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ABSTRACT

Franciscanas are small coastal cetaceans threatened by human activities, such as by-catch in driftnet fisheries. During the last two decades, research and conservation actions for this species have been delineated based on management units named Franciscana Management Areas (FMA). Recently, genetic data provided preliminary evidence that FMAII comprises two population units. The present study presents new evidence in favour of subdivision of FMAII from genetic, organochlorine compounds and stable isotopes data.

INTRODUCTION

The franciscana (*Pontoporia blainvillei*) is a small coastal cetacean that is threatened by human activities, notably incidental capture in fishing gear (Crespo 2009, Secchi 2010, Zerbini *et al.* 2017). Research and conservation actions for the franciscana have been devised based on Franciscana Management Areas (FMA), which were first proposed in 2003, based on multiple lines of evidence, such as genetic, morphology, life history and parasite load data (Secchi *et al.* 2003). Since then, genetic data have been used to refine the number and limits of FMA (Lázaro *et al.* 2004, Mendez *et al.* 2008, 2010, Costa-Urrutia *et al.* 2012, Cunha *et al.* 2014, Gariboldi *et al.* 2016).

Cunha *et al.* (2014) was the only study that analysed the entire range of the species, using mitochondrial control region sequences. One of the main conclusions was a deep evolutionary break between franciscanas from Espirito Santo and northern Rio

de Janeiro and those from southern Rio de Janeiro southwards to Argentina. That divergence justified the split of the species in two Evolutionarily Significant Units (ESU), the North ESU (Espírito Santo, ES and northern Rio de Janeiro, RJN) and the South ESU (southern Rio de Janeiro, RJS to Argentina, ARG). In addition, analyses revealed population differentiation in FMAI, between franciscanas from ES and RJN, and in FMAII, between franciscanas from SPN+RJS and SPC to SC (Figure 1). Due to the low number of samples from SPN (N = 8) and RJS (N = 2) available at the time, results were considered preliminary. Nevertheless, taking into account that franciscanas face a high risk of extinction, and that the area is under considerable human impact, the authors proposed the subdivision of FMAII in two management units, FMAIIa (RJS and SPN) and FMAIIb (SPC to SC), until new data could be analysed.

The presence of franciscanas in RJS was unknown until 2002 (Azevedo *et al.* 2002). Records of the species in this region remained scarce until recently, when 16 franciscanas stranded in RJS, between 2017 and 2019 (Azevedo AF and Lailson-Brito J, *personal observation*). Those new samples were incorporated in genetic, contaminant and stable isotope analyses, which also included samples from SPC and other localities, with the aim of testing the hypothesis of population differentiation between FMAIIa and FMAIIb.

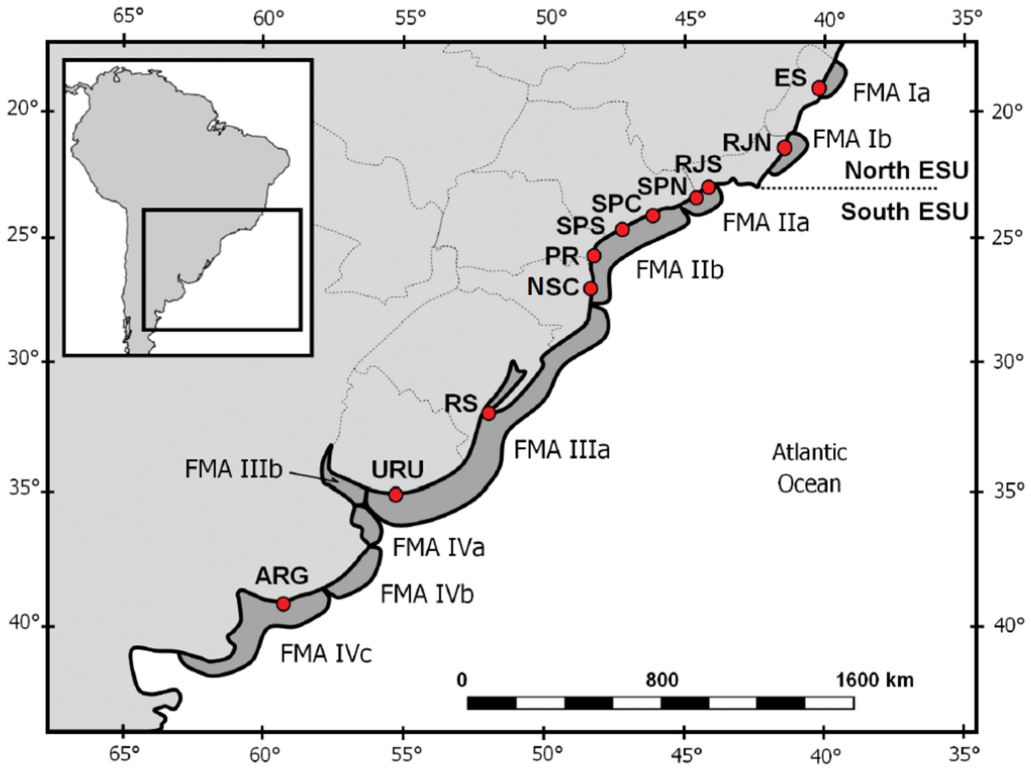

Figure 1: The FMA proposal of Secchi *et al.* (2003) as refined by Cunha *et al.* (2014). ES: Espírito Santo; RJN: northern Rio de Janeiro; RJS: southern Rio de Janeiro; SPN: northern São Paulo; SPC: central São Paulo; SPS: southern São Paulo; PR: Paraná; NSC: northern Santa Catarina; RS: Rio Grande do Sul; URU: Uruguay; ARG: Argentina. From Cunha *et al.* (2014)

MATERIALS AND METHODS

Sampling

Samples were collected from stranded or by-caught franciscanas. For genetic analysis, eight new skin samples collected in RJS were used. Published sequences from RJS and other localities were also included in genetic analyses (ES = 14, RJN = 10, RJS = 2, SPN = 8, SPC = 19, SPS = 7, NSC = 17, RS = 15, URU = 38, ARG = 31). For organochlorine analyses, blubber samples from 34 franciscanas were used (RJS, N = 9; SPC, N = 25). Muscle from 27 individuals were used for determination of stable carbon and nitrogen isotopes (RJS, N = 7; SPC, N = 20).

Genetic analyses

DNA was extracted using the phenol/chloroform protocol (Sambrook *et al.* 1989). A fragment of the mitochondrial DNA control region was amplified using the primers RCPb-F and RCPb-R, according to the protocol described by Cunha *et al.* (2014). PCR products were purified and sequenced in both directions in an ABI3500xl DNA Analyzer (Applied Biosystems). Sequences were edited with program SeqMan 7 (Lasergene Inc.) and visually aligned in MEGA 5.0 (Tamura *et al.* 2007).

Previously published sequences (Secchi *et al.* 1998; Cunha *et al.* 2014) were added to the alignment. Diversity indices (h, π) were estimated in DnaSP (Librado and Rozas 2009). Population structure analyses (pairwise F_{ST}, ϕ_{ST} and AMOVA) were done in Arlequin 3.5 (Excoffier and Lischer 2010). A median joining haplotype network was constructed using PopArt (Leygh and Bryant 2015).

Analysis of organochlorine compounds

Methodology was adapted from Lailson-Brito et al. (2010). Briefly, 0.5g of blubber was spiked with internal standards (ISTD; PCB 103 + PCB 198) and extracted via soxhlet using dichloromethane and n-hexane (1:1) for 8 hours. An aliquot was used to determine the lipid content gravimetrically. The extract underwent an acidic clean up, followed by elution in neutral aluminum oxide column in two steps: with dichloromethane and n-hexane (2:1) and dichloromethane and methanol (9:1). Analyses of organochlorine contaminants were performed in a Gas Chromatographer (Agilent Technologies 6890) with an automatic injector (Agilent Technologies 7683B) coupled to a Mass Spectrometer (Agilent Technologies 5975) operating in the electrons impact (EI) source on Selected Ion Monitoring (SIM) mode. Chromatograms were analyzed in the Enhanced ChemStation® software (Agilent Technologies). Finally, the integration results were transformed based on the lipid weight of each sample. Thirty five organochlorinated compounds were determined in the present study, among them 27 PCB congeners (IUPAC numbers: 8, 28, 31, 44, 49, 52, 70, 74, 97, 99, 101, 105, 118, 132, 138, 141, 151, 153, 158, 169, 170, 177, 180, 183, 194, 195 and 206) and eight pesticides (p.p'-DDT, p.p'-DDE and p.p'-DDD; mirex; HCB; α-HCH, β-HCH and γ-HCH).

An analytical blank was added to the analysis in order to detect possible contamination sources from the procedure. No compounds were detected in the blank.

Mean ISTD recovery IN this study was 91.0% ± 8.7%. The compounds herein analyzed were validated based on Standard Reference Material® 1945 (SRM®1945, NIST), pilot-whale (*Globicephala melas*) adipose tissue, and recovery for each compound was accepted between 65 and 135%. Ultimately, detection and quantification limits (DL and QL) for each compound were calculated. Organochlorine compounds found in concentrations above the QL were considered for statistical analysis.

A discriminant function analysis was performed using all individual compounds (PCB congeners, DDT and its metabolites, HCB and HCH isomers) for investigating possible differences in the organochlorine accumulation patterns of franciscanas from Rio de Janeiro (RJS) and São Paulo (SPC). From 35 compounds (PCBs, DDTs, HCB and HCHs), 24 were used in the test, since they fulfilled the requirements of the model.

Analysis of $\delta^{13}C$ and $\delta^{15}N$

Muscle samples were dried at 60°C for 72h and then ground into a homogeneous powder. Dried samples (0.3 mg) were weighed and placed in tin capsules, and carbon and nitrogen stable isotope measurements were performed on a DELTA V isotope ratio mass spectrometer coupled to an Flash 2000 NCS elemental analyzer (Thermo Fisher Scientific). Stable isotope ratios were expressed in delta notation as parts per thousand according to the following equation: $\delta X = [(R_{sample}=R_{standard}) -1] \times 1000$, where X is ^{13}C or ^{15}N and R is the corresponding ratio of $^{13}C/^{12}C$ or $^{15}N/^{14}N$. Carbon and nitrogen ratios were expressed in relationship to the V-PDB standard and to atmospheric nitrogen, respectively. Reference materials (USGS24 and IAEA-N2) were also analyzed. The standard deviation on replicated measurements from a single sample was ±0.3%. Because lipids have been shown to be depleted in ^{13}C and lipid tissue content can be variable, we measured the elemental content and calculated the sample C:N ratio to verify the lipid content of each sample (Post *et al.*, 2007). Only one sample presented C:N > 3.5; therefore, we normalized the $\delta^{13}C$ values according to the following equation (Post *et al.*, 2007): $\delta^{13}C_{normalized} = \delta^{13}C_{untreated} - 3.32+0.99*C:N$.

RESULTS

Genetic analyses

In total, 168 sequences were analysed. Sequences were 455bp-long and 41 polymorphic sites were observed, defining 33 haplotypes. The new sequences from RJS belonged to two haplotypes. The haplotype network shows that all franciscanas from RJS and all but one from SPN belong to haplotypes that are closely related (Figure 2). Concerning the new sequences from RJS, one individual had the same haplotype observed before in two franciscanas from RJS, and from other localities in FMAII (SPN and SPC). The other seven franciscanas from RJS shared a haplotype also previously found in individuals from FMAII (SPN, SPC and NSC). Haplotypes found in FMAIIa and FMAIIb tend to form a cluster, but some individuals from FMAIIb have haplotypes closer to those observed in FMAIII and FMAIV (Figure 2).

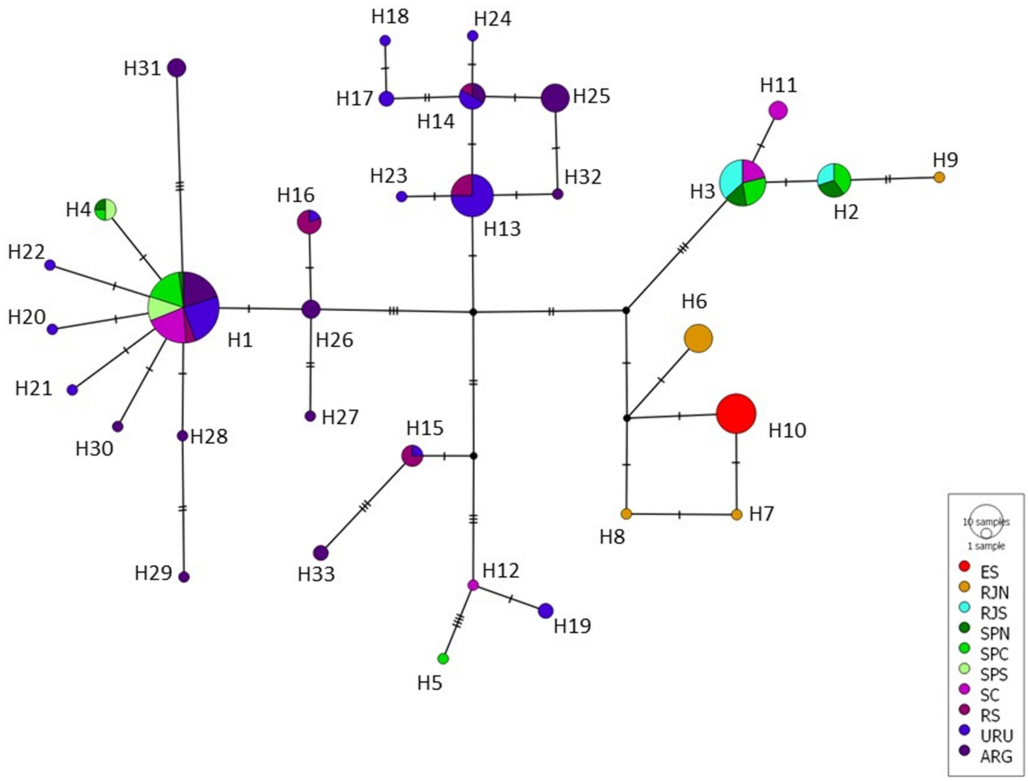

Figure 2: Median-joining haplotype network (455bp, N = 168) of mtDNA control region sequences of franciscanas.

Because the goal of this study was to test differentiation within the South ESU, sequences from the North ESU (ES and RJN) were not included in population structure analyses. Pairwise F_{ST} analysis suggested four populations: ARG, URU+RS, NSC+SPS+SPC, and SPN+RJS (Table 1). Φ_{ST} results failed to detect differentiation between ARG, URU and RS (FMAIV and FMAIII), but showed separation of NSC+SPS+SPC, and SPN+RJS. The two analyses thus agreed about the genetic distinctiveness of FMAIIa and FMAIIb.

AMOVA analysis rejected the hypothesis of panmixia in the South ESU (RJS, SPN, SPC, SPS, NSC, RS, URU and ARG; Φ_{ST} = 0.251, P = 10^{-5}). When all the possible subdivisions were tested, the scenario with higher significant Φ_{CT} was the one separating FMAIIa (RJS and SPN) from all other localities (FMAIIb, FMAIII, and FMAIV) (Φ_{CT} = 0.340, P = 0.034). None of the scenarios that considered RJS and SPN as part of a single population including SPC, SPS and NSC, was significant (data not shown). The hypothesis of four populations corresponding to FMAIIa, FMAIIb, FMAIII and FMAIV had a relatively low but significant Φ_{CT} (Φ_{CT} = 0.224, P = 0.015) (Table 2).

Table 1: Pairwise F_{ST} (top diagonal) and Φ_{CT} (lower diagonal) values. Significant values after Bonferroni correction are in bold (alfa = 0.0018).

	ARG	URU	RS	NSC	SPS	SPC	SPN	RJS
ARG		**0.078**	**0.116**	0.091	0.114	0.084	0.140	**0.296**
URU	0.044		0.032	0.114	0.138	0.107	**0.163**	**0.313**
RS	0.061	0.014		**0.195**	**0.247**	**0.168**	**0.178**	**0.340**
SC	0.151	**0.216**	0.175		0.062	0.013	0.148	**0.306**
SPS	0.142	0.302	0.388	0.266		0.084	0.273	**0.529**
SPC	**0.182**	**0.235**	0.174	0.001	0.294		0.001	0.178
SPN	**0.346**	**0.382**	**0.322**	0.093	0.627	0.013		0.005
RJS	**0.558**	**0.576**	**0.598**	0.408	**0.938**	**0.306**	0.112	

Table 2: Detailed AMOVA results for the six scenarios with highest significant Φ_{CT}.

	Sum of squares	Variance components	Percentage Variation	Φ Statistics	P
a) 2 populations: AR+UR+RS+SC+SPS+SPC/SPN+RJS				0.340	0.034
Among groups	43.801	1.20276	34		
Among populations/within groups	46.879	0.34927	9.87		
Within populations	270.049	1.98566	56.13		
b) 5 populations: AR+UR+RS / SC/ SPS/SPC/ SPN+RJS				0.273	0.006
Among groups	82.416	0.77781	27.28		
Among populations/within groups	11.264	0.08764	3.07		
Within populations	270.049	1.98566	69.65		
c) 3 populations: AR+UR+RS/SC+SPS+SPC/ SPN+RJS				0.267	0.003
Among groups	71.441	77546	26.72		
Among populations/within groups	22.238	0.14102	4.86		
Within populations	270.049	1.98566	68.42		
d) 4 populations: AR+UR+RS / SC+SPS/ SPC/ SPN+RJS				0.247	0.004
Among groups	74.891	0.70157	24.69		
Among populations/within groups	18.789	0.15391	5.42		
Within populations	270.049	1.98566	69.89		
e) 3 populations: AR+UR+RS/SC+SPS/SPC+SPN+RJS				0.226	0.001
Among groups	65.051	0.64267	22.58		
Among populations/within groups	28.629	0.22159	7.77		
Within populations	270.049	1.98566	69.65		
f) 4 populations: AR/UR+RS / SC+SPS+ SPC/ SPN+RJS				0.224	0.015
Among groups	78.016	0.61155	22.36		
Among populations/within groups	15.664	0.13764	5.03		
Within populations	270.049	1.98566	72.61		

Analysis of organochlorine compounds

PCBs were the predominant compounds in franciscanas from both RJS and SPC, followed by DDTs, Mirex and HCB. For specimens from RJS (n=9), median values were: 6706 ng.g^{-1} lip for ∑PCB, 2117 ng.g^{-1} lip for ∑DDT, 46 ng.g^{-1} lip for Mirex and 27 ng.g^{-1} lip for HCB. For SPC (n=25), median values were: 4955 ng.g^{-1} lip for ∑PCB, 1787 ng.g^{-1} lip for ∑DDT, 45 ng.g^{-1} lip for Mirex and 41 ng.g^{-1} lip for HCB. This pattern was similar to that reported in other studies with cetaceans from Brazilian waters (e.g. Alonso *et al.* 2010, Lailson-Brito *et al.* 2011, Santos-Neto *et al.* 2014).

Despite the similar pattern, individuals from FMAIIa (RJS) and FMAIIb (SPC) presented significantly different profiles for organochlorine compounds accumulation (Wilks Lambda = 0.22; F (13.20) = 5.28; p = 0.0005). Amongst the 24 variables input in the model, 13 were accepted (*pp*-DDD, *pp*-DDT, *pp*-DDE, Mirex, HCB, PCB 28, PCB 52, PCB 49, PCB 74, PCB 99, PCB 132, PCB 105 e PCB 177). The variables with stronger effect in group separation along canonical axis 1 were: positively – PCB 28 and PCB 49; and negatively – Mirex and PCB 52.

The *Mahalanobis* square distance between the two groups was 16.62 (F= 5.28, p=0.0004). Classification was correct for 97.05% of the samples, and only one individual collected in RJS had a profile similar to that from SPC.

Analysis of $\delta^{13}C$ and $\delta^{15}N$

The values of carbon and nitrogen stable isotopes were different between the two areas (RJS and SPC; Table 3). Franciscanas from FMAIIa showed 13C-depleted values compared to specimens from FMAIIb (-16.5‰; Student´s T-Test, t = -3.9, p=0.0007, n=7 e n=20, respectively). Also Franciscanas from FMAIIa displayed higher mean δ15N value (14.4 ‰) than FMAIIb specimens (13.8 ‰; t = -2.3, p = 0.03).

Table 3: Mean (±SD) $\delta^{13}C$ and $\delta^{15}N$ values in franciscana muscle from FMAIIa and FMAIIb, southeastern Brazil.

area	n	δ13C (‰)		δ15N (‰)	
		mean±SD	min/max	mean±SD	min/max
FMAIIa (RJS)	7	-17.1±0.4	-17.6/-16.6	14.4±0.9	13.3/15.5
FMAIIb (SPC)	20	-16.5±0.3	-17.1/-15.6	13.8±0.2	13.4/14.3

DISCUSSION

The subdivision of FMAII was first suggested by Cunha *et al.* (2014), based on a smaller sample size. Considering the relevance of that finding for the conservation of franciscanas, the authors proposed the recognition of FMAIIa and FMAIIb, until further analyses could be performed. The present study aimed to test that hypothesis using a larger sample size for genetic analyses, as well as organic pollutants and stable isotope

ratios data. All the analyses supported the genetic and ecological differentiation of franciscanas from FMAIIa and FMAIIb.

Genetic analyses confirmed that specimens from RJS and SPN do not belong to the same population that inhabits the area from SPC to NSC. This conclusion was supported by pairwise F_{ST} and Φ_{ST}, and AMOVA analyses. Consistent separation of the two areas in all analyses was also reported by Cunha *et al.* (2014), but in the present study the AMOVA scenario with higher support showed the separation of FMAIIa from the others, but not the differentiation of FMAIIb from FMAIII and FMAIV. It means that the separation of RJS and SPN (FMAIIa) had higher support then other accepted divisions.

Organochlorine compounds profiles and stable carbon and nitrogen isotopes provided evidence that franciscanas from RJS and SPC belong to two ecological populations, further justifying the recognition of FMAIIa and FMAIIb. The discriminant function analysis suggests that franciscanas from the RJS and SPC feed on different areas (Aguilar 1987, Lailson *et al.* 2010), since food intake is the main entry route for organochlorine compounds in cetaceans (Ross *et al.* 2000, Hansen *et al.* 2004). Likewise, stable isotopes also showed that franciscanas from RJS and SPC have different foraging habitats, with specimens from RJS having ^{13}C-depleted values. $\delta^{13}C$ values point to two distinct ecological populations in FMAIIa and FMAIIb areas.

The abundance of franciscanas in FMAII was estimated through aerial surveys during 2008/2009 (Sucunza *et al.* 2020). The estimates were 1,915 (CV = 0.32) for FMAIIa, and 4,353 (CV = 0.24) for FMAIIb. The authors used data on by-catch to calculate a minimum mortality in FMAII at that time. The by-catch data, despite incomplete, resulted in an unsustainable mortality of 4.4 to 7.3% of the census size (Sucunza *et al.* 2020). Taking into account that the available data is from a decade ago and that since then abundance has probably declined while by-catch did not decrease, the conservation of franciscanas in FMAII is at risk, even disregarding other threats that exist in the area, such as habitat degradation caused by human developments, chemical pollution and noise. With the recognition of those two FMA, research can be designed to assess demographic information for each, enabling the evaluation of their vulnerability and the definition of proper conservation actions.

REFERENCES

Aguilar A (1987) Using organochlorine pollutants to discriminate marine mammal populations: a review and critique of the methods. Marine Mammal Science, 3(3), 242-262.

Alonso MB, Marigo J, Bertozzi CP, Santos MCO, Taniguchi S, Montone RC (2010) Occurrence of chlorinated pesticides and polychlorinated biphenyls (PCBs) in Guiana dolphins (*Sotalia guianensis*) from Ubatuba and Baixada Santista, São Paulo, Brazil. *Latin America Journal of Aquatic Mammals*, 8, 123-130.

Azevedo AF, Fragoso AB, Lailson-Brito JJ, H.A. C (2002) Records of the franciscana (*Pontoporia blainvillei*) in the Southwestern Rio de Janeiro and Northernmost São

Paulo State coasts – Brazil. Latin American Journal of Aquatic Mammals 1: 191-192.

Crespo EA (2009) Franciscana dolphin *Pontoporia blainvillei*. In W. F. Perrin, B. Würsig, & J. G. M. Thewissen (Eds.), Encyclopedia of marine mammals (2nd ed., pp. 466–469). New York, NY: Academic Press.

Excoffier L, Lischer HEL (2010) Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Molecular Ecology Resources 10: 564-567.

Hansen LJ, Schwacke LH, Mitchum GB, Hohn AA, Wells RS, Zolman ES, Fair PA (2004) Geographic variation in polychorinated biphenyl and organochlorine pesticide concentrations in the blubber of bottlenose dolphins from the US Atlantic coast. Science of the Total Environment, 319(1-3), 147-172.

Lailson-Brito J, Dorneles PR, Azevedo-Silva CE, Azevedo AF, Vidal LG, Zanelatto RC, Lozinski CPC, Azeredo A, Fragoso ABL, Cunha HA, Torres JPM, Malm O (2010) High organochlorine accumulation in blubber of Guiana dolphin, *Sotalia guianensis*, from Brazilian coast and its use to establish geographical differences among populations. Environmental Pollution, 158(5), 1800-1808.

Lailson-Brito J, Dorneles P., Azevedo-Silva CE, Azevedo AF, Vidal LG, Marigo J, Bertozzi C, Zanelatto RC, Bisi TL, Malm O, Torres JPM (2011) Organochlorine concentrations in franciscana dolphins, *Pontoporia blainvillei*, from Brazilian Waters. *Chemosphere* 84(7), 882-887.

Lázaro M, Lessa EP, Hamilton H (2004) Geographic genetic structure in the franciscana dolphin (*Pontoporia blainvillei*) Marine Mammal Science 20: 201-214.

Leigh JW, Bryant D (2015). PopART: Full-feature software for haplotype network construction. Methods Ecol Evol 6(9):1110–1116.

Librado P, Rozas J (2009) DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25: 1451-1452.

Post DM, Layman CA, Arrington DA, Takimoto G, Quattrochi J, *et al.* (2007). Getting to the fat of the matter: models, methods and assumptions for dealing with lipids in stable isotope analyses. Oecologia 152: 179–189.

Ross PS, Ellis GM, Ikonomou MG, Barrett-Lennard LG, Addison RF (2000) High PCB concentrations in free-ranging Pacific killer whales, Orcinus orca: effects of age, sex and dietary preference. Marine Pollution Bulletin, 40(6), 504-515.

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: a Laboratory Manual: Cold Spring Harbor Lab. Press.

Santos-Neto E, Azevedo-Silva CE, Bisi TL, Santos J, Meirelles ACO, Carvalho, VL, Azevedo AF, Guimarães JE Lailson-Brito J (2014) Organochlorine concentrations (PCBs, DDTs, HCHs, HCB and Mirex) in delphinids stranded at the northeastern Brazil. *Science of the Total Environment* 472, 194-203.

Secchi ER (2010) Review on the threats and conservation Status of Franciscana *Pontoporia blainvillei* (Cetacea, Pontoporiidae). In: Shostell JM, Ruiz-Garcia M, editors. Biology, Evolution and Conservation of River Dolphins within South America and Asia. New York: Nova Science Publishers Inc. pp. 323-339.

312 Sucunza F, Danilewicz D, Andriolo A, Azevedo AF, Secchi ER, Zerbini A (2020)
313 Distribution, habitat use, and abundance of the endangered franciscana in
314 southeastern and southern Brazil. Marine Mammal Science 36:421–435
315 Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4 Molecular evolutionary genetics
316 analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24: 1596-
317 1599
318 Wade TL, Cantillo AY (1994) Use of standards and reference materials in the
319 measurement of chlorinated hydrocarbon residues. Chemistry Workbook. NOAA
320 Technical Memorandum NOS ORCA 77, Silver Spring, Maryland, 59p.
321 Zerbini AN, Secchi E, Crespo E, Danilewicz D, Reeves R (2017) *Pontoporia*
322 *blainvillei*. The IUCN Red List of Threatened Species.