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COMPARING TWO ALTERNATIVE METHODS FOR SAMPLING SMALL CETACEANS FOR MOLECULAR ANALYSIS

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During the last decade, non-destructive tissue sampling has been increasingly used to support the conservation and management of cetaceans. Biopsy sampling has permitted remote collection of small cores of skin and blubber to address questions on population size and structure, toxicological burdens, and feeding ecology for both large (*e.g.*, Brown *et al.* 1991, Palsbøll *et al.* 1991, Barrett-Lennard *et al.* 1996) and small (*e.g.*, Weller *et al.* 1997, Fossi *et al.* 2000) cetaceans (see Bearzi 2000 for review). Concern about the possible disturbance and physical impact caused by biopsy sampling has led to the development of less-invasive methods of tissue sampling (*e.g.*, Harlin *et al.* 1999, Parsons *et al.* 1999). However, the relative success of these alternative sampling approaches has not been examined. Here, we provide a direct comparison of the success and cost effectiveness of invasive and non-invasive methods for obtaining tissue samples from free-swimming bottlenose dolphins (*Tursiops truncatus*).

Tissue sampling was conducted from June to October, 1998–2000. Our objective was to obtain tissue samples for use in molecular analyses from known individuals using both remote biopsy sampling and collection of dolphin feces. Skin and blubber biopsy samples were obtained using the pneumatic darting system described in Barrett-Lennard *et al.* (1996). This system uses a variable-power dart projector (Pneudart Inc., Model 196) to deploy a lightweight, hollow aluminum dart body terminating with a nylon “stopper” and a stainless steel biopsy tip. Darts were modified for use on subtropical bottlenose dolphins by decreasing the tip length to limit the depth of penetration to 17 mm (based on ultrasonically measured skin/blubber thickness of bottlenose dolphins in Sarasota Bay, Florida.¹

¹ Personal communication from R. S. Wells, Mote Marine Laboratory, Sarasota, FL, 2 March 1998.

Corresponding to the decrease in tip length, the length of the aluminum dart body was shortened from 138 mm to 104 mm, thereby decreasing the overall mass of the dart by 1.22 g and minimizing the striking energy (Barrett-Lennard *et al.* 1996), while providing sufficient volume to ensure floatation.

Sampling was conducted from vessels ranging from 5 to 7 m in length, powered by single outboard engines. Sampling attempts began only after all individual dolphins within the group had been photographed (Durban *et al.* 2000), and the behavioral state (*e.g.* feeding, foraging, socializing, travelling, or resting) and composition of both the group and the target individual recorded. This ensured that any animal being targeted for biopsy sampling was photographed before the sampling attempt, facilitating individual identification and subsequent monitoring of both behavioral reactions and wound healing. All biopsy-sampling attempts were directed at the dorsal-lateral target region directly below, and extending posterior to, the dorsal fin. Following impact and recoil from the target animal, the floating dart was retrieved, labelled, wrapped in sterile foil, and stored in a cooler for subsequent processing. The behavioral reaction of the target animal, and all other non-target animals, as well as the outcome of every biopsy attempt were recorded. The target animal was reapprached, and a postbiopsy photograph was obtained whenever possible.

A systematic biopsy sampling protocol was developed and strictly adhered to throughout the study to minimize risk to both target and non-target animals. This protocol ensured that biopsy sampling would be attempted only on non-calf animals that were positioned and behaving in such a way that the path to the target area was unimpeded. Sampling was not attempted in rough waters (sea state > Beaufort 3), when the behavior of the animals was deemed unpredictable, or when animals were tightly grouped and synchronous. Furthermore, a minimum of two personnel practised in biopsy darting, dolphin photo-identification, and boat handling were present for all biopsy attempts. Adherence to this biopsy protocol resulted in many dolphin encounters where sampling could not be attempted, and restricted the total number of biopsies collected throughout the study. Out of 376 dolphin encounters, only 217 (57.7%) were with groups not comprised solely of a female and calf nor a previously biopsied animal, and also met the criteria for both sea state and personnel. In addition, bottlenose dolphins in the study area often engaged in activities where surfacing profiles were typically shallow and, consequently, the biopsy target region remained submerged. Due to these behavioral constraints, only 51 of the 217 (24%) encounters were determined to be suitable for biopsy attempts, and samples were successfully collected on 25 occasions.

During encounters when it was not possible to obtain skin and blubber biopsies, an alternative fecal sampling strategy was adopted. As with the biopsy sampling, we adhered to the photo-identification protocol prior to fecal collection. We obtained fecal samples by towing a snorkeller alongside the boat while following focal groups for extended periods of observation. When defecation was observed, a sample of the sinking feces was collected in a sterile 150-ml plastic vial (Parsons *et al.* 1999). All fecal samples were labelled with encounter information and the dolphin's identification number *only* when identification was

deemed unequivocal by an experienced observer. Following collection, excess seawater was decanted and fecal samples were preserved in a salt-saturated DMSO solution and stored at -20°C (Parsons *et al.* 1999, Parsons 2001). When following a dolphin group, we attempted to minimize repeated sampling of the same individuals.

Just as with biopsy sampling, collection of dolphin feces was subject to encounter-specific constraints. Fecal collection was attempted during encounters when dolphins were travelling relatively slowly (≤ 4.6 km/h) in sea state of Beaufort ≤ 4 . However, defecation was not observed during every fecal sampling attempt, nor was it always possible to collect feces when defecation was observed (*e.g.* if feces sank too quickly in shallow waters, or was too diffuse to collect). Detailed encounter records were examined for 77 encounters between May 1999 and October 2000. During this period, fecal collection was attempted on 31 encounters, and samples were successfully collected during 15 (48%) of these attempts. Although defecation was not observed during every attempt, 42% of successful attempts resulted in the collection of multiple samples ($\bar{X} = 1.83 \pm 1.20$ SD).

Over the study period 25 biopsy samples and 44 fecal samples were collected. Only 29 (66%) of the fecal samples could be assigned to individually identified dolphins at the time of sampling (and later confirmed by examining photographs), however, this was increased to 37 (84%) following molecular analyses. These eight additional individual assignments were made possible because encounter information recorded at the time of collection enabled the identification of duplicate samples *post hoc* with reference to molecular-determined sex, mitochondrial control region haplotypes, and microsatellite genotypes from fecal-extracted dolphin DNA. Fecal samples were obtained from 23 different dolphins based on field identifications, but subsequent molecular assignment of unidentified samples revealed an additional five individuals, for a total of 28 individual dolphins. From both the field-based and molecular assignments it was evident that some individuals were sampled more than once (Fig. 1), as it was not always possible to "target" and collect samples from specific animals. In contrast, multiple biopsy samples were never obtained from the same individual because it was always possible to predetermine the sampled animal.

Biopsy samples were obtained from 25 different individuals, 16 males and 9 females. In addition to skin samples, 72% of tissue biopsies contained blubber cores. Although all biopsy dart impacts were within the intended target area, the dart struck the target animal but did not retain a sample on seven occasions. All but two of these incidences occurred during the first year of sampling. Prior to the second season, manufacturing of new stainless steel biopsy tips with a more precise internal bevel angle (inclusive angle = 8°) resulted in a greater sample-per-hit success rate in subsequent years (64% in 1998 compared to 85% and 83% in 1999 and 2000, respectively).

All biopsy dart impacts ($n = 32$) were within the target area on the intended target animal, and non-target animals were never contacted with a biopsy dart. Reactions to the dart impact varied among the individuals (Table 1); however, the intensity and variety of the behavioral reaction was comparable in those at-

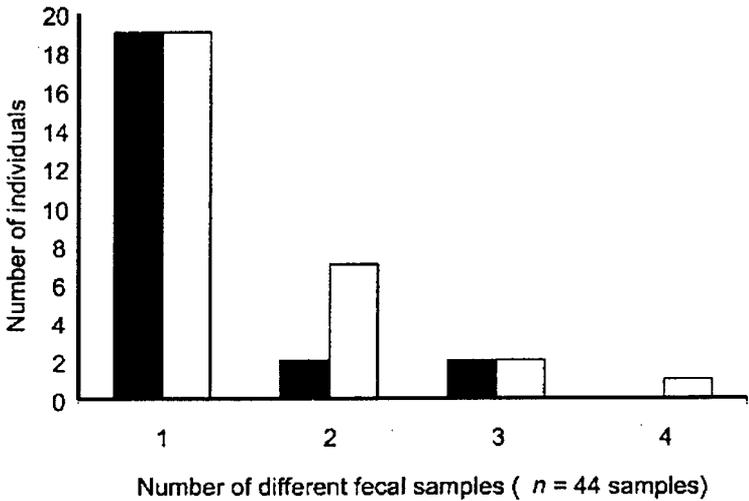


Figure 1. Frequency distribution of number of different fecal samples collected from individual bottlenose dolphins determined by field identifications (black bars) and by molecular identification of samples assigned to photographically documented dolphin groups, but individual identification at time of sample collection was unknown (gray bars).

tempts that did and did not pull a sample. The majority (22 out of 25) of the sampled individuals exhibited a minor response that ranged from no visible reaction to a small tail flick and immediate dive, but resumed their previous surfacing pattern after <4 surfacings (Table 1). This reaction was very similar to, and often less than, the startle reaction displayed by an animal when the dart did not make contact with the targeted animal, but struck the water nearby. Three animals displayed a strong reaction (a breach) to a biopsy dart that did not immediately recoil, however, even these reactions were immediate and of short duration (<3 min). All three of these animals were approached and photographed immediately following the biopsy attempt, and repeatedly during subsequent encounters. No visible reaction was detected among the non-target dolphins present during any of the biopsy encounters.

Only one biopsy attempt resulted in a dart that struck the target region on an adult animal and stuck for a prolonged period of time. In this instance, the dart remained attached to the animal by a very thin tissue fragment, and was observed hanging limp and lying parallel to the dolphin's body on subsequent surfacings. The dolphin exhibited no visible reaction to the dart's presence and was observed interacting with other animals during the 56-min post-biopsy focal follow (the focal follow was terminated at this point due to deteriorating weather conditions). When we re-encountered the individual 19 h later, the dart was no longer present, and the dolphin's behavior was consistent with the rest of the group, exhibiting no discernible negative reaction to the biopsy vessel.

In total, 28 out of 32 (88%) of the animals that were struck with a biopsy dart were subsequently documented with a high quality photograph on at least

Table 1. Summary of biopsy attempts and apparent behavioral reactions. Reactions were defined as: no visible = no visible reaction; slight = flinch and/or or immediate dive; minor = tail flick/kick and immediate dive; moderate = tail slap and acceleration away from vessel; strong = breach; persistent = reaction to biopsy vessel persists beyond immediate encounter.

	Behavioral Reaction					
	No visible	Slight	Minor	Moderate	Strong	Persistent
Sample ($n = 25$)	3	10	8	2	2	0
No sample ($n = 7$)	2	1	3	0	1	0

one different day following the biopsy attempt. The number of different days on which individual dolphins were encountered following the biopsy event varied from one to 16 d ($\bar{X} = 5.70 \pm 4.66$), spanning a period of up to 22 mo. Repeated photo-documentation of darted animals enabled us to document biopsy wounds and monitor their healing rates postbiopsy. Based upon high-quality photographs, wounds appear to be "closed" (covered by epidermal tissue) but pale in coloration after approximately 30 d, and visually undetectable after one year (Fig. 2). The observed pattern and timing of healing was consistent with the rates of surgical biopsy wound healing reported by Weller *et al.* (1997). This type of individual-based monitoring proved valuable in examining both the rate of wound healing and postbiopsy behavioral responses.

Both fecal collection and biopsy sampling provided useful tissue samples from bottlenose dolphins, but the success of these two methods differed in several key aspects. When conducted simultaneously with photo-identification, biopsy sampling permits analysis of samples with respect to individual-based social and ecological data. In contrast, a maximum of only 84% of fecal samples could be assigned to individually identified dolphins and 93% of fecal samples were of sufficient quality and quantity to permit successful amplification of dolphin mitochondrial DNA. Furthermore, of those fecal samples that yielded high quality mtDNA, some did not yield sufficient quantity to enable reliable microsatellite genotyping at all 17 loci screened ($\bar{X} = 13 \pm 3.97$). In contrast, 100% of skin biopsy samples yielded DNA of sufficient quality and quantity to provide reliable mitochondrial and nuclear molecular data (mean number of microsatellite loci typed = 16.6 ± 0.957).

Because of the difference in sample type, the two methods differ markedly in laboratory sample analysis costs. The cost of molecular analysis of fecal samples is approximately four times that of skin biopsy samples, and considerably more time consuming, when striving to obtain reliable genetic data from fecal DNA of comparatively low quality and quantity (Parsons *et al.* 1999, Parsons 2001). The two methods also differ in sample collection costs. Ignoring costs of personnel time and boat surveys (which are assumed constant) the expenses incurred for fecal sampling are approximately US\$1.70 per sample for equipment and sample preservation. In contrast, biopsy sampling (using the described darting system) costs approximately US\$28 per sample, however this cost will depreciate with

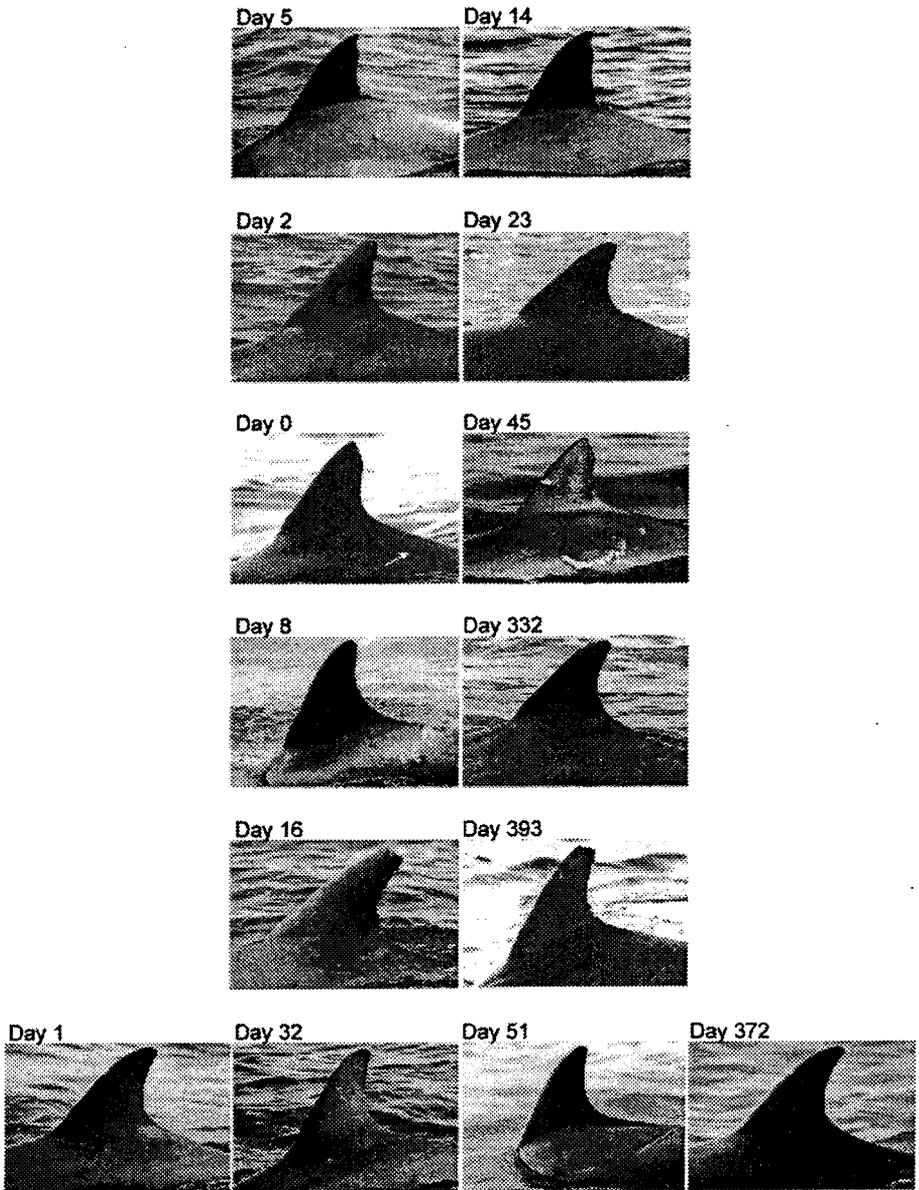


Figure 2. Repeated photo-documentation of biopsy darted individual dolphins. Photographs of six different individuals displayed, illustrating healing of biopsy wounds over varying time periods. Labels above each photograph correspond to number of days elapsed postbiopsy.

every additional sample obtained as much of the cost is due to the initial expense of the dart projector and dart manufacture. Therefore, despite its relative inefficiency, low collection costs initially make fecal sampling the most cost-effective method of collecting usable molecular data. However, owing to the comparatively low sample analysis costs, the biopsy method will become the most cost-effective method as the sample number increases.

While cost may be a factor when adopting a sampling strategy, it is important to consider the range of analyses and the breadth of information that can be obtained from samples. In addition to molecular genetic applications, tissue samples can be valuable for a variety of applications. Seventy-two percent of the biopsy samples collected during this study yielded blubber cores in addition to skin samples. These blubber samples proved useful in a preliminary assessment of the organochlorine contaminant burden of Bahamas bottlenose dolphins (unpublished data). In addition to organochlorine contaminants analyses (e.g., Aguilar *et al.* 2002), blubber cores can yield valuable information on foraging distribution and diet through fatty acid signature analysis (e.g., Iverson *et al.* 1997). If preserved appropriately, skin subsamples can be used for cytochrome P450 (Troisi and Mason 1997) and stable isotope assays (Hooker *et al.* 2001), in addition to molecular analyses and population genetic applications. While fecal samples may not prove useful in the same assays as biopsy samples, the potential exists for their use in alternative applications such as fecal glucocorticoid assays to assess reproductive status and measure physiological stress (Wasser *et al.* 2000, Foley *et al.* 2001).

The two sampling methods employed in this study differ considerably with respect to the unique costs and benefits of each strategy. In addition to the nature of the sample collected and the potential uses of the sample, the study location and behavior of the study population will play an important role in determining which method can be adopted. In situations where biopsy sampling is neither desirable nor possible, alternative non-invasive methods, such as fecal sampling, can be successful. Furthermore, conducting more than one sampling method concurrently can prove useful for maximizing the number of different individuals sampled, particularly where individual behavior or encounter location restrict biopsy sampling opportunities.

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