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Article · January 2017

DOI: 10.5597/00225

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Latin American Journal
of Aquatic Mammals
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Online ISSN: 2236-1057

Effective population size of an offshore population of bottlenose dolphins, *Tursiops truncatus*, from the São Pedro and São Paulo Archipelago, Brazil

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ARTICLE INFO

Manuscript type	Article
Article history	
Received	22 November 2011
Received in revised form	11 February 2014
Accepted	19 November 2014
Available online	29 December 2016

Keywords: Delphinidae, genetic sex identification, effective population size, sex ratio

Responsible Editor: Luciana Möller

Citation: Oliveira, L.R., Ott, P.H., Moreno, I.B., Tavares, M., Siciliano, S. and Bonatto, S.L. (2016) Effective population size of an offshore population of bottlenose dolphins, *Tursiops truncatus*, from the São Pedro and São Paulo Archipelago, Brazil. *Latin American Journal of Aquatic Mammals* 11(1-2): 162-169. http://dx.doi.org/10.5597/lajam00225

Abstract. The São Pedro and São Paulo Archipelago (SPSPA) (00°56'N, 29°22'W) lies approximately 1010km northeast off the coast of Rio Grande do Norte State in Brazilian waters. Recently, through photo-identification and group size analysis, around 20-30 individual bottlenose dolphins, *Tursiops truncatus,* from SPSPA were recognized as a resident, and potentially genetically isolated, population. The effective population size (N_e), not the census number (N_c), as well the sex ratio, are of primary concern from an evolutionary and conservation management perspectives. The estimate of N_e reflects the number of individuals responsible for the maintenance of genetic diversity of a species or population as well its evolutionary potential. For this reason, we present here the first N_e and sex ratio estimates for the bottlenose dolphin population from SPSPA. Sex was molecularly determined for 19 biopsy samples collected from bottlenose dolphins from SPSPA between January and February 2005. The N_e was estimated by direct counting of reproductive adults sexed by DNA analysis. The resulting N_e was 12 individuals and the sex ratio was 1.11 male to 1 female, however, it was not significantly different from the expected 1:1 ratio (χ^2 test, $\alpha = 0.05$; df = 1). The effective population size based on the genetic diversity of 19 sequences of the mtDNA control region resulted in a female effective population size of 223 individuals, and the total long-term effective size of ~470 individuals. We believe that the estimated N_e for the SPSPA population is a critical value, because it is significantly lower than the mean minimum viable population (MVP) suggested for vertebrates (around 5000 breeding age adults). This small N_e is of great concern and should be taken into account in future management plans to ensure the conservation and protection of this small population at SPSPA.

Resumo. O Arquipélago de São Pedro e São Paulo (SPSPA) (00°56'N, 29°22'W) encontra-se distante cerca de 1010km da costa nordeste brasileira. Recentemente, a partir das análises de foto-identificação e dos tamanhos de grupo avistados, verificou-se a existência na região de uma população residente de cerca de 20 a 30 espécimes do golfinho-nariz-de-garrafa (*Tursiops truncatus*), a qual possivelmente está isolada geneticamente. Para o cenário evolutivo, o tamanho efetivo (N_e) de uma população, e não o número total de indivíduos (N), é o fator fundamental. A estimativa do número de indivíduos responsáveis pela produção da próxima geração é determinante para a manutenção da variabilidade genética. Neste sentido, apresentamos a primeira estimativa de N_e e da proporção sexual da população do golfinho-nariz-de-garrafa do SPSPA, através da determinação genética do sexo de seus espécimes. Biópsias de 19 espécimes do golfinho-nariz-de-garrafa foram coletadas entre janeiro e fevereiro de 2005 no SPSPA. A proporção sexual observada foi de 1,11 macho para 1 fêmea, porém como não foi estatisticamente diferente da proporção esperada de 1:1 (teste χ^2, α= 0,05; gl = 1), não se pode refutar a hipótese de que esta proporção deva-se ao acaso. O tamanho efetivo estimado pela contagem direta foi de 12 espécimes reprodutivamente adultos. Contudo, esta mesma estimativa feita através da análise da diversidade genética de 19 sequências da região controladora do mtDNA de espécimes coletados em SPSPA resultou num tamanho efetivo de fêmeas de 223 indivíduos, o que totalizou ~470 golfinhos (considerando machos e fêmeas). Nós acreditamos que os valores estimados de tamanho efetivo para os golfinhos de SPSPA são críticos para conservação desta população, porque são significativamente menores que o mínimo populacional viável (MPV) estimado para vertebrados (cerca de 5000 adultos em idade reprodutiva). Neste sentido, este resultado pode ser considerado preocupante e deve ser levado em consideração em qualquer futuro plano de manejo da região para que sejam asseguradas a conservação e viabilidade da pequena população do golfinho-nariz-de-garrafa do SPSPA.

Introduction

The São Pedro and São Paulo Archipelago - SPSPA (also known as St. Paul's Rocks) is a small and isolated group of rocky islets just north of the Equator (00°56'N, 29°22'W) that has as total land area, and shallow waters surrounding them, less than 0.5km^2 (Edwards and Lubbock, 1983). The archipelago lays approximately 1010km northeast of the coast of Rio Grande do Norte State, Brazil, and 1824km southwest of Guinea-Bissau, Africa, in the equatorial Atlantic Ocean. The nearest landmass is the Fernando de Noronha Archipelago, 630km to the southwest, while Cape Verde Islands, off northwestern Africa, are about 1850km to the north-northeast (Campos *et al.,* 2009). In order to provide a comprehensive review about the cetacean species around the SPSPA, dedicated surveys were undertaken from 1999 to 2005[1], with special attention to the bottlenose dolphin, *Tursiops truncatus* (Montagu, 1821) (Moreno *et al.*, 2009; Ott *et al.*, 2009). This offshore bottlenose dolphin population was studied mainly by photo-identification and genetic analysis[2,3] (Moreno *et al.*, 2009; Ott *et al.*, 2009).

The bottlenose dolphin is a highly social species with a worldwide distribution in cold temperate to tropical waters, as well as inshore and offshore areas (Wells and Scott, 2009). Differences between nearshore and offshore populations have been found for this species in many geographic locations[4] (Ross, 1977; 1984; Duffield *et al.,* 1983; Ross and Cockroft, 1990; Van Waerebeck *et al.,* 1990; Mead and Potter, 1995; Hoelzel *et al.,* 1998). This great geographical variation has led some authors in the past to divide the genus *Tursiops* into as many as 20 different species (Hershkovitz, 1966; Rice, 1998). Nevertheless, only two species are currently recognized, *T. truncatus,* the 'common bottlenose dolphin', and *T. aduncus* (Ehrenberg, 1982), the 'Indian Ocean bottlenose dolphin' (Wang *et al.,* 1999; 2000*a*, *b*; Natoli *et al.,* 2004; Wells and Scott, 2009). Recently a third potential species of *Tursiops* was formally described for southern Australian coastal waters, *T. australis*, the 'Burrunan dolphin' (Charlton-Robb *et al.,* 2011). However, it was not considered as a valid species of *Tursiops* by the Committee on Taxonomy of The Society for Marine Mammalogy (2016). Despite the broad geographic coverage of all these studies, bottlenose dolphins have mostly been studied in peri-continental and shallow waters, and very little is known about offshore populations (Klatsky *et al.*, 2007; Quérouil *et al.*, 2007; Baird *et al.*, 2009).

The bottlenose dolphins from the SPSPA have been studied since 1999 and the first genetic data from mitochondrial DNA (mtDNA) analysis of this population indicated an extremely low gene diversity in the control region of the mtDNA (h = 0.1053 e π = 0.0007) (Oliveira *et al.*, 2008; Moreno *et al.*, 2009; Ott *et al.,* 2009). The 19 sampled animals from the SPSPA presented only two haplotypes, which were not shared with other populations along the Brazilian coast (Oliveira *et al.*, 2008; Moreno *et al.*, 2009; Ott *et al.,* 2009). These results suggest that the bottlenose dolphins from SPSPA are likely to be genetically isolated from Brazilian populations. In addition, the sighting

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[1] Project *Ocurrence and sazonality of cetaceans in the vicinity of St Paul's Rocks* conducted by GEMARS with support of SECIRM and CNPq (Proc. # 480037/2004-3).

[2] Oliveira, L.R., Ott, P.H., Moreno, I.B., Tavares, M., Danilewicz, D., Almeida, R., Siciliano, S. and Bonatto, S. (2008) Variabilidade genética e estrutura populacional do golfinho-nariz-de-garrafa, *Tursiops truncatus*, em águas brasileiras. Page 80 *in* Resumos, *XIII Reunião de Trabajo de Especialistas en Mamíferos Acuáticos de América del Sur* y *7 Congreso SOLAMAC*, 13-17 October 2008, Montevideo, Uruguay.

[3] Ott, P.H., Oliveira, L.R., Almeida, R.S., Tavares, M., Moreno, I.B., Danilewicz, D., Siciliano, S., Apolinário, M. and Bonatto, S.L. (2006) Os golfinhos-nariz-de-garrafa do Arquipélago de São Pedro e São Paulo são uma população isolada? Page 196 *in* Resumos, *52º Congresso Brasileiro de Genética*, 3-6 September 2006, Foz do Iguaçu, PR, Brazil.

[4] Walker, W.A. (1981) *Geographic variation in morphology and biology of bottlenose dolphins* (Tursiops) *in the eastern North Pacific.* NOAA/NMFS Southwest Fisheries Science Center Administrative Report no. LJ-81-3c. 21 pp.

data about group size and the photo-identification results indicated that this population contains approximately 20-30 individuals, which are potentially resident to the region (Caon *et al.*, 2009; Moreno *et al.*, 2009; Ott *et al.*, 2009).

Therefore, the census size (N_c) estimated for this population is extremely small and is a matter of concern. Small populations are more vulnerable to inbreeding depression and loss of genetic diversity that may reduce the evolutionary potential to respond to stochasticities (such as environmental changes or the emergence of new diseases) that make them more prone to extinction (*e.g.* Frankham *et al.*, 2002). Nevertheless, according to Wright (1931), from evolutionary and conservation management perspectives, the effective population size (N_e), not the census number (N_c), is of prime concern. The N_e is envisioned as an 'ideal' population (stable, random mating) of *N* diploid individuals reconstructed each generation from a random sample of 2N gametes that has the same rate of increase in homozygosity or gene frequency change as the actual population under consideration (Wright, 1931). In other words, N_e is the real number of reproductive adults that will produce the next generation and will be determinant for keeping the genetic variability. Thus, the effective population size is normally substantially lower than census sizes (N_c), N_e usually being around 1/3 to 1/10 of the N_c (Frankham *et al.,* 2002).

Nevertheless, the census population size (N_c) is usually the only information available for most wild populations. According to Hedrick (2000), factors such as variation in the sex ratio of breeding individuals, variation of population size in different generations, and variation of mating system (*e.g.* polygynous versus polyandrous) may be important, and can be accounted for the estimate of N_e. In addition, the estimation of the sex ratio in natural populations of mammals is essential for understanding population dynamics, behavior, breeding systems, demography, as well as it is important for management and conservation actions (Brown *et al.,* 1991*a*; Gompper *et al.,* 1998; Hughes, 1998). In fact, early detection of N_e reduction is critical, since immediate management actions may be necessary to avoid population endangerment or extinction (Schwartz *et al.*, 1998).

In many wild populations the number of females and males are not equal, and the sex ratio deviates from 1:1. Deviations in the sex ratio accelerate the effects of inbreeding on extinction rate because of the effect of sex ratio on effective population size (Mills and Smouse, 1994). However, for both estimates, N_e and sex ratio, the identification of the sex of each individual in a population is difficult for some species, such as some free-ranging cetaceans, since usually there is no remarkable evidence of sexual dimorphism. The direct observation of the genital region is extremely opportunistic. For this reason, gender identification through analyses of molecular markers is becoming a powerful tool in the study of wild populations of cetaceans (*e.g.* Shaw *et al.*, 2003; Oliveira *et al.*, 2009).

However, both parameters N_e and sex ratio have proven difficult to estimate for wildlife populations, even though it can be estimated using demographic or genetic methods. For that reason, we present the first estimate of N_e and sex ratio for the potentially isolated bottlenose dolphin population from the SPSPA.

Material and Methods

Sample collection and DNA extraction

Nineteen biopsy samples were collected from live bottlenose dolphins, between January and February 2005 in offshore waters of the SPSPA. Skin biopsies were collected using a crossbow and lightweight darts (CETA-DART©) (Brown *et al.*, 1991*b;* Ott *et al.*, 2009). Samples were stored in NaCl-saturated solution of 20% dimethyl sulfoxide (Amos and Hoelzel, 1991) and frozen at -20°C until DNA was extracted. Biopsies were taken from a research vessel, and most biopsied dolphins were photographically identified at the same time. All 19 samples used in these analyses represent 19 different individuals, according to the photo-identification data as well by the screening of eight microsatellite loci (unpublished results).

DNA extraction followed the phenol/chloroform method described by Sambrook *et al.* (1989) and modified by Shaw *et al.* (2003). The sex of each individual was identified by the amplification of introns from ZFX and ZFY genes, which are located on the X and Y mammalian chromosomes, respectively (Page *et al.,* 1987), using primers ZFY1204 and ZFY0097 under PCR conditions described in Pasbøll *et al.* (1992). Amplification products were digested by Taq I restriction endonuclease and visualized in 2% agarose gel with 100 base-pair ladder (GE Healthcare) for fragment size estimation.

Gender determination

Gender identification was based on the number of bands for a given sample. Females have a single band that corresponds to the ZFX intron on the X chromosome, whereas males have two bands, one corresponding to the X intron and the other to the ZFY intron on the Y chromosome (Page *et al.,* 1987). DNA from bottlenose dolphins of known gender (*e.g.* stranded animals in southern Brazil) were amplified, digested and visualized in gel as positive controls to validate the technique.

Sex ratio was calculated by the division between the total number of males and females identified. The Chi-square test was used to verify if the resulting sex ratio deviates significantly from an expected 1:1 ratio.

N_e calculations

We calculated the contemporary N_e after the estimate of sex ratio, because in organisms with separate sexes one sex may be more common than other and need an equation that accounts for the effects of an unequal sex ratio (Wright, 1931). However, there was no statistically significant deviation from 1:1 (see Results), and therefore the estimate of N_e was calculated by counting all the adult males and females genetically sexed (Wright, 1931). The age classes of the dolphins were visually estimated based on body size (Siciliano *et al.*, 2007) relative to the boat and the amount of tooth rake scarring (*e.g.* Scott *et al.*, 2005). Individuals estimated to be longer than 2.5m and 'heavily' scarred were classified as adults, as well as animals accompanied by a calf.

We also estimated the long-term effective population size based on genetic diversity of the 19 sequences from the mtDNA control region (316bp) (Oliveira *et al.*, 2008; Moreno *et al.*, 2009), collected from specimens of SPSPA. Haplotype (*h*) and nucleotide (π) diversities (Nei, 1987) were estimated using Arlequin 3.5 (Excoffier and Lischer, 2010). Based on mtDNA diversities we estimated the female effective population size (N_{ef}) for this population using the formula: $N_{ef} = \theta/2\mu g$, where μ = mutational substitution rate per generation and θ = genetic diversity (estimated here by π) (Avise *et al.*, 1988). Generation time (g) estimated for bottlenose dolphin and used for calculation was 10 (Cassens *et al.,* 2005) with a mutation rate of $1.5E^{-7}$ (Hoelzel *et al.*, 1991).

Results

From the 19 bottlenose dolphins biopsied, nine were genetically sexed as females and ten as males. The observed sex ratio was 1.11 male to one female, which was not significantly different from the expected ratio of 1:1 (χ^2 test, α=0.05; df=1).

The contemporary N_e from bottlenose dolphins of the SPSPA was 12 individuals, accounting for the effects of an equal sex ratio since 12 from the 19 individuals were potential breeding adults from both sexes (seven males and five females). Sequence analysis of the mitochondrial DNA control region (316 base pairs) of the 19 individuals revealed a total of two polymorphic sites defining two different haplotypes, leading to extremely low genetic diversities (*h* = 0.1053 and π = 0.00067). This resulted in a female effective population size of 223 individuals, and considering the 1.11:1 sex ratio estimated above, to a total long-term effective size of ~470 individuals. None of the haplotypes were shared between SPSPA and the other known Brazilian populations (Oliveira *et al.*, 2008; Ott *et al.*, 2009).

Discussion

We present the first estimates of sex ratio and effective population size for the SPSPA population of bottlenose dolphins and suggest that the slight predominance of male individuals (1.11:1) is likely random.

Despite the significant amount of studies on bottlenose dolphins, most of them are on coastal populations, and very little is known about sex ratio of bottlenose dolphin populations in distant offshore regions. The only similar study conducted at an offshore area was made by Quérouil *et al.* (2007), who analyzed the population structure of bottlenose dolphins in two of the most isolated archipelagos of the North Atlantic: the Azores and Madeira. Sex ratio estimated by molecular sexing for bottlenose dolphins in the Azores clearly indicated a sampling bias in favor of males. Excluding the samples from stranded animals (two males from Madeira), there were 61 males and 22 females in the Azores (sex ratio = 2.77:1) and 13 males and 12 females in Madeira (sex ratio = 1.08:1). The authors attributed this result probably to a sampling artifact, as it seemed that adult females tended to avoid the boat, especially when accompanied by young calves. However, in the case of the dolphins from SPSPA, the presence of females and calves was relatively common in the groups sighted around the archipelago, including during sampling.

Our estimate of a contemporary N_e of 12 dolphins for the SPSPA population does not account for two other possible factors: i) the variation in reproductive success among individuals, and ii) gene flow, *i.e.* the possibility of peripheral males or females (from other geographically close populations) being reproductively active. In both cases, it is very difficult to obtain empirical information. If individual variance in reproductive success exists, its effect will reduce the estimate of N_e to a more critical value. Regarding gene flow, the relationship between the dolphins from SPSPA and other offshore populations is still unclear. We did not find sharing of mtDNA haplotypes between SPSPA and some Brazilian inshore populations from southern region (Oliveira *et al.*, 2008; Moreno *et al.*, 2009; Ott *et al.*, 2009). However, bottlenose dolphins have been occasionally sighted in other oceanic regions, such as the biological reserve of Rocas Atoll (03°50'S; 33°49'W) (Baracho *et al.*, 2008), located about 720km from the SPSPA. Furthermore, our long-term N_e result based on genetic diversity from specimens of SPSPA was much larger (~470 individuals), reinforcing the possibility of gene flow, at least in the past. Therefore, we can not presently reject the hypothesis that some gene flow exists or existed between SPSPA and other populations.

The data from group size and photo-identification indicated that the bottlenose dolphin population in the SPSPA contains about 20-30 that are potentially residents to the area (Caon *et al.*, 2009; Moreno *et al.*, 2009; Ott *et al.*, 2009). Therefore, it is unlikely that the current N_e for this population would be much higher than estimated here. Nevertheless, fewer than ten dolphins were resighted between 1999 and 2005 in the area (Caon *et al.*, 2009; Moreno *et al.*, 2009; Ott *et al.*, 2009).

Interestingly, contrary to the usual situation (Hare *et al.*, 2011), the SPSPA contemporary N_e estimated by direct count is much lower than the long-term N_e estimated by mtDNA nucleotide diversity. However, long-term N_e is usually not a reliable indicator of short-term or contemporary N_e for a population (Hare *et al.*, 2011), since the latter is more similar to a N_e averaged over tens to hundreds of generations

(Avise, 2000) and may be heavily affected by a past migration event, for example.

Unfortunately, there is very few information on N_e for vertebrates in general and even less so for delphinids, with available information generally focused on the diversity of genetic markers as an alternative method to the demographic estimation by counting (*e.g.* Galov *et al.,* 2011; Caballero *et al.,* 2012; Martien *et al.*, 2012). These previous studies concentrated on species or populations of the continental shelf and found a large range of values for N_e. For example, for north Pacific killer whales (*Orcinus orca*) N_e could vary from high (>1000) to very low (<50) (Hoelzel *et al.,* 2007). For bottlenose dolphins, there are very few studies that present molecular estimates of N_e, and one presents this estimation for the Black Sea population, with the value oscillating between 162 and 2273, according to the mutation rate used (Viaud-Martinez *et al.,* 2008).

Considering data from other studies, as well as the small size and geographic isolation of the SPSPA, the very small N_e estimated for bottlenose dolphins in this area is not unexpected and does not seem to result from anthropogenic factors. Nevertheless, the extremely low N_e for the SPSPA population represents a critical value, since it is significantly lower than the MVP estimated for vertebrates (*e.g.* Reed *et al.*, 2003). Originally, Franklin (1980) proposed the so-called '50/500' rule, whereby an N_e of 50 adult individuals is required to prevent damaging effects of inbreeding, while a long-term N_e of 500 individuals is required to ensure overall genetic variability. However, more recent assessments suggest that this number should be higher, usually approaching 5000 adult individuals (Reed *et al.*, 2003; Traill *et al.*, 2007; 2010).

The small N_e for the bottlenose dolphin population from SPSPA, as well as its very low genetic diversity, are reasons of great concern and should be taken into account in future management plans to ensure the conservation and protection of this population at the SPSPA. In addition, the available genetic information (Oliveira *et al.*, 2008; Moreno *et al.*, 2009; Ott *et al.*, 2009) strongly indicates that this population is an 'evolutionary significant unit' (ESU, *sensu* Moritz, 1994) and as such should be considered 'distinct' for conservation management purposes.

Acknowledgments

We greatly acknowledge the logistic and financial support from the Secretaria da Comissão Interministerial para os Recursos do Mar – SECIRM, the Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq (National Council for Scientific and Technological Development) (CNPq Proc. 480037/2004-3 and CNPq Proc. 557176-2009-3) and 'Projeto Mamíferos e Quelônios Marinhos', coordinated and supported by 'Gerência de Avaliação e Monitoramento Ambiental do CENPES/PETROBRAS'. We are also grateful to the crew of the fishing vessels *Transmar I, II* and *III* for the logistic support during the sampling expeditions. We also thank Matheus Williams de Oliveira, Marcus Rodrigo Guidoti and Cladinara Sarturi Roberts for their laboratory help. L.R. Oliveira was supported by CNPq (Proc. 151307/2005-9). This paper is dedicated to the memory of Raquel Santos de Almeida, who was involved in the laboratory work of the present study. With Raquel's passing, we have lost an excellent student and very good friend.

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