 4).

Cluster analysis among all areas revealed that they could be grouped in three main clusters. Marine areas were clearly separated from the Amazon River and branched in two groups, one including the three main estuaries (AmE, RS and URU) and ES and the other with the remaining regions (CE, NRJ, NSP, SC and SP/PR). This latter was in turn, separated in a group containing CE and NSP and a second one including NRJ, SC and SP/PR (Figure 6). Cluster analysis among species within regions demonstrated the high separation of the freshwater species from the marine ones. Species inhabiting the main estuaries (AmE, RS and URU), ES, franciscanas from NRJ and Guiana dolphins from SP/PR clustered together. The remaining areas (CE, NSP and

SC), Guiana dolphins from NRJ and franciscanas from SP/PR were included in another subgroup. In this latter, a separation of dolphins from CE and NSP was also evident (Figure 7).

Ba/Ca and Ba/Sr individual elemental profiles

Elemental profiles for Ba/Ca and Ba/Sr were related to age for 9 bottlenose-dolphins from RS (Figure 8). The profiles showed some individuals presenting almost no variation in either Ba/Ca or Ba/Sr along their lives, while others appeared to have considerable variation throughout the years. Among the latter, three animals experimented high Ba/Ca and Ba/Sr values after their first year of age ($Ba/Ca > 0.03$ and $Ba/Sr > 0.20$). Ba/Ca and Ba/Sr profiles of the rough-toothed dolphin did not show a variation in their values from birth to death.

DISCUSSION

Laser ablation ICP-MS was proved to be a reliable tool for assessing the elemental composition of dolphin teeth. This technique offers the advantages of minimal sample preparation procedures, no sample size requirements, increased sample throughput and the spatial characterization of solid samples with limited surface damage (Russo et al. 2002, Durrant & Ward 2005, Mokgalaka & Gardea-Torresdey 2006). On the other hand, the difficulty in finding appropriate solid standard reference materials, preclude the quantification of the amount of the element present in the sample, thus data obtained are of semi-quantitative nature. However, as demonstrated in this study, values obtained by both methods are highly correlated.

Elemental Ba/Ca and Ba/Sr ratios in teeth from different dolphin species were highly diagnostic of the saline characteristics of the environment they lived. Freshwater dolphins presented Ba/Ca and Ba/Sr ratios more than tenfold higher than marine species. Furthermore,

Ba/Ca and Ba/Sr decreased with decreasing freshwater discharge in different areas along the coasts of Brazil and Uruguay.

Values of Ba/Ca and Sr/Ca did not vary with age and length. Temporal variations in those values would be expected in migratory species whenever areas visited differ in their salinity characteristics (Elsdon & Gillanders 2005, Fowler et al. 2005). Guiana dolphins, tucuxis, franciscanas and Amazon dolphins are resident and/or small home range species (e.g. Flores & Bazzalo 2004, Martin & da Silva 2004, Bordino et al. 2008, Flores & da Silva 2009, Oshima et al. 2010) which can explain the lack of influence of age on both ratios. In the case of bottlenose dolphins, populations that perform seasonal long distance movements have been described in literature (e.g. Rosel et al. 2009). Although a resident population inhabits the Patos Lagoon estuary and its adjacent coastal areas, movements of some identified individuals between the latter and Uruguay have been reported (Laporta et al. 2008). These movements, however, are not likely related to the age of the individuals, as they are probably performed for feeding purposes, thus explaining the lack of influence of age on Ba/Ca or Sr/Ca ratios.

Moreover, it was demonstrated that Ba/Ca and Sr/Ca were not related to predators' trophic level as no correlation was found among $\delta^{15}\text{N}$ values and those elemental compositions. Therefore, even in the case of an ontogenetic shift in diet in any of the species/populations, no variation among age classes would be expected. Ba/Ca and Sr/Ca have been extensively used in the archaeological field for studying the diet of ancient populations, mainly seeking for variations in the amount of vegetables *versus* meat in their diets (Burton et al. 2003). Differences among carnivore consumers might not be reflected in measurable variations of Ba/Ca or Sr/Ca ratios in their tissues.

Elemental differences among areas

The highly significant difference in the Ba/Ca and Ba/Sr content in teeth of the freshwater species with respect to the marine dolphins evidenced the potential of this approach for studying the habitat use of these aquatic mammals. This finding demonstrates the potential for applying these tracers not only for studying habitat use patterns in extant cetacean populations but also for extracting useful information about the habitat of fossil specimens.

Mean Sr/Ca ratio found in freshwater dolphins was not significantly different from marine individuals. This element have been extensively used for studying habitat use and migrations in fish (Elsdon & Gillanders 2006, Albuquerque et al. 2010) as its deposition in the otolith matrix is in agreement with environment levels (Campana 1999, Hamer et al. 2006). Although the intestinal absorption ratio for Ca, Sr and Ba is 10:5:1, respectively (Gilbert et al. 1994 *apud* Szostek et al. 2009) there is no information available for those elements deposition in the tooth matrix. Furthermore, although Sr/Ca ratios in marine environments are relatively constant, freshwater end-members show considerable geographic and temporal variability (Elsdon et al. 2008), thus underlying differences in water chemistry may be another factor influencing the lack of a variation found in Sr/Ca among freshwater and marine species. Nevertheless, both physiological and environmental effects on Sr/Ca variability should be better explored in the future.

Dolphins collected in areas along a salinity gradient, from estuaries with high freshwater discharge (e.g. AmE , URU) to areas with minimal runoff (e.g. NSP, CE) showed decreasing Ba/Ca and Ba/Sr ratios. This trend was also observed in the grouping resulted from the cluster analysis among areas. Mean Ba/Ca and Ba/Sr values found for dolphins from AmE, therefore,

were expected to be higher than RS or URU. This estuary presents a mean freshwater discharge of $180,000 \text{ m}^3 \cdot \text{s}^{-1}$, which is several times higher than the La Plata River and the Patos Lagoon estuaries (Table 1). However, due to the influence of the North Brazil Current, the runoff from the Amazon river flows northwestward (Lentz 1995, Santos et al. 2008). The lower than expected values found in AmE dolphins might suggest that they preferentially use adjacent areas to the southeast of the estuary mouth, with less influence of freshwater discharge. Stable isotope compositions in teeth of the specimens showing values typical of marine coastal food webs corroborate this (Botta et al. Annex I, this thesis).

Differences in habitat use between species inhabiting the same area, could also be evidenced by their discrepant Ba/Ca and Ba/Sr ratios. Indeed, although samples from NRJ were clustered with the low freshwater influenced areas, cluster analysis of species per area showed that franciscanas from this area presented Ba/Ca and Ba/Sr ratios more similar to those found in areas with higher freshwater discharge. In NRJ, the main input of freshwater comes from the Paraíba do Sul river, and both franciscanas and Guiana dolphins used its adjacent coastal areas. While franciscanas are seen year round, Guiana dolphins are mostly observed during autumn/winter seasons (Di Benedetto et al. 2001). Therefore, this different pattern of habitat utilization might be reflected in higher Ba/Ca and Ba/Sr ratios in franciscanas. Guiana dolphins from SP/PR also showed values more similar to more freshwater influenced areas, than franciscanas from this area. In this area, Guiana dolphins use the Cananéia estuary year round, with some resident individuals (Oshima et al. 2010). Conversely, franciscanas only occasionally use this estuary (Santos et al. 2007), occurring predominantly in adjacent coastal waters.

Individual Ba/Ca and Ba/Sr profiles

Elemental profiles of Ba/Ca and Ba/Sr in teeth of nine bottlenose dolphins from RS showed at least three different patterns of ontogenetic variation. Two animals showed almost no variation in elemental values throughout their lives, with Ba/Ca and Ba/Sr values remaining low until death. Profiles and values of these individuals resemble that of the rough-toothed dolphin analyzed, thus indicating a probable distinct aquatic environment from that of the estuary-influenced coastal waters. At least one of these dolphins (indicated by a dashed line in Figure 8) was supposed to belong to an offshore ecotype of bottlenose dolphins which is corroborated by the carbon and nitrogen stable isotope compositions and cranial morphology (Botta et al. *in press*). The remaining specimens presented variable temporal variations in Ba/Ca and Ba/Sr values, displaying peaks and troughs in the age-related profiles. In this group, Ba/Ca and Ba/Sr peaks do not exceed the values of 0.02 or 0.20. Three animals showed extreme high peaks of these ratios early in their lives (>1yr), remaining high until death. Although this peaks and troughs might be interpreted either as movements between environments with different freshwater influence, they can also reflect variations in freshwater input. Indeed at least three periods with high freshwater discharge of the Patos Lagoon estuary associated with strong "El Niño" events were recorded during the period when these individuals were alive (1980-2008). Changes in magnitudes of flood events were recorded in Ba/Ca values in structures such as corals (Alibert et al. 2003, Jupiter et al. 2008), and shells (Gillikin et al. 2006). Nevertheless, the role of physiological regulation in controlling the uptake of Ba into the cetacean tooth matrix was not yet studied, thus precluding further conclusions. The confirmation that tooth elemental composition reflects that of the ambient water and a future comprehensive study relating

oscillating patterns in Ba/Ca and Ba/Sr ratios to the amount of freshwater discharge will help to elucidate this issue.

Conclusions

Although a large and increasing number of studies have been conducted using Ba and Sr as tracers for environmental histories of fish, this is the first time that an attempt is made to apply them to aquatic mammals. We have proved that discriminating among aquatic environments (e.g. freshwater, large estuaries and coastal areas with minimal freshwater discharge) is possible based on differences in Ba/Ca and Ba/Sr values in dolphin teeth. However, due to the variable patterns of freshwater discharge, we stress the need of more detailed studies on local estuaries in order to validate the use of these ratios for studying individual environmental histories of local populations, especially for distinguishing movements from variations in freshwater inputs. Furthermore, studies under controlled conditions are needed in order to validate and quantify the extent of the relationship among ambient water and deposited elements in teeth. Nevertheless, results presented here encourage the application of this technique as a potential new tool for studying habitat use in aquatic mammals.

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Figure captions

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

Figure 2. Representative age estimation slide (a), laser scan (b) and age-related Ba/Ca profile (c) of a bottlenose dolphin (*Tursiops* sp.).

Figure 3. Ba/Ca, Sr/Ca and Ba/Sr in teeth of Amazon dolphins (*Inia geoffrensis*) from the Amazon River (AmR), Guiana dolphins (*Sotalia guianensis*) from the Amazon estuary (AmE) and from Ceará (CE). Relationship among values obtained by Laser Ablation ICP-MS and by solution-based ICP-MS.

Figure 4. Mean log(Ba/Ca) (a), log(Ba/Sr) (b) and log(Sr/Ca) (c) found in teeth dentine of freshwater and marine dolphins from Brazil and Uruguay.

Figure 5. Mean log(Ba/Ca) and log(Ba/Sr) in teeth dentine of dolphins from Brazil and Uruguay: Amazon River (AmR), Amazon Estuary (AmE), Ceará (CE), Espírito Santo (ES), Northern Rio de Janeiro (NRJ), Northern São Paulo (NSP), Southern São Paulo/Northern Paraná (SP/PR), Babitonga Bay (SC), Patos Lagoon estuary and adjacent coastal areas (RS) and La Plata River estuary and adjacent coastal areas, Uruguay (URU).

Figure 6. Tree diagram of areas from Brazil and Uruguay resulted from cluster analysis of log Ba/Ca and log Ba/Sr values from teeth of dolphins (see Table 1 for codes).

Figure 7. Tree diagram of species per areas from Brazil and Uruguay resulted from cluster analysis of log Ba/Ca and log Ba/Sr values from teeth of dolphins (see Table 1 for codes).

Figure 8. Individual profiles of age-related Ba/Ca (a) and Ba/Sr (b) values in teeth of bottlenose dolphins (*Tursiops* sp.) and a rough-toothed dolphin (*Steno bredanensis*).

Table 1. Teeth samples analyzed for Ba, Ca and Sr from freshwater/coastal areas in Brazil and Uruguay: Amazon River (AmR), Amazon Estuary (AmE), Ceará (CE), Espírito Santo (ES), Northern Rio de Janeiro (NRJ), Northern São Paulo (NSP), Southern São Paulo/Northern Paraná (SP/PR), Babitonga Bay (SC), Patos Lagoon estuary and adjacent coastal areas (RS) and La Plata River estuary and adjacent coastal areas, Uruguay (URU). Species included Amazon dolphins (*Inia geoffrensis*), the tucuxis (*Sotalia fluviatilis*), Guiana dolphins (*Sotalia guianensis*), franciscanas (*Pontoporia blainvillei*) and bottlenose dolphins (*Tursiops* sp.). Mean freshwater discharge (FD) of main freshwater inputs are indicated and expressed as m³s⁻¹.

Area	FD	Environment	Species	Age ranges	n
AmR		River	Amazon dolphin	-	16
			Tucuxi	-	25
AmE	180,000 ^a	Estuary	Guiana dolphin	-	16
CE	11 ^b	Coastal	Guiana dolphin	1-23	19
ES	1139 ^c	Coastal	Guiana dolphin	5-33	17
			Franciscana	1-7	5
NRJ	796 ^d	Coastal	Guiana dolphin	6-30	21
			Franciscana	3-9	19
NSP	24-127 ^e	Coastal	Franciscana	2-14	19
SP/PR	435 ^f	Estuary	Guiana dolphin	3-29	17
			Franciscana	2-18	16
SC	<20 ^g	Estuary (bay)	Guiana dolphin	2-24	13
			Franciscana	1-10	8
RS	2000 ^h	Estuary	Bottlenose dolphin	5-30	17
			Franciscana	3-8	27
URU	23000 ⁱ	Estuary	Franciscana	1-7	24

^aAmazon River (Geyer et al. 1996)

^bPacoti River, Fortaleza (Lacerda et al. 2007)

^cDoce River (Coelho 2006)

^dParaíba do Sul River (Nicolite et al. 2009)

^eBaixada Santista Estuary (Moser et al. 2005)

^fCananéia Estuary (Schaeffer-Noveli et al. 1990)

^gBabitonga Bay (Barros et al. 2010)

^hPatos Lagoon Estuary (Calliari et al. 2009)

ⁱLa Plata River Estuary (Piola and Romero 2004)

Table 2. Mean Ba/Ca, Ba/Sr and Sr/Ba in teeth of Amazon dolphins (*Inia geoffrensis*), tucuxis (*Sotalia fluviatilis*), Guiana dolphins (*Sotalia guianensis*), franciscanas (*Pontoporia blainvillei*) and bottlenose dolphins (*Tursiops truncatus*) from freshwater and marine coastal regions in Brazil and Uruguay (see Table 1 for codes).

Area	Ba/Ca ($\pm$ SD)	Ba/Sr ($\pm$ SD)	Sr/Ca ($\pm$ SD)	n
AmR				
Amazon dolphin	0.18 ($\pm$ 0.17)	1.29 ($\pm$ 0.49)	0.15 ($\pm$ 0.15)	17
Tucuxi	0.17 ($\pm$ 0.10)	1.07 ($\pm$ 0.28)	0.16 ($\pm$ 0.10)	24
Mean freshwater	0.17 ($\pm$0.29)	1.16 ($\pm$0.39)	0.16 ($\pm$0.12)	41
AmE				
Guiana dolphin	0.014 ($\pm$ 0.012)	0.121 ($\pm$ 0.114)	0.125 ($\pm$ 0.045)	16
CE				
Guiana dolphin	0.005 ($\pm$ 0.003)	0.041 ($\pm$ 0.023)	0.126 ($\pm$ 0.014)	19
ES	0.012 ($\pm$ 0.009)	0.100 ($\pm$ 0.075)	0.125 ($\pm$ 0.046)	22
Guiana dolphin	0.012 ($\pm$ 0.010)	0.114 ($\pm$ 0.079)	0.101 ($\pm$ 0.014)	17
Franciscana	0.011 ($\pm$ 0.005)	0.053 ($\pm$ 0.022)	0.204 ($\pm$ 0.011)	5
NRJ	0.008 ($\pm$ 0.005)	0.068 ($\pm$ 0.032)	0.126 ($\pm$ 0.070)	40
Guiana dolphin	0.006 ($\pm$ 0.003)	0.080 ($\pm$ 0.031)	0.073 ($\pm$ 0.021)	21
Franciscana	0.010 ($\pm$ 0.006)	0.054 ($\pm$ 0.027)	0.184 ($\pm$ 0.059)	19
NSP				
Franciscana	0.004 ($\pm$ 0.001)	0.041 ($\pm$ 0.011)	0.088 ($\pm$ 0.019)	19
SP/PR	0.008 ($\pm$ 0.006)	0.073 ($\pm$ 0.029)	0.105 ($\pm$ 0.051)	33
Guiana dolphin	0.010 ($\pm$ 0.008)	0.078 ($\pm$ 0.026)	0.123 ($\pm$ 0.061)	17
Franciscana	0.006 ($\pm$ 0.004)	0.067 ($\pm$ 0.032)	0.086 ($\pm$ 0.029)	16
SC	0.006 ($\pm$ 0.003)	0.056 ($\pm$ 0.020)	0.109 ($\pm$ 0.037)	21
Guiana dolphin	0.006 ($\pm$ 0.004)	0.055 ($\pm$ 0.021)	0.109 ($\pm$ 0.046)	13
Franciscana	0.006 ($\pm$ 0.003)	0.058 ($\pm$ 0.020)	0.109 ($\pm$ 0.016)	8
RS	0.017 ($\pm$ 0.011)	0.124 ($\pm$ 0.066)	0.135 ($\pm$ 0.030)	44
Bottlenose dolphin	0.018 ($\pm$ 0.015)	0.125 ($\pm$ 0.081)	0.138 ($\pm$ 0.025)	17
Franciscana	0.016 ($\pm$ 0.008)	0.123 ($\pm$ 0.057)	0.134 ($\pm$ 0.033)	27
URU				
Franciscana	0.022 ($\pm$ 0.017)	0.200 ($\pm$ 0.134)	0.112 ($\pm$ 0.033)	24
Mean marine	0.011 ($\pm$0.010)	0.094 ($\pm$0.079)	0.118 ($\pm$0.046)	243

Table 3. Tukey`s HSD test p values of multiple comparisons of Ba/Ca and Ba/Sr values for teeth dentine of marine dolphins from Amazon Estuary (AmE), Ceará (CE), Espírito Santo (ES), Northern Rio de Janeiro (NRJ), Northern São Paulo (NSP), Southern São Paulo/Northern Paraná (SP/PR), Babitonga Bay (SC), Patos Lagoon estuary and adjacent coastal areas (RS) and La Plata River estuary and adjacent coastal areas, Uruguay (URU). Up-right values from Ba/Sr comparisons and down-left values from Ba/Ca comparisons.

	AmE	CE	ES	NRJ	NSP	SP/PR	SC	RS	URU
AmE		**	1.00	0.19	**	0.63	*	0.97	*
CE	**		**	*	1.00	**	0.46	**	**
ES	1.00	**		0.48	**	0.93	0.14	0.51	**
NRJ	0.14	0.44	0.31		0.10	1.00	0.98	**	**
NSP	**	0.87	**	**		*	0.78	**	**
SP/PR	0.13	0.57	0.29	1.00	*		0.76	**	**
SC	*	0.99	0.06	0.97	0.27	0.99		**	**
RS	0.79	**	0.22	**	**	**	**		0.09
URU	0.30	**	*	**	**	**	**	0.97	

*p<0.05

**p<0.001

Table 4. Tukey`s HSD test p values of multiple comparisons of Ba/Ca and Ba/Sr values for teeth dentine of Guiana dolphins, *Sotalia guianensis* (SG), franciscanas, *Pontoporia balinvillei* (PB) and bottlenose dolphins, *Tursiops* sp. (TT) from Amazon Estuary (AmE), Ceará (CE), Espírito Santo (ES), Northern Rio de Janeiro (NRJ), Northern São Paulo (NSP), Southern São Paulo/Northern Paraná (SP/PR), Babitonga Bay (SC), Patos Lagoon estuary and adjacent coastal areas (RS) and La Plata River estuary and adjacent coastal areas, Uruguay (URU). Up-right values from Ba/Sr comparisons and down-left values from Ba/Ca comparisons.

		AmE	CE	ES		NRJ		NSP	SP/PR		SC		RS		URU
		SG	SG	SG	PB	SG	PB	PB	SG	PB	SG	PB	TT	PB	PB
AmE	SG		**	1.00	0.54	1.00	*	**	1.00	0.64	0.13	0.63	1.00	1.00	0.06
CE	SG	**		**	1.00	**	0.94	1.00	**	0.19	0.91	0.84	**	**	**
ES	SG	1.00	*		0.43	0.98	*	**	0.99	0.46	0.07	0.49	1.00	1.00	0.09
	PB	1.00	0.35	1.00		0.95	1.00	1.00	0.96	1.00	1.00	1.00	0.21	0.08	**
NRJ	SG	*	1.00	0.14	0.68		0.35	*	1.00	1.00	0.71	0.99	0.78	0.32	**
	PB	0.99	0.10	1.00	1.00	0.46		1.00	0.45	0.99	1.00	1.00	**	**	**
NSP	PB	**	0.97	**	*	0.56	**		*	0.47	0.99	0.96	**	**	**
SP/PR	SG	0.99	0.17	1.00	1.00	0.59	1.00	**		1.00	0.78	0.99	0.82	0.41	**
	PB	*	1.00	0.10	0.58	1.00	0.37	0.85	0.48		1.00	1.00	0.16	*	**
SC	SG	0.06	1.00	0.24	0.71	1.00	0.60	0.82	0.70	1.00		1.00	*	**	**
	PB	0.59	1.00	0.87	0.97	1.00	0.99	0.62	0.99	1.00	1.00		0.21	0.06	**
RS	TT	0.99	**	0.79	1.00	**	0.31	**	0.29	**	**	*		1.00	0.35
	PB	0.96	**	0.60	0.99	**	0.13	**	0.13	**	**	*	1.00		0.42
URU	PB	0.47	**	0.11	0.86	**	*	**	*	**	**	**	1.00	1.00	

*p<0.05

**p<0.001

Figure 1.

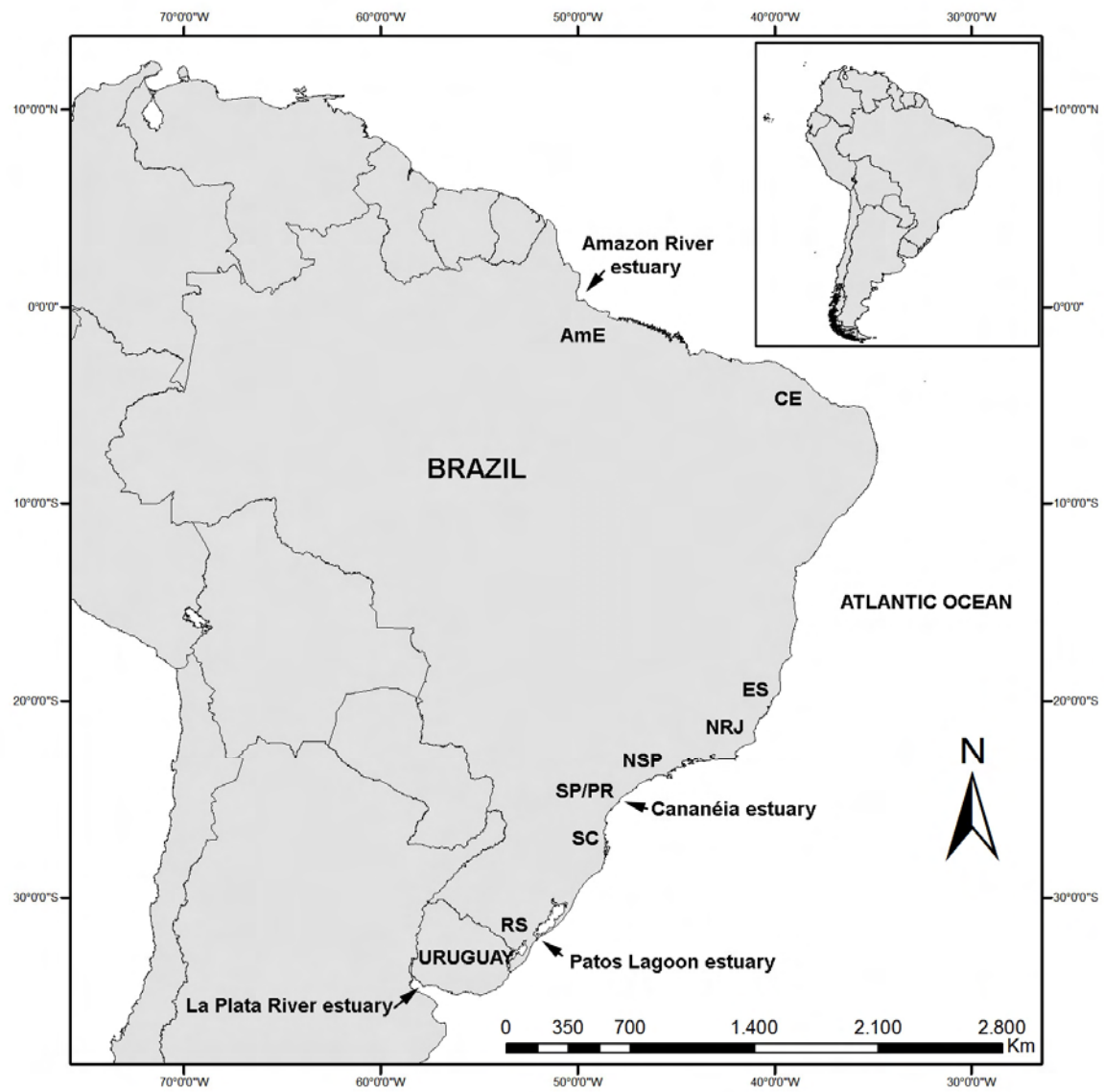


Figure 2

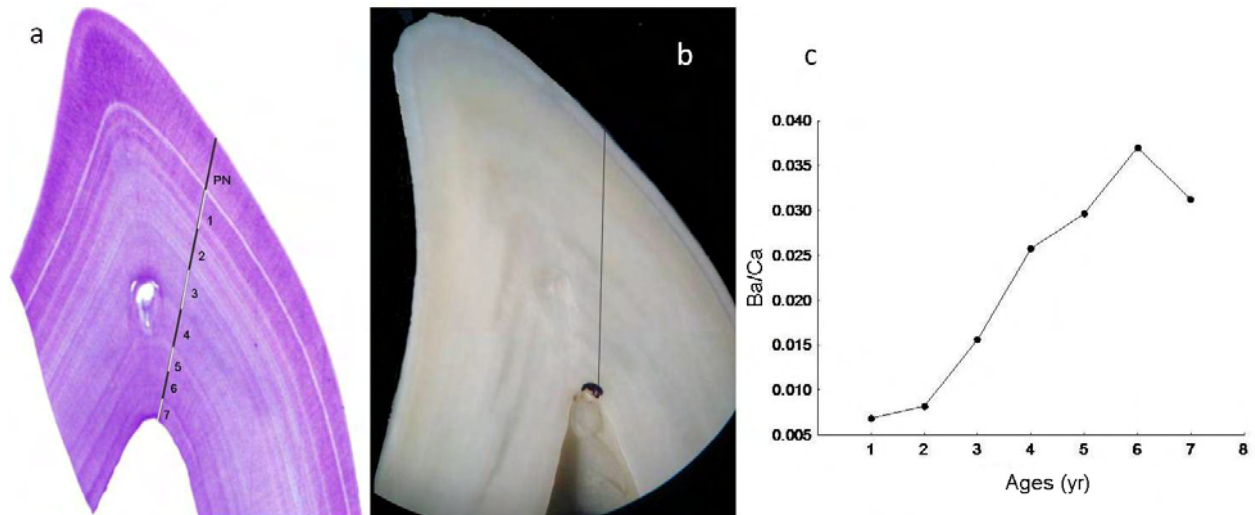


Figure 3.

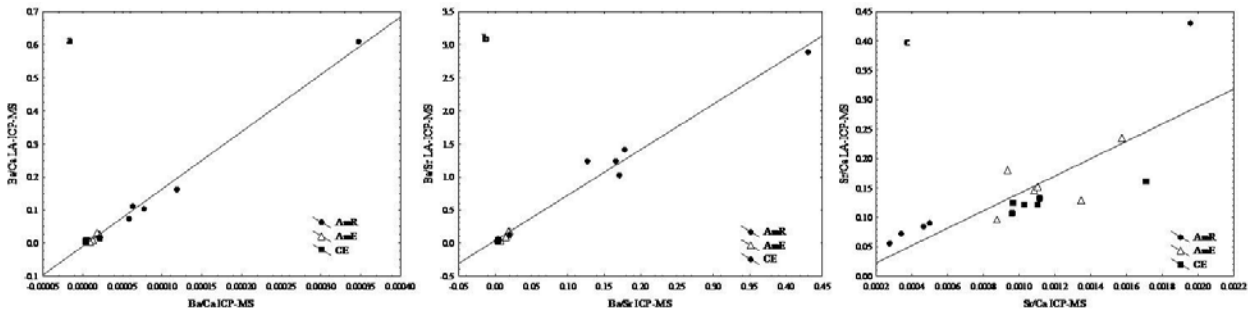


Figure 4

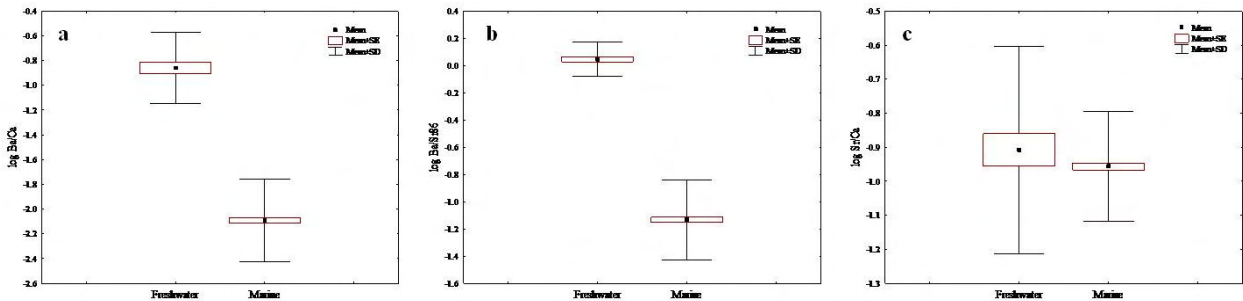


Figure 5 .

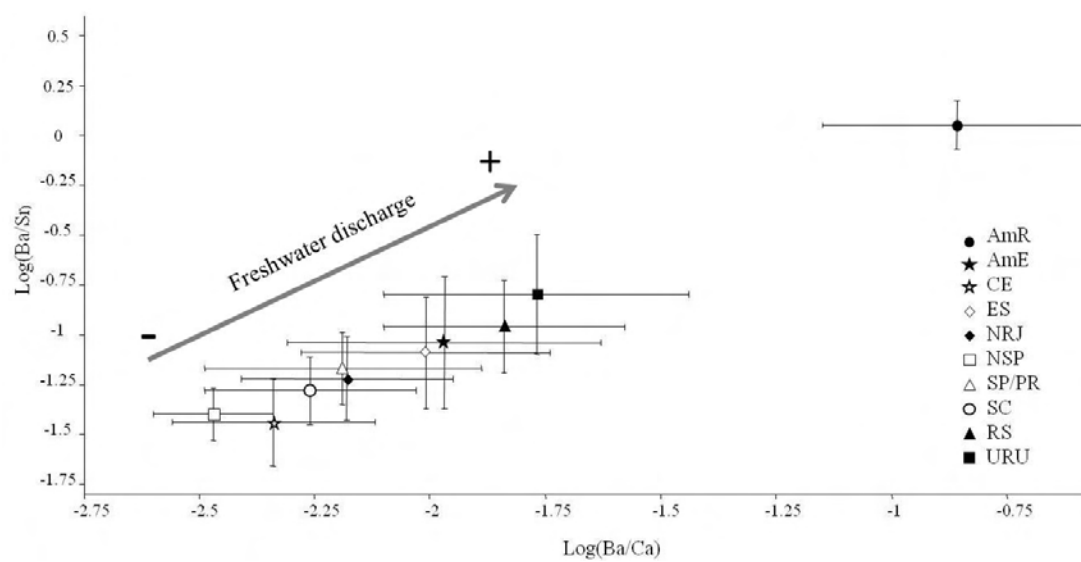


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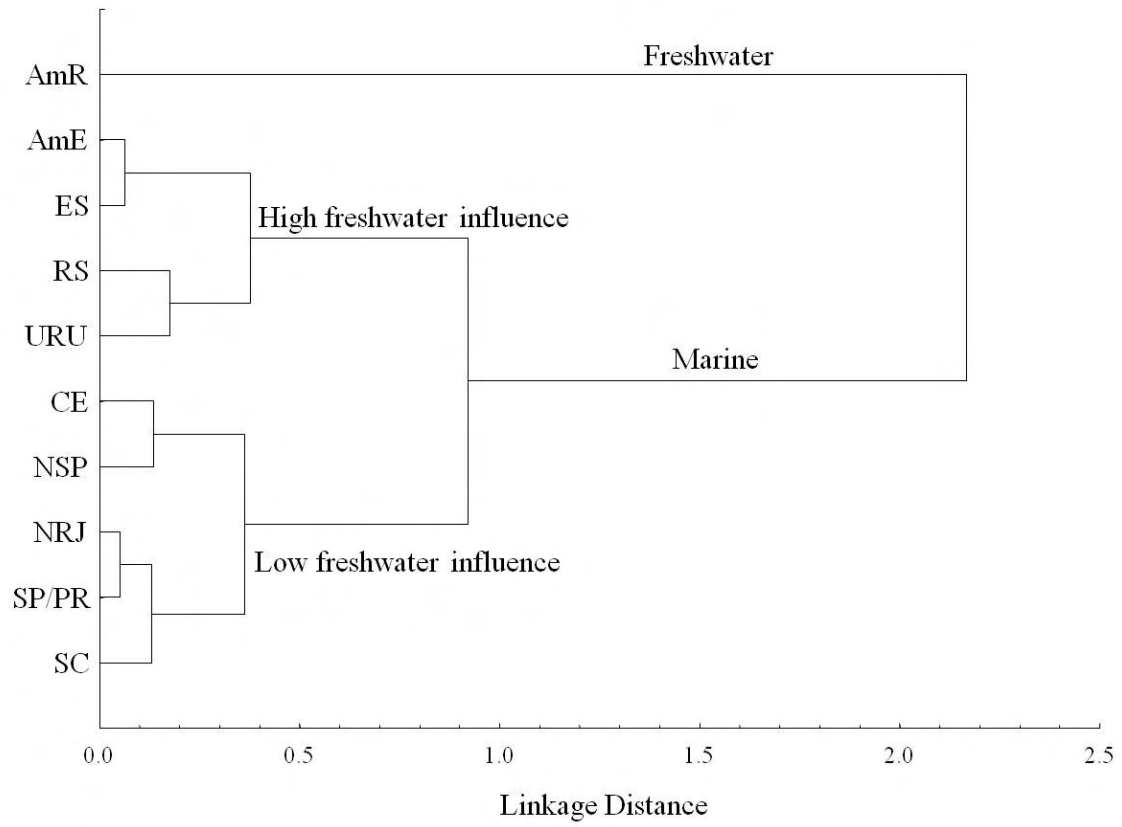


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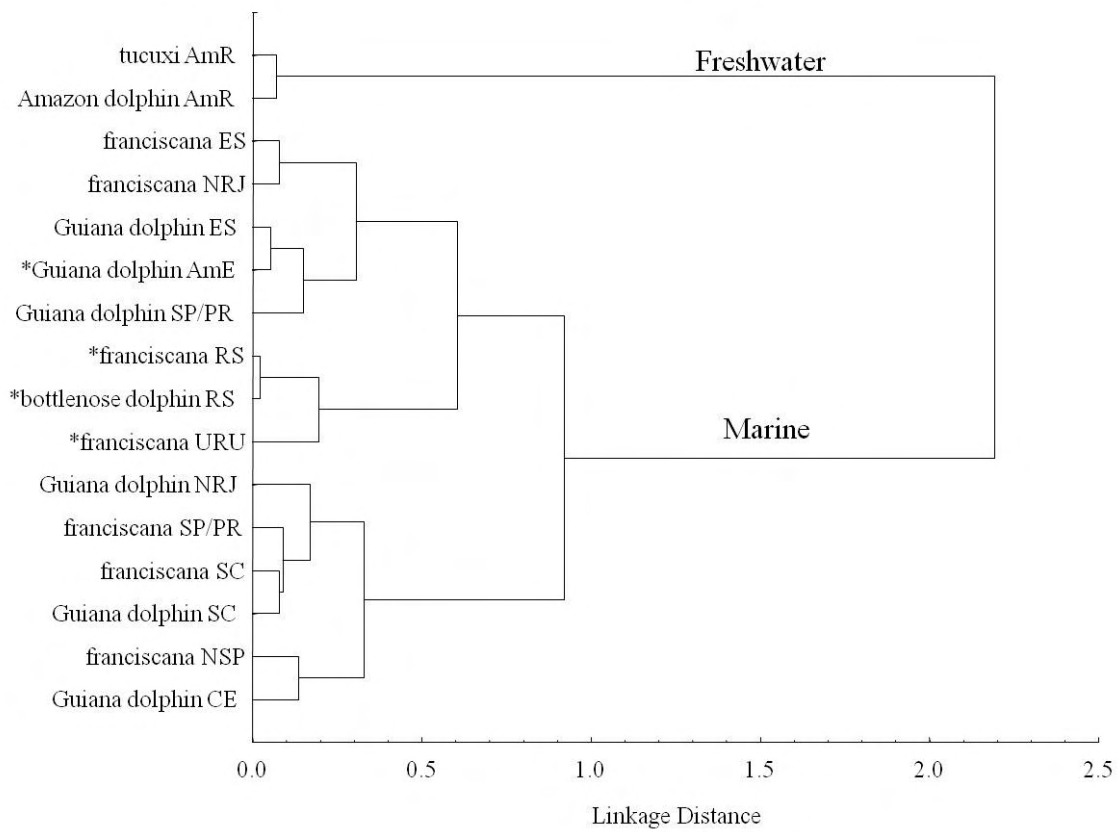
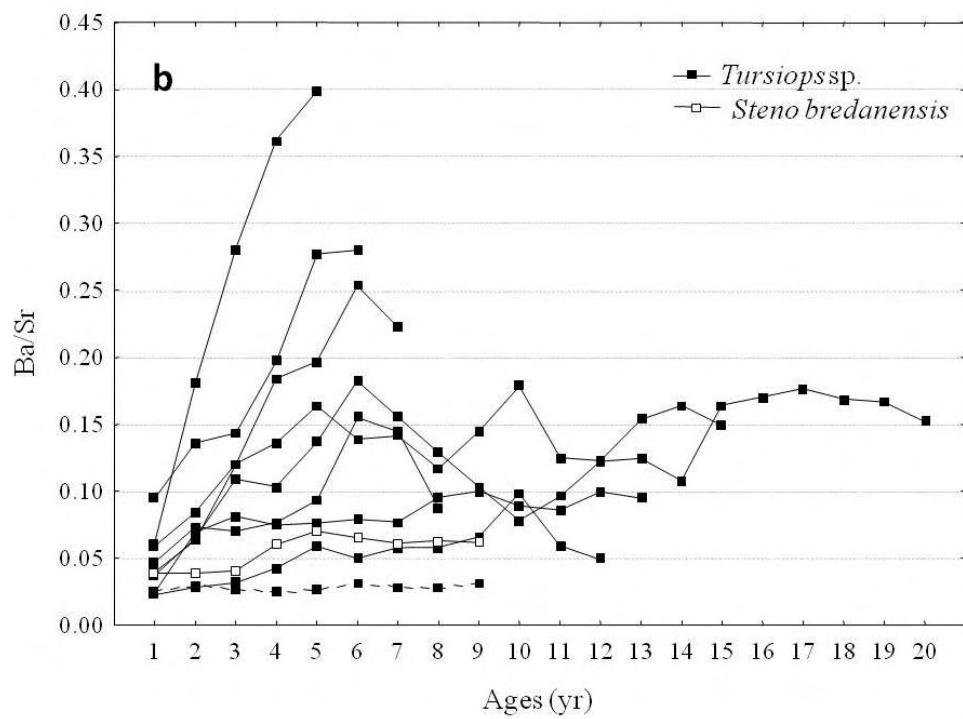
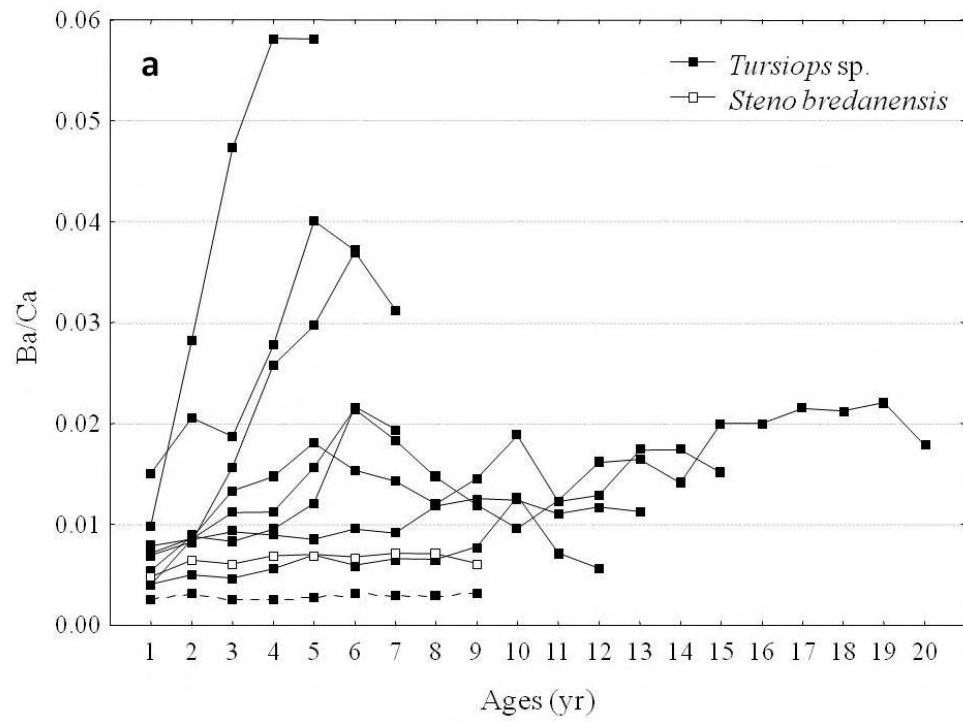


Figure 8.



Apêndice 1

Instituições e grupos de pesquisa parceiros do projeto, espécies cujas amostras foram cedidas, área de coleta e responsável pela colaboração.

Instituição/Grupo	Espécies	Área	Responsável
INPA	<i>Inia geoffrensis</i> <i>Sotalia fluviatilis</i> <i>Sotalia guianensis</i>	Amazonas Pará	Dra. Vera Maria da Silva
AQUASIS (ONG)	<i>Sotalia guianensis</i>	Ceará	Dra. Ana Carolina Meirelles
ORCA (ONG)	<i>Sotalia guianensis</i> <i>Pontoporia blainvillei</i>	Espírito Santo	Lupércio Barbosa
UENF	<i>Sotalia guianensis</i> <i>Pontoporia blainvillei</i>	Rio de Janeiro	Dra. Ana Paula Madeira Di Beneditto
BIOPESCA (ONG)	<i>Pontoporia blainvillei</i>	São Paulo	Dra. Carolina Bertozzi
USP	<i>Sotalia guianensis</i> <i>Pontoporia blainvillei</i> <i>Tursiops truncatus</i>	São Paulo	Dr. Marcos César de Oliveira Santos
UNIVILLE	<i>Sotalia guianensis</i> <i>Pontoporia blainvillei</i>	Santa Catarina	Dra. Marta Cremer
Cetáceos Uruguay/Universidad de la República	<i>Pontoporia blainvillei</i>	Uruguai	M.Sc. Valentina Franco-Trecu
Cethus (ONG)	<i>Pontoporia blainvillei</i>	Argentina	Miguel Iñiguez Mauricio Failla
MACN	<i>Pontoporia blainvillei</i>	Argentina	Dr. Luis Capozzo