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Biopsy darting of common bottlenose dolphins (*Tursiops truncatus*) in southern Brazil: evaluating effectiveness, short-term responses and wound healing

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Abstract. Cetacean biopsy sampling is a widely used technique with undisputable scientific value. Although it is generally considered as a harmless technique with no apparent long-lasting effects, studies have recommended examining behavioral responses to evaluate potential impacts on individuals, groups and sampled populations. In this study, we evaluated individual behavioral reactions and wound-healing in common bottlenose dolphins (*Tursiops truncatus*) during a biopsy sampling program carried out in southern Brazil from 2003 to 2012, and compared sampling effectiveness between dedicated and opportunistic sampling surveys. Two hundred and fifty-two biopsy attempts were made, resulting in 118 hits (48% of attempts) and 134 samples (52% of attempts) collected successfully. Responses to biopsy sampling were low-level, of short-term duration, and elicited similar reactions on the dolphins, irrespective of shot distance, sex of individuals, dolphins' group size and pre-behavioral state. Dolphins subjected to multiple biopsy attempts reacted in a similar manner as in previous attempt(s), with no evidence of increasing the intensity of the reaction. Wounds could be monitored in 18 animals and healed over 18 to 35 days. Generally, wounds appeared to be covered by epidermis in about three weeks with no observed signs of skin infection. Our results agree with previous studies suggesting that biopsy sampling does not cause significant disturbance to the behavior of dolphins. At a local level, this study demonstrates that biopsy sampling of bottlenose dolphins in the Patos Lagoon Estuary is more effective, less costly and less intrusive when conducted opportunistically, but that long-term sampling is required to achieve a relatively good sample size from photo-identified individuals in the population.

Resumo. Amostras de pele e gordura de cetáceos coletadas no ambiente selvagem através do uso de sistemas de biopsia possuem um valor científico inquestionável. Embora a biopsia seja geralmente considerada uma técnica inofensiva e sem aparentes efeitos de longa duração, estudos recomendam avaliar as respostas comportamentais dos animais durante as amostragens para acessar potenciais impactos sobre os indivíduos, grupos ou populações. Neste estudo avaliamos as reações comportamentais individuais e o processo de cicatrização nos botos (*Tursiops truncatus*) amostrados durante um programa de coleta de biopsias realizada no sul do Brasil entre 2003 e 2012, e comparamos a eficiência das coletas entre saídas de campo dedicadas e oportunísticas. No total realizamos 252 disparos resultando em 118 acertos (48% das tentativas) e 134 amostras (52% das tentativas) coletadas com sucesso. De uma maneira geral, as reações foram fracas e de curta duração e tiveram efeitos semelhantes sobre o comportamento dos botos, independentemente da distância do disparo em relação ao animal, do sexo do animal, do tamanho do grupo e do comportamento dos indivíduos antecedente ao disparo. Indivíduos submetidos a várias tentativas de biopsia reagiram de maneira semelhante a tentativa(s) anterior(es), sem qualquer evidência de aumento da intensidade da reação. A cicatrização das feridas variou de 18 a 35 dias (n = 18 indivíduos), mas geralmente estavam cobertas por epiderme em cerca de três semanas, sem sinais de infecções da pele. Nossos resultados corroboram estudos anteriores indicando que este tipo de amostragem não causa uma perturbação significativa sobre o comportamento dos botos. A nível local, este estudo demonstrou que a biopsia no estuário da Lagoa dos Patos é mais eficaz, possui um menor custo e é menos intrusiva quando conduzida de forma oportunística; contudo, torna-se necessário uma amostragem a longo prazo para que um número significativo de amostras seja coletado de indivíduos identificados na população.

Introduction

Remote biopsy sampling is a widely recognized technique for obtaining skin and blubber tissues from free-ranging cetaceans (*e.g.* Weinrich *et al.*, 1991; Krützen *et al.*, 2002; Bilgmann *et al.*, 2007; Noren and Mocklin, 2012). Skin samples have been used in genetic and stable isotope analyses addressing questions regarding stock identity, social structure, phylogeography and trophic ecology (*e.g.* Baker *et al.*, 1990; Möller and Beheregaray, 2001; Knoff *et al.*, 2008; Caballero *et al.*, 2012). Furthermore, important population parameters such as dispersal, effective population size (*e.g.* Waples, 1991; Möller and Beheregaray, 2004) and population sex ratio can be estimated through molecular sex determination (Quérouil *et al.,* 2010). Blubber samples have provided information on pollutant concentrations, stable isotope and fatty acid signatures, which have been widely used to monitor population health and investigate feeding ecology (*e.g.* Berrow *et al.*, 2002; Herman *et al.*, 2005; Kiszka *et al.*, 2011). Pregnancy status from individuals can also be determined from blubber (Mansour *et al.,* 2002). Therefore, biopsy samples have proved to be a powerful source of information in marine mammal science.

Remote biopsy sampling also has advantages over sampling stranded or bycaught animals. Studies using such samples may be limited because the origin of the animal sampled is usually unknown and research may be restricted to certain species or areas where stranded animals wash ashore or are caught by fishing gear, which may be biased towards certain age or sex classes, or animals with specific health conditions.

Cetacean biopsy samples are generally collected with modified darts and tips with barbed dental broach or hook fired from crossbows, spear guns and 0.22 or CO_2 rifles. Samples from live animals can also be obtained from less-invasive techniques such as skin swabbing (Harlin *et al.,* 1999), or from non-invasive techniques such as dolphin feces (Parsons, 2001) and sloughed skin (Whitehead *et al.,* 1990). However, it is not possible to collect blubber using these approaches and, in some circumstances, the quality and amount of skin is not enough to use in genetic, biomarker or isotopic studies (Parsons *et al.*, 1999; 2003). Furthermore, these methods are not applicable when dolphins do not get close enough or do not ride the bow wave of a boat.

Studies evaluating the effects of remote biopsy sampling have been carried out for at least six species of small delphinids (Table 1), but few have addressed possible effects of group size, pre-behavior state or sex on the dolphin's reactions. Overall, these studies suggest that when carried out responsibly, sampling effects on the animals are minimal, of short-term duration (Tezanos-Pinto and Baker, 2012) and similar between species regardless of body and group sizes (*e.g.* smaller delphinids and groups do not seem to be more reactive than larger ones; Kiszka *et al.*, 2010). However, initial behavioral state seems to influence the level of reaction for some species (*e.g. Stenella longirostris*; Kiszka *et al.*, 2010). Despite this, studies have recommended to evaluate behavioral responses to assess potential interspecific and interpopulation differences in behavioral responses. Furthermore, the report of a common dolphin (*Delphinus delphis*) death after a biopsy dart penetrated beyond the stop point (Bearzi, 2000) reinforces the need for continued studies when intrusive techniques are used to collect skin samples from delphinids, especially when dealing with small and/or at risk populations.

About 85 common bottlenose dolphins (*Tursiops truncatus*) inhabit the Patos Lagoon Estuary (PLE) and adjacent coastal waters in southern Brazil (Dalla Rosa, 1999; Fruet *et al.*, 2011). Adult males can reach up to 3.8m and females up to 3.4m in this population (Fruet *et al.*, 2012). Here, bottlenose dolphins usually do not bow-ride on small boats. Previous studies showed that dolphins of this population are year-round residents, form small groups (generally between two and five individuals), and tend to occupy mostly waters at the estuary's mouth (Mattos *et al.*, 2007; Di Tullio *et al.*, 2015).

As part of a long-term population monitoring program of bottlenose dolphins initiated in 2002, biopsy samples were required to investigate population and social structure, and for contributing to a major project aimed at defining the dolphins' management units and contamination levels along the Southwest Atlantic Ocean (SWAO). In this study, we describe and compare the effectiveness of remote biopsy sampling between two different types of survey methodologies, and evaluate the short-term impacts of sampling on dolphin's

Table 1. Behavioral reaction levels of small delphinids to biopsy sampling and hit rates obtained from different studies carried out around the world, including this study. Behavioral reactions refer to hits only.

Source	Species	Study area	Equipment	Attempts	Hit rate	Reaction levels			
						None	Low-level	Moderate	Strong
Weller *et al.* (1997)	*T. truncatus*	Galveston Bay	45kg Crossbow	13	61.5%	0%	0%	100%	0%
Krützen *et al.* (2002)	*Tursiops* sp.	Shark Bay	Rifle	414	75.8%	0%	59.3%	33.7%	6.9%
Krützen *et al.* (2002)	*T. aduncus*	Jervis Bay	Rifle	42	52.4%	40.9%	50%	9.1%	0%
Krützen *et al.* (2002)	*T. aduncus*	Port Stephens	Rifle	71	52.1%	8.1%	73%	18.9%	0%
Krützen *et al.* (2002)	*T. truncatus*	Patos Lagoon	Rifle	34	35%	25%	8.3%	58.3%	8.3%
Parsons *et al.* (2003)	*T. truncatus*	Bahamas	Rifle	51	62.7%	13.2%	26.9%	6.2%	9.4%
Gorgone *et al.* (2007)	*T. truncatus*	Southeast coast, USA	68kg Crossbow	475	67.4%	2.5%	84.4%	10.6%	2.5%
Jefferson and Hung (2008)	*S. chinensis*	Pearl River Estuary	68kg Crossbow	87	56.3%	27%	55%	18%	0%
Kiszka *et al.* (2010)	*S. attenuata*	Mayotte Island	68kg Crossbow	77	65%	-	-	-	-
Kiszka *et al.* (2010)	*S. longirostris*	Mayotte Island	68kg Crossbow	137	70%	-	-	-	-
Kiszka *et al.* (2010)	*T. aduncus*	Mayotte Island	68kg Crossbow	22	77%	-	-	-	-
Kiszka *et al.* (2010)	*P. electra*	Mayotte Island	68kg Crossbow	23	78%	-	-	-	-
Tezanos-Pinto and Baker (2011)	*T. truncatus*	Bay of Islands	Rifle	215	73%	1.2%	71.3%	26.4%	1.1%
Tezanos-Pinto and Baker (2011)	*T. truncatus*	Doubtful Sound	Rifle	39	46%	-	-	-	-
This study	*T. truncatus*	Patos Lagoon	68kg Crossbow	252	46.8%	1.7%	75.7%	21.7%	0.9%

Levels of reactions from the literature surveyed were re-allocated according to levels of reactions defined in this study (see Table 2).

behavior. In addition, we investigate the effects of pre-biopsy behavioral state, target distance, group size and sex on the reaction to sampling, and describe the process of wound healing for some of the sampled individuals.

Material and Methods

Surveys and sampling procedures

Surveys were conducted in the PLE and adjacent coastal waters (Figure 1). Surveys took place between 2003 and 2004 onboard a 6m aluminum boat powered by a two-stroke 60hp outboard engine, while between 2009 and 2012 surveys were onboard an inflatable boat powered by a two-stroke 90hp outboard engine. The boat driver and the biopsy sampler were the same in all surveys. A third person was responsible for collecting behavioral data and retrieving the floating darts from the water. Biopsies were taken from two different types of surveys: i) dedicated surveys to collect biopsy samples and ii) opportunistic surveys carried out to investigate dolphin abundance and habitat use. During opportunistic surveys, attempts took place only after standard ecological and photo-identification data collection had ceased. Basically, while collecting data, we looked for the presence of marked animals in the group which had never been sampled (confirmed through visualization of digital photographs of the dorsal fin and comparing to a field photo-id catalogue), and evaluated dolphin's group behavior before making the decision to spend time biopsying the animals. Groups engaged in activities or areas that make biopsy sampling difficult to carry out (*e.g.*

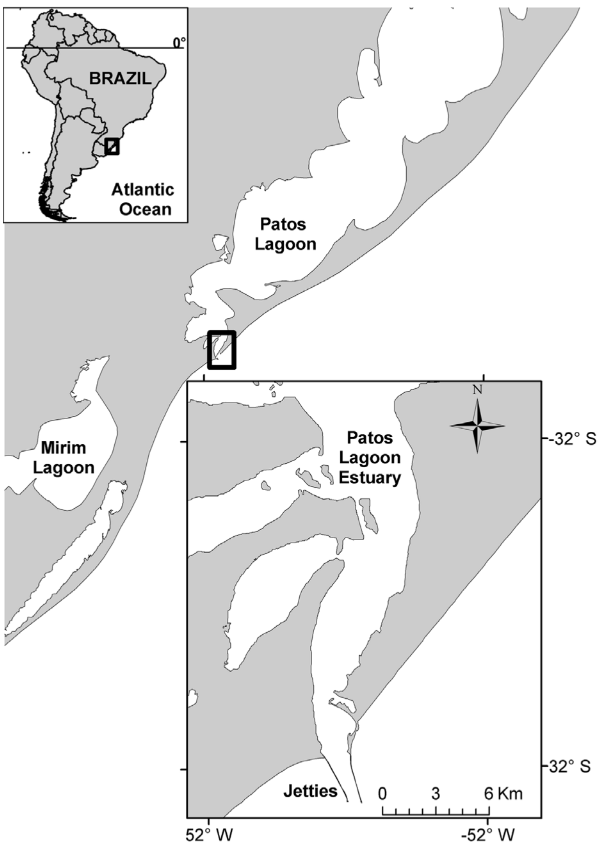

Figure 1. Map of study area in the Patos Lagoon Estuary and adjacent coastal waters, southern Brazil.

feeding in the surfing zone, spending little time on the safe navigation channel or dolphins with unpredictable surface behavior), even in the presence of target animals, were not pursued and surveys returned to the standard sampling routine. During dedicated surveys, we randomly searched for dolphins and would spend up to many hours towards sampling the same group of individuals, irrespective of dolphin pre-biopsy behavior state. This survey design generally constrains the use of collected data in other analyses that are critical for the long-term monitoring program, such as abundance estimation and habitat use. However, regardless of survey type, for each sighted group we maneuvered the boat for traveling parallel to the animals and photo-identification took place. Data regarding geographical position, behavioral state [feeding, traveling, socializing or resting; see Shane (1990) for details], group composition, sea state (Beaufort scale) and group size estimation were collected.

Biopsies were taken using stainless steel cylindrical sampling tips specially designed to collect skin and blubber samples for small cetaceans. Tips measured 25mm in length and 8mm in diameter and had a cylindrical punch fitted with three internal barbs (to hold a sample in place) attached to modified darts[1]. A cylindrical foam stopper caused the bolt to rebound after impact and limited the penetration depth to 20mm. Darts were fired from a crossbow with fixed power of 68kg draw weight. Shots were made only when dolphins were perpendicular to the sampler, allowing a more likely hit to the side of the animal, below the dorsal fin (Figure 2). After shooting, behavioral reactions of the target animal were recorded *ad libitum* by onboard researchers for a minimum of five minutes (Altmann, 1974). Reactions of target animals were defined following Weinrich *et al.* (1991) modified by Berrow *et al.* (2002) (Table 2). In situations where simultaneous photo-identification failed, experienced researchers were able to identify marked individuals visually. All dorsal fin photographs were examined for 'marked' animals by one of the authors (PFF) using the bottlenose dolphin reference catalogue created during a previous photo-identification study (Dalla Rosa, 1999), and periodically maintained since 2005 by the Laboratório de Mamíferos Marinhos, Museu Oceanográfico 'Prof Eliézer de C. Rios'. Skin samples were placed in a saturated saline solution (dimethyl sulphoxide/DMSO) and blubber stored in a –20°C freezer.

Laboratory work consisted of extracting total DNA from tissue samples, identifying sex through PCR amplification following methods described by Gilson *et al.* (1998) and using PCR conditions reported in Möller *et al.* (2001).

Table 2. Definitions of behavioral reactions of bottlenose dolphins (*Tursiops truncatus*) subjected to biopsy sampling in southern Brazil (following Weirinch *et al.*, 1991, and modified by Berrow *et al.*, 2002).

Reaction level	Individual behavior reaction
None	Dolphins showed no modification of pre-biopsy behavior
Low	Slight change in behavioral state such as an increase in swimming speed and/or immediate dive
Moderate	Dolphins modified behavior in a more forceful manner, such as a tail slap, but showed no prolonged evidence of behavioral disturbance
Strong	Rough movements and succession of forceful activities, retarding and/or not returning to prior behavioral state (breaches, multiples tail slaps)

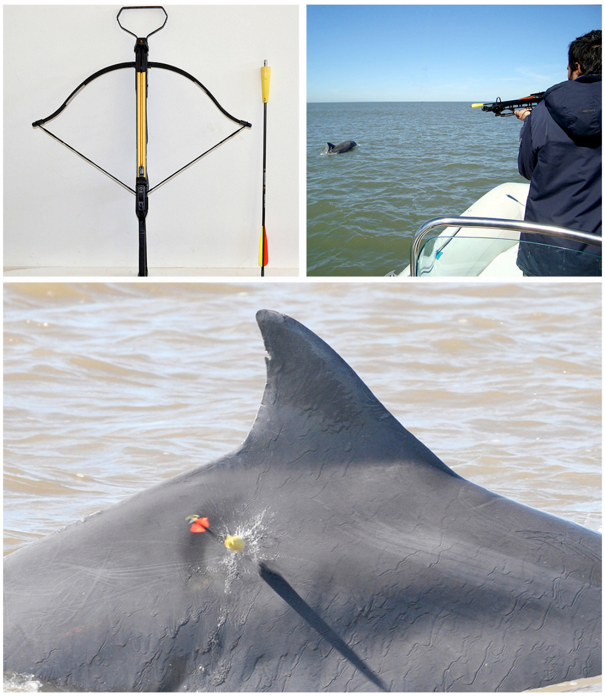

Figure 2. Standard biopsy darting protocol during the study. Upper left: biopsy equipment used (68kg crossbow and modified dart and tip). Upper right: general boat approach during a biopsy attempt. Below: typical dolphin position in relation to the darter and location of successful sampling collection.

[1]Ceta-Dart, Copenhagen, Denmark

Table 3. Summary of survey effort, group size and sampling success in bottlenose dolphins (*Tursiops truncatus*) biopsied in the Patos Lagoon Estuary and adjacent coastal waters, southern Brazil. Standard deviations are given in parentheses.

Type of survey	Survey (n)	Effort (h:min)	Attempts (n)	Hit rate (%)	Groups (n)	Mean group size	Average time spent per group (h:min)	Average biopsies per group
Dedicated	15	114:51	151	41.7	44	4.3 (3.8)	1:47 (0:58)	1.5 (0.5)
Opportunistic	36	72:31	101	54.4	41	6.4 (5.5)	0:56 (0:32)	1.2 (0.5)
Total	51	187:22	252	46.8	85	4.8 (4.2)	1:02 (0:42)	1.3 (0.8)

Estimating Target Distance

Digital images from biopsied dolphins were uploaded in a Range Finder application (http://www.codeproject.com/Articles/35029/Range-Finder) from where distances of sampling attempts were estimated. Briefly, this software can be used to estimate the size of an object or the distance to objects of known sizes based on four different variables: the size of the object (Y), the distance of the object from the lens (X1), the size of the image on the sensor (Y2), and the distance between the sensor and the lens (X2). The program automatically recognizes variables Y2 and X2 from digital camera files; therefore, the distance estimation is sensitive to the size of the object, variable Y (in this case the dorsal fin size). As observational data have suggested that bottlenose dolphins in this region may present sexual dimorphism, with adult males apparently presenting higher dorsal fin height than females (P.F. Fruet, unpub. data), we measured the mean height of the dorsal fins of previously stranded adults (see Fruet *et al.*, 2012 for maturity class definition in stranded carcasses) and used as input parameter (Y) to estimate the distances to target (X1) for each sex separately. According to the software's manufacturer, accuracy of this method depends on camera sensor size, quality of lens and noise of the image, but generally it is higher than 95% (http://www.codeproject.com/Articles/35029/Range-Finder). Distances were estimated only when simultaneous photography of arrow and targeted dolphin was available.

Statistical analysis

Yates' chi-square test for contingency tables was used to investigate potential differences in the frequencies (none versus low-level, moderate and strong pooled) and intensity (none and low-level pooled versus moderate and strong) of reactions between hits and misses as well as to compare the frequencies of occurrence of reactions in different group sizes (lone individuals; small groups: 2-4 individuals; or large groups: more than four individuals). A pairwise 2x2 contingency table with a Fisher's exact test was used to test the frequency of responses among pre-biopsy behavioral states (traveling, feeding and socializing), and if behavior, sex or group size affected the intensity of reactions of animals that were hit. Significance level for all tests was set at $\alpha = 0.05$.

Results

Effectiveness of remote biopsy sampling

Fifty-one surveys were undertaken during the study, totaling 187:22hs of sampling effort. Overall, 252 biopsy attempts were made on 85 groups, resulting in 118 hits (47% of attempts) and 104 samples (41% of attempts) collected successfully (Table 3). In 14 occasions, the dart hit the dorsal fin and did not collect any tissue. Sampler hitting success rate increased as number of attempts increased (Figure 3). From the sampled animals, seven (7%) were juveniles and the remaining were adults.

Mean time elapsed between initial encounter with a given group and first biopsy attempt was 39min (SD = 13min). The number of samples collected during dedicated surveys was slightly greater than that collected during opportunistic surveys, but time spent was twice as long (Table 3). At the individual level, the number of attempts per identified dolphin varied from one to five, but the majority (73%) of animals were subjected to biopsy attempts no more than once. At least 20 resident animals were subjected to ≥ 2 biopsy attempts. From these, 15 (75%) reacted similarly in all biopsy attempts, even when more than one attempt was conducted successively in the same sampling session (Table 4). Interestingly, from seven dolphins that were hit twice, only one responded in a more forceful manner in the second hit, while the others had weak reactions in both hits.

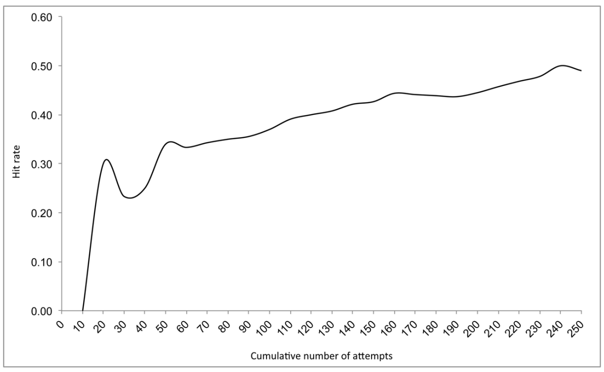

Figure 3. Sampler effectiveness. Hit rate was re-calculated for every 10 attempts.

Table 4. Detailed comparison of individual reactions of bottlenose dolphins (*Tursiops truncatus*) subjected to multiple biopsy attempts. ID = dolphin code based on the bottlenose dolphin photo-identification catalogue maintained by Laboratório de Mamíferos Marinhos/Museu Oceanográfico 'Prof. Eliézer de C. Rios'.

ID	Sex	Attempts (n)	Surveys (n)	Attempt 1	Reaction Level	Attempt 2	Reaction Level	Attempt 3	Reaction Level	Attempt 4	Reaction Level	Attempt 5	Reaction Level
LP001	F	4	2	Miss	Low	Miss	Low	Miss	Low	Hit	Moderate	-	-
LP003	F	2	1	Miss	Moderate	Miss	Moderate	-	-	-	-	-	-
LP005	F	5	4	Miss	Low	Miss	Moderate	Miss	Low	Miss	Low	Hit	Moderate
LP006	M	3	2	Miss	Low	Miss	Low	Miss	Low	-	-	-	-
LP014	M	3	2	Miss	Low	Miss	None	Miss	Low	-	-	-	-
LP018	M	2	2	Hit	Low	Hit	Moderate	-	-	-	-	-	-
LP020	M	2	1	Miss	Low	Miss	Low	Hit	Low	-	-	-	-
LP022	M	2	2	Miss	Low	Miss	Low	Hit	Low	-	-	-	-
LP023	F	2	1	Miss	Low	Hit	Low	-	-	-	-	-	-
LP036	M	5	4	Hit	Low	Miss	Low	Miss	Low	Miss	Low	Miss	Low
LP039	M	2	2	Miss	Low	Hit	Moderate	-	-	-	-	-	-
LP041	F	3	2	Miss	Low	Hit	Low	Hit	Low	-	-	-	-
LP043	F	2	2	Miss	Low	Hit	Low	Hit	Low	-	-	-	-
LP044	M	2	2	Miss	Low	Hit	Low	Hit	Low	-	-	-	-
LP045	F	2	2	Hit	Low	Hit	Low	Miss	Low	-	-	-	-
LP049	F	2	2	Miss	Low	Hit	Low	Hit	Low	-	-	-	-
LP064	F	2	2	Miss	Low	Miss	Low	-	-	-	-	-	-
LP/ID	M	3	2	Hit	Low	Hit	Low	Miss	Low	-	-	-	-
C004	M	3	2	Miss	Low	Miss	Low	Hit	Low	-	-	-	-
NM	F	2	2	Miss	Low	Hit	Low	-	-	-	-	-	-

Instant reactions to biopsy sampling

We collected data on behavioral responses for 246 biopsy attempts (115 hits and 131 misses). Overall, bottlenose dolphins reacted to 98% of hits and 78% of misses, and this difference was statistically significant (x^2 = 21.3; $p < 0.05$, df = 1). Low-level reactions were the most frequently observed response to both hits and misses (Figure 4). However, dolphins displayed stronger reactions to hits than they did to misses (x^2 = 7.48, $p < 0.05$, df = 1). Moderate reactions were very similar among individuals, consisting of instantaneous acceleration caused by a rapid and a sharp movement of the tail peduncle, resulting in a strong impact in the water's surface followed by an arched back and a short dive. During these observations, dolphins were resighted in close proximity to the boat a few minutes (< 3min) after biopsied, with no observed sign of boat avoidance. In one occasion, the dart got stuck for 33min on the side of an adult male (LP#036), but the dolphin had a weak reaction to the hit, executing short dives and smooth movements. No sample was retrieved after the dart dropped from the animal. A strong reaction was observed in one particular situation. This consisted of accelerated swimming and multiple jumps of a group of four dolphins (including the target biopsied adult female and its presumed 1.5yr calf). The dolphins were feeding at the mouth of the estuary and left the area towards the coastal zone. The animals moved out at high speed for about 2min (traveling approximately 1.2km), slowing down when in the coastal zone. We did not observe the group return to the estuary during our final 25 minutes of observations for that day.

Dolphins were biopsied at estimated distances ranging between 3 and 32m (n = 84; mean = 15.7; SD = 5.4) and reacted in a similar fashion irrespective of target distance (Figure 5). Genetic analysis showed that, of the 103 samples collected, 61 samples were females (59.2%) and 42 males (40.8%), with both sexes showing similar types of reactions (χ^2 = 0.02, $p > 0.05$, df = 1). Data on behavioral states prior to biopsy attempts were recorded for 241 attempts (113 hits and 128 misses). Low-level reactions (n = 176) were the most

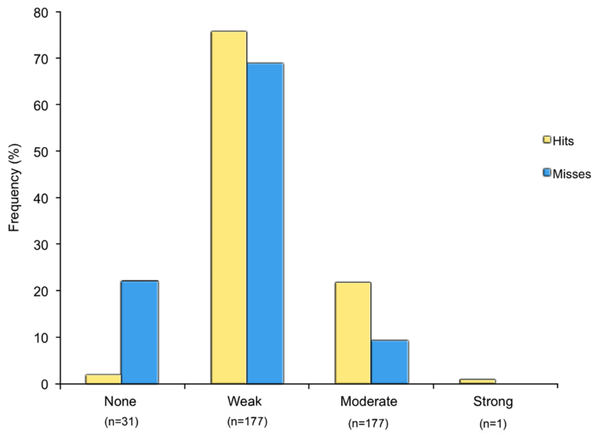

Figure 4. Individual behavioral reactions of bottlenose dolphins (*Tursiops truncatus*) to biopsy hits (n = 115) and misses (n = 131).

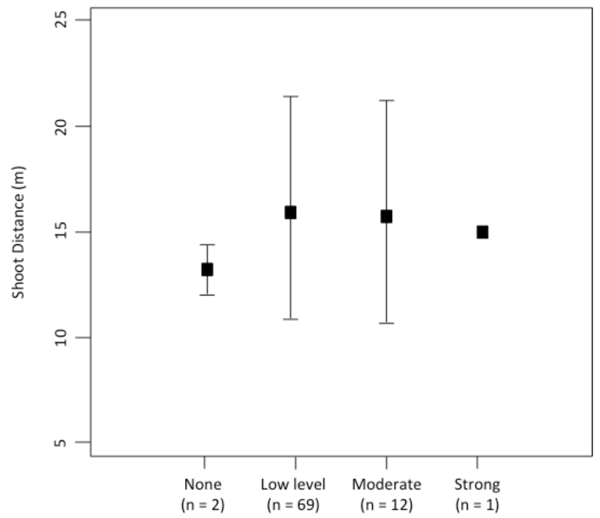

Figure 5. Mean target distances and intensity of reactions of bottlenose dolphins (*Tursiops truncatus*). Lines represent standard deviations. Data presented for hits only (n = 84).

common for all pre-biopsy behavioral states (Figure 6). We did not find a significant effect of initial behavioral state on the level of the reaction (Fisher's exact test: all pairwise comparisons p > 0.05). Intensity of response (none/low vs. moderate) from sampled animals was not statistically different according to pre-behavioral state (Fisher's exact test: all pairwise comparisons p > 0.05). Response frequencies were also not affected by group size (n = 249; $\chi^2 = 0.45$, $p > 0.05$, df = 2), and no clear evidence of an association between group size and intensity of reactions was found when tested for hits only (Fisher's exact test: all pairwise comparisons p > 0.05).

Wound Healing

We could monitor the progress of wound healing in 18 biopsied animals from which high quality photographs were acquired in subsequent surveys after biopsy. The number of different days individuals were resighted varied from one to ten, with a maximum of 26 months elapsed. Healing process was very similar between individuals with no apparent signs of skin infections. After biopsy, wounds presented red coloration instantly; few days after biopsying a dark circle around the biopsy point appeared (Figure 7). The time needed for wounds to heal varied from 18 to 35 days, but generally in about three weeks wounds appeared to be completely covered by epidermis. The pale color started to gradually change towards the standard skin coloration, but wounds were still visually detectable for biopsied animals even more than two years later. When a dart hit the dorsal fin, the wound took longer to heal (between one and three months), and wound swelling was also observed (Figure 8).

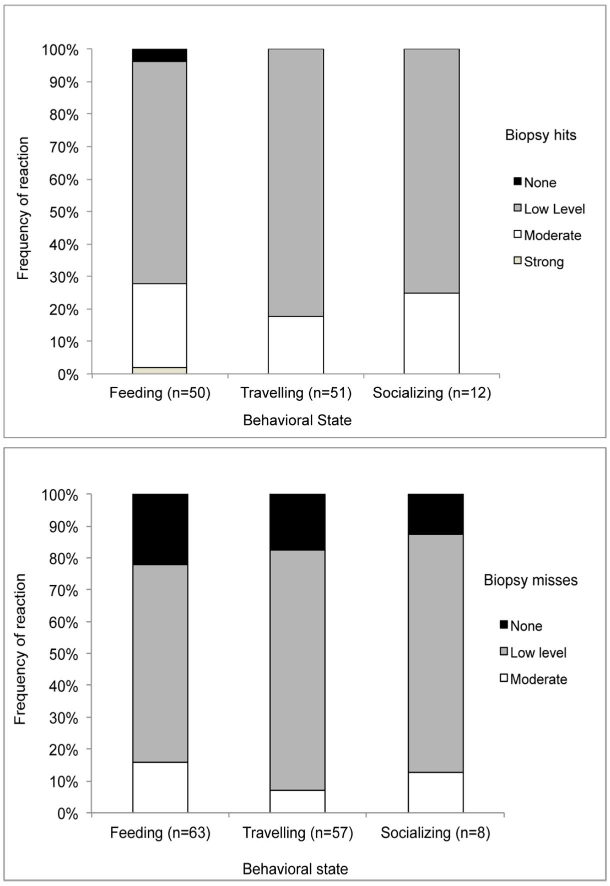

Figure 6. Relationship between pre-biopsy behavior and instant reactions of bottlenose dolphins (*Tursiops truncatus*) to biopsy hits (n = 113) and misses (n = 128).

Discussion

Sampling success rate and survey type

Although our success hit rate (46.8%) is within the range of values reported for other studies, it is the third lowest rate

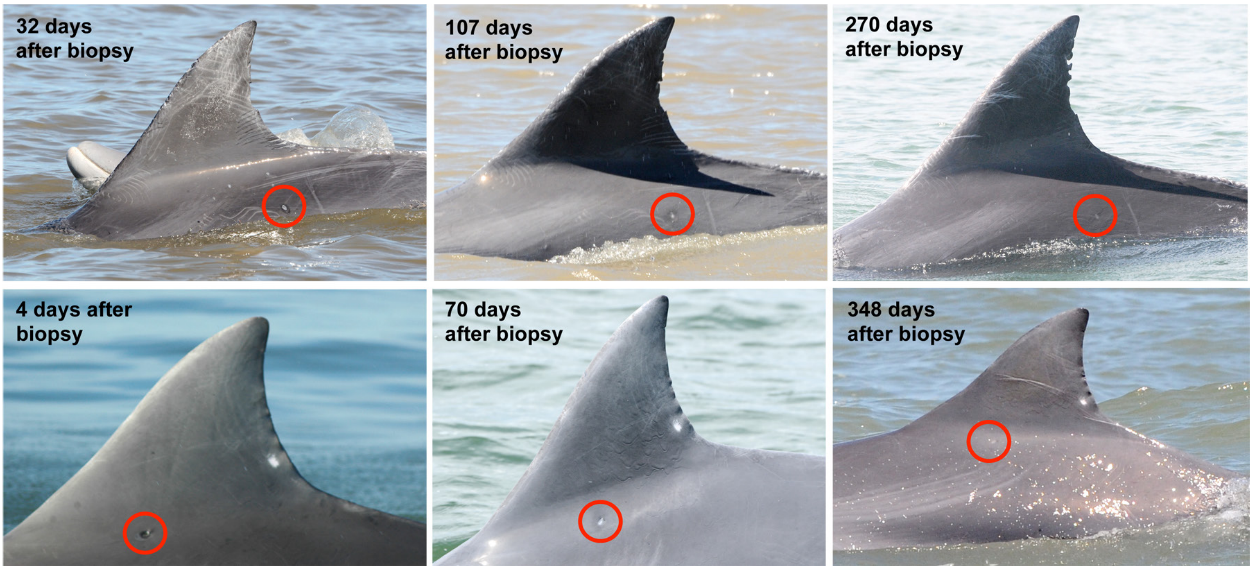

Figure 7. Typical wound healing documented in two wild bottlenose dolphins (*Tursiops truncatus*) biopsy-sampled in southern Brazil. Red circles indicate biopsy scars. Time (days) between sightings after biopsy is shown in the upper left of each picture.

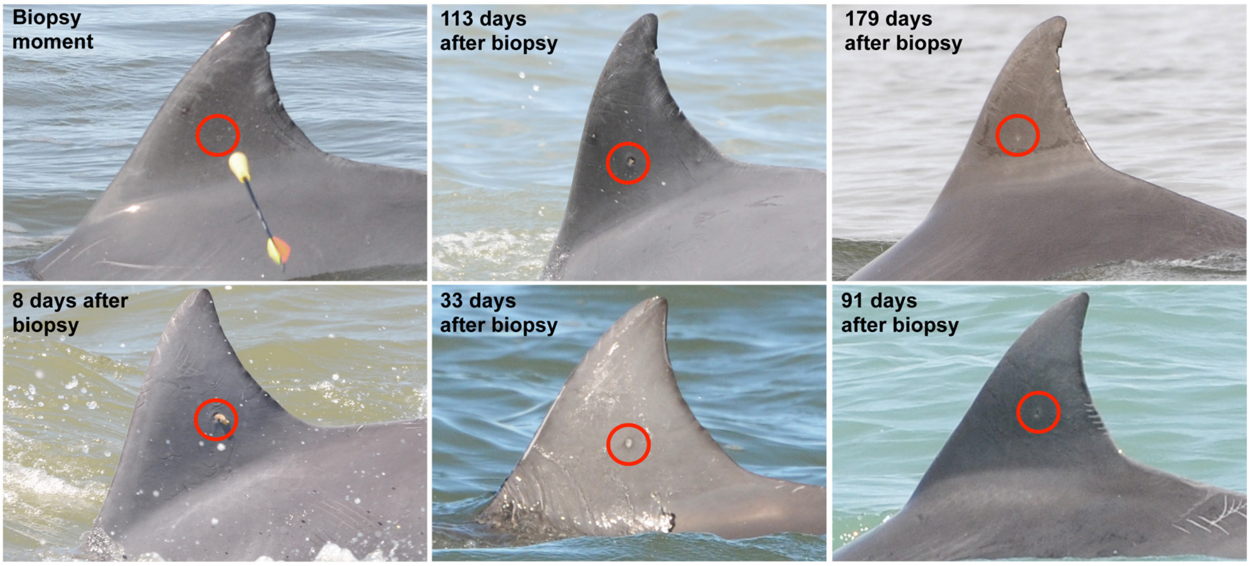

Figure 8. Examples of wound healing in the dorsal fin of two juvenile bottlenose dolphins (*Tursiops truncatus*) biopsy-sampled in southern Brazil. Red circles indicate biopsy scars. Time (days) between sightings after biopsy is shown in the upper left of each picture.

reported for small delphinids, irrespective of the equipment used (Table 1). The inexperience of the sampler, particularly in the early stages of this study (see Figure 3) should not be excluded as one of the reasons contributing to such a low hit rate, but it is unlikely the main one. High hit rates obtained by experienced samplers in some regions dropped to similar low rates as that found in the present study when conducting biopsy sampling in bottlenose dolphin populations inhabiting turbid waters and where individuals do not usually bow-ride (see Krützen *et al.*, 2002 and Tezanos-Pinto and Baker, 2012; Table 1). Our study area imposes difficulties to sampling. High levels of water turbidity and speed currents along the year make it very hard to predict surfacing behavior of dolphins in the estuarine mouth, where dolphins tend to occur (Mattos *et al.*, 2007; Di Tullio *et al.*, 2015). When in coastal areas, dolphins spend a great amount of time milling in the surf zone, where waves are regular and sea conditions are rarely below sea state Beaufort 3 for more than a few hours. During our sampling program, we attempted to biopsy only marked animals to maximize data quality and reliability, and to avoid re-sampling individuals. Therefore, in many situations we missed the opportunity to sample animals that were in appropriate and favorable conditions for sampling because they could not be reliably identified. In summary, many factors such as differences in dolphin's behavior, environmental conditions and sampling protocols may have contributed to the low sampling success rate in our study area and limit further comparisons with other studies.

An important finding of our study, especially when considering the resources invested in sampling surveys and animal's welfare, is that time with targeted groups (a measure of disturbance to the animals) and the total time spent on survey (a measure of cost) are reduced during opportunistic surveys when compared to dedicated surveys, with a similar number of samples collected (see Table 2). Therefore, data originated from these surveys can be used to answer other relevant research questions. This means that biopsy sampling in our area was effective, less costly and less intrusive when conducted opportunistically, although a long-term program is required to achieve a relatively good sample size from identified individuals for population studies.

Instant reactions to biopsy sampling and wound healing

Several factors can influence the reactions of dolphins to biopsy sampling, but generally the response to boat type and approach have been suggested as confounding effects (*e.g.* Brown *et al.*, 1994; Bilgmann *et al.*, 2007). As part of our protocol, photo-identification always took place prior to any biopsy attempt and as a consequence the amount of time spent per group until the first biopsy attempt was relatively long (mean = 39min). In addition, we attemped to maintain a distance of about 15m from the animals, minimizing the influence of boat presence on the reaction of dolphins to the biopsy procedure. Unfortunately, due to only a few field trips conducted with the aluminum boat, we could not test for differences in the reactions according to boat type, but we did not detect any obvious differences during our field observations. We observed the dolphin's behavior for at least five minutes after biopsy attempts and dolphins often returned to their pre-biopsy behavior within less than three minutes after attempts, suggesting that sampling caused only short-term responses on the animals. Furthermore, in three opportunities that darts were fired from land in the estuarine waters, behavioral responses were identical to that reported when using boats for biopsy sampling.

Our results show that biopsy sampling using modified darts fired from a 68kg crossbow is an efficient and safe method of obtaining tissue samples from bottlenose dolphins in the Patos Lagoon population. This technique results in similar effects on dolphin behavior, irrespective of the distance to target animals, sex, group size and pre-biopsy behavior, which is useful when conducting population studies. A few studies have investigated the effects of similar variables on the behavior response of bottlenose dolphins to biopsy sampling (Krützen *et al.*, 2002; Jefferson and Hung, 2008; Kiszka *et al.*, 2010), and our results seem to corroborate this published information. In addition, the results presented here agree with prior observations that remote biopsy sampling causes mostly minimal and short-term disturbance on the dolphin's behavior, where low-level reactions predominate and strong reactions are rare (Table 1). In only one situation we observed a strong reaction to a biopsy hit, similarly to that previously described for bottlenose dolphins elsewhere, where single or multiple breaches out of the water were reported (*e.g.* Krützen *et al.*, 2002; Gorgone *et al.*, 2008). Although these four dolphins did not return to the pre-biopsy behavior, the female and calf were resighted in the estuary at a later date and closely approached the boat in subsequent surveys, suggesting that the strong reaction observed was not long-lasting. The reason for the unusual strong reaction may potentially relate to an individual variation in behavior response. Our data show that animals subjected to multiple biopsy attempts usually reacted in a similar manner after an attempt (see Table 4), suggesting that individual variations may exist. However, our sample size is too small to test this hypothesis.

Interestingly, we found a significant difference in the response frequency and intensity of reactions to hits and misses. Responses to misses have been suggested as potentially analogous to the instantaneous reactions of bottlenose dolphins in captivity when encountering unexpected or unusual situations (Weller *et al.*, 1997). On the other hand, the reaction intensity was significantly stronger for hits, with a higher rate of moderate responses when the dart hit the animal, probably as a consequence of the physical impact of the dart.

We could identify 84 biopsied dolphins, from which 55 were year-round residents in the PLE, giving us the opportunity to track them in a non-systematic way during consecutive surveys. From these, one adult male (LP015) biopsied in 2004 died in 2008 victim of a fishing net entanglement, and an adult female (LP033), also biopsied in 2004, has not been seen in the area since 2010; 53 dolphins are still alive. There is no indication of any long-term effects of biopsy sampling, such as avoidance of the boat or sampling area. The research boat easily approached all dolphins for photo-identification purposes and they were still observed using the estuarine waters frequently.

Wounds healed well for the 18 dolphins from which it was possible to observe the wound healing progress, and our observations are in agreement with wound healing pattern and timing reported in the literature (Weller *et al.*, 1997; Krützen *et al.*, 2002; Parsons *et al.*, 2003; Jefferson and Hung, 2008). However, when the dart hit the dorsal fin, a swelling around the wound was observed. To our knowledge no long-term negative effects of biopsy sampling on the behavior or health of any cetacean species have yet been reported (*e.g.* Noren and Mocklin, 2012; Tezanos-Pinto and Baker, 2012), even considering those living in a polluted environment (Jefferson and Hung, 2008). Our study supports these observations.

In conclusion, results presented here showed that reactions of bottlenose dolphins to biopsy sampling were mostly low-level and short-term regardless of sex, target distance, pre-biopsy behavior and group size. Biopsy sampling of the bottlenose dolphin population inhabiting the PLE using a crossbow is therefore viable and likely does not cause any long-term effect on dolphin behavior. We suggest that biopsying should be carried out by trained personnel only (both sampler and boat pilot) and that the power of crossbows should not exceed 68kg. Traveling animals should be preferred over other behavioral states because they are easier for approaching and predicting their surface behavior, decreasing the chance of hits in undesirable areas.

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References

Altmann, J. (1974) Observational study of behavior: sampling methods. *Behaviour* 49(3): 227-267. http://dx.doi.org/10.1163/156853974X00534

Baker, C.S., Palumbi, S., Lambertsen, R., Weinrich, M., Calambokidis, J. and O'Brien, S. (1990) Influence of seasonal migration on geographic distribution of mitochondrial DNA haplotypes in humpback whales. *Nature* 344(6263): 238-240. http://dx.doi.org/10.1038/344238a0

Bearzi, G. (2000) First report of a common dolphin (*Delphinus delphis*) death following penetration of a biopsy dart. *Journal of Cetacean Research and Management* 2(3): 217-221.

Berrow, S., McHugh, B., Glynn, D., McGovern, E., Parsons, K., Baird, R. and Hooker, S. (2002) Organochlorine concentrations in resident bottlenose dolphins (*Tursiops truncatus*) in the Shannon estuary, Ireland. *Marine Pollution Bulletin* 44(11): 1296-1303. http://dx.doi.org/10.1016/S0025-326X(02)00215-1

Bilgmann, K., Griffiths, O.J., Allen, S.J. and Möller, L.M. (2007) A biopsy pole system for bow-riding dolphins: Sampling success, behavioral responses, and test for sampling bias. *Marine Mammal Science* 23(1): 218–225. http://dx.doi.org/10.1111/j.1748-7692.2006.00099.x

Brown, M., Corkeron, P., Hale, P., Schultz, K. and Bryden, M. (1994) Behavioral responses of east Australian humpback whales *Megaptera novaeangliae* to biopsy sampling. *Marine Mammal Science* 10(4): 391–400. http://dx.doi.org/10.1111/j.1748-7692.1994.tb00496.x

Caballero, S., Islas-Villanueva, V., Tezanos-Pinto, G., Duchene, S., Delgado-Estrella, A., Sanchez-Okrucky, R. and Mignucci-Giannoni, A.A. (2012) Phylogeography, genetic diversity and population structure of common bottlenose dolphins in the Wider Caribbean inferred from analyses of mitochondrial DNA control region sequences and microsatellite loci: conservation and management implications. *Animal Conservation* 15(1): 95-112. http://dx.doi.org/10.1111/j.1469-1795.2011.00493.x

Dalla Rosa, L. (1999) *Estimativa do tamanho da população de botos,* Tursiops truncatus*, do estuário da Lagoa dos Patos, RS, a partir da foto-identificação de indivíduos com marcas naturais e da aplicação de modelos de marcação-recaptura*. M.Sc. Thesis. Fundação Universidade Federal do Rio Grande, Instituto de Oceanografia. Rio Grande, Brazil.104 pp.

Di Tullio, J.C., Fruet, P.F. and Secchi, E.R. (2015) Identifying critical areas to reduce bycatch of coastal common bottlenose dolphins *Tursiops truncatus* in artisanal fisheries of the subtropical western South Atlantic. *Endangered Species Research* 29(1): 35–50. http://dx.doi.org/10.3354/esr00698

Fruet, P.F., Secchi, E.R., Di Tullio, J.C. and Kinas, P.G. (2011) Abundance of bottlenose dolphins, *Tursiops truncatus* (Cetacea: Delphinidae), inhabiting the Patos Lagoon estuary, southern Brazil: implications for conservation. *Zoologia* 28(1): 23-30. http://dx.doi.org/10.1590/S1984-46702011000100004

Fruet, P.F., Kinas, P.G., Da Silva, K.G., Di Tullio, J.C., Monteiro, D.S., Dalla Rosa, L., Estima, S.C. and Secchi, E.R. (2012) Temporal trends in mortality and effects of by-catch on common bottlenose dolphins, *Tursiops truncatus*, in southern Brazil. *Journal of the Marine Biological Association of the United Kingdom* 92(8): 1865-1876. http://dx.doi.org/10.1017/S0025315410001888

Gilson, A., Syvanen, M., Levine, K. and Banks, J. (1998) Deer gender determination by polymerase chain reaction: validation study and application to tissues, bloodstains, and hair forensic samples from California. *California Fish and Game* 84(4): 159-169.

Gorgone, A.M., Haase, P.A., Griffith, E.S. and Hohn, A.A. (2008) Modeling response of target and nontarget dolphins to biopsy darting. *Journal of Wildlife Management* 72(4): 926-932. http://dx.doi.org/10.2193/2007-202

Harlin, A., Würsig, B., Baker, C.S. and Markowitz, T.M. (1999) Skin swabbing for genetic analysis: application to dusky dolphins (*Lagenorhynchus obscurus*). *Marine Mammal Science* 15(2): 409-425. http://dx.doi.org/10.1111/j.1748-7692.1999.tb00810.x

Herman, D., Burrows, D., Wade, P., Durban, J., Matkin, C., LeDuc, R., Barrett-Lennard, L. and Krahn, M. (2005) Feeding ecology of eastern North Pacific killer whales *Orcinus orca* from fatty acid, stable isotope, and organochlorine analyses of blubber biopsies. *Marine Ecology Progress Series* 302: 275-291. http://dx.doi.org/10.3354/meps302275

Jefferson, T.A. and Hung, S.K. (2008) Effects of biopsy sampling on Indo-Pacific humpback dolphins (*Sousa chinensis*) in a polluted coastal environment. *Aquatic Mammals* 34(3): 310-316. http://dx.doi.org/10.1578/AM.34.3.2008.310

Kiszka, J., Simon-Bouhet, B., Charlier, F., Pusineri, C. and Ridoux, V. (2010) Individual and group behavioural reactions of small delphinids to remote biopsy sampling. *Animal Welfare* 19(4): 411-417.

Kiszka, J., Simon-Bouhet, B., Martinez, L., Pusineri, C., Richard, P. and Ridoux, V. (2011) Ecological niche segregation within a community of sympatric dolphins around a tropical island. *Marine Ecology Progress Series* 433: 273-288. http://dx.doi.org/10.3354/meps09165

Knoff, A., Hohn, A. and Macko, S. (2008) Ontogenetic diet changes in bottlenose dolphins (*Tursiops truncatus*) reflected through stable isotopes. *Marine Mammal Science* 24(1): 128-137. http://dx.doi.org/10.1111/j.1748-7692.2007.00174.x

Krützen, M., Barré, L., Möller, L.M., Heithaus, M., Colleen, S. and Sherwin, W. (2002) A biopsy system for small cetaceans: Darting success and wound healing in *Tursiops* spp. *Marine Mammal Science* 18(4): 863-878. http://dx.doi.org/10.1111/j.1748-7692.2002.tb01078.x

Mansour, A., Mkay, D., Lien, J., Orr, J., Banoub, J., Oien, N. and Stenson, G. (2002) Determination of pregnancy status from blubber samples in minke whales (*Balaenoptera acutorostrata*). *Marine Mammal Science* 18(1): 112-120. http://dx.doi.org/10.1111/j.1748-7692.2002.tb01022.x

Mattos, P.H., Dalla Rosa, L. and Fruet, P.F. (2007) Activity budgets and distribution of bottlenose dolphins (*Tursiops truncatus*) in the Patos Lagoon estuary, southern Brazil. *Latin American Journal of Aquatic Mammals* 6(2): 161-169. http://dx.doi.org/10.5597/lajam00121

Möller, L.M. and Beheregaray, L.B. (2001) Coastal bottlenose dolphins from southeastern Australia are *Tursiops aduncus* according to sequences of the mitochondrial DNA control region. *Marine Mammal Science* 17(2): 249-263. http://dx.doi.org/10.1111/j.1748-7692.2001.tb01269.x

Möller, L.M. and Beheregaray, L.B. (2004) Genetic evidence for sex-biased dispersal in resident bottlenose dolphins (*Tursiops aduncus*). *Molecular Ecology* 13(6): 1607-1612. http://dx.doi.org/10.1111/j.1365-294X.2004.02137.x

Möller, L.M., Beheregaray, L.B., Harcourt, R. and Krützen, M. (2001) Alliance membership and kinship in wild male bottlenose dolphins (*Tursiops aduncus*) of southeastern Australia. *Proceedings of the Royal Society of London B* 268(1479): 1941-1947. http://dx.doi.org/10.1098/rspb.2001.1756

Noren, D.P. and Mocklin, J.A. (2012) Review of cetacean biopsy techniques: factors contributing to successful sample collection and physiological and behavioral impacts. *Marine Mammal Science* 28(1): 154-199. http://dx.doi.org/10.1111/j.1748-7692.2011.00469.x

Parsons, K.M. (2001) Reliable microsatellite genotyping of dolphin DNA from faeces. *Molecular Ecology Notes* 1(4): 341-344. http://dx.doi.org/10.1046/j.1471-8278.2001.00098.x

Parsons, K.M., Dallas, J., Claridge, D.E., Durban, J.W., Balcomb, J., Thompson, P. and Noble, L. (1999) Amplifying dolphin mitochondrial DNA from faecal plumes. *Molecular Ecology* 8(10): 17661768. http://dx.doi.org/10.1046/j.1365-294x.1999.00723-8.x

Parsons, K.M., Durban, J.W. and Claridge, D.E. (2003) Comparing two alternative methods for sampling small cetaceans for molecular analysis. *Marine Mammal Science* 19(1): 224-231. http://dx.doi.org/10.1111/j.1748-7692.2003.tb01104.x

Quérouil, S., Freitas, L., Dinis, A., Alves, F., Cascão, I., Prieto, R., Silva, M.A., Magalhães, S., Matos, J.A. and Santos, R.S. (2010) Sex bias in biopsy samples collected from free-ranging dolphins. *European Journal of Wildlife Research* 56(2): 151-158. http://dx.doi.org/10.1007/s10344-009-0299-7

Shane, S. (1990) Behavior and ecology of the bottlenose dolphin at Sanibel Island, Florida. Pages 245-265 *in* Leatherwood, S. and Reeves, R.R. (Eds) *The Bottlenose Dolphin*. Academic Press, San Diego, USA.

Tezanos-Pinto, G. and Baker, C.S. (2012) Short-term reactions and long-term responses of bottlenose dolphins (*Tursiops truncatus*) to remote biopsy sampling. *New Zealand Journal of Marine and Freshwater Research* 46(1): 13-29. http://dx.doi.org/10.1080/00288330.2011.583256

Waples, R. (1991) Genetic methods for estimating effective population size of cetacean populations. Pages 279-300 *in* Hoelzel, R.A. (Ed.) *Genetic ecology of whales and dolphins*. International Whaling Commission (Special Issue 13), Cambridge, UK.

Weinrich, M., Lambertsen, R., Baker, C.S., Schilling, M. and Belt, C. (1991) Behavioural responses of humpback whales (*Megaptera novaeangliae*) in the southern Gulf of Maine to biopsy sampling. Pages 91-97 *in* Hoelzel, R.A. (Ed) *Genetic ecology of whales and dolphins*. International Whaling Commission (Special Issue 13), Cambridge, UK.

Weller, D.W., Cockcroft, V.G., Würsig, B., Lynn, S.K. and Fertl, D. (1997) Behavioral responses of bottlenose dolphins to remote biopsy sampling and observations of surgical biopsy wound healing and observations of surgical biopsy wound healing. *Aquatic Mammals* 23(1): 49-58.

Whitehead, H., Gordon, J., Mathews, E. and Richard, K. (1990) Obtaining skin samples from living sperm whales. *Marine Mammal Science* 6(4): 316-326. http://dx.doi.org/10.1111/j.1748-7692.1990.tb00361.x