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DNA evidence of the consumption of short-beaked common dolphin *Delphinus delphis* by the shortfin mako shark *Isurus oxyrinchus*

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ABSTRACT: Stomachs of shortfin mako sharks *Isurus oxyrinchus* caught in the northeastern Atlantic by Iberian longliners were analyzed. A number of juveniles, 6 out of 96 individuals with non-empty stomachs, had consumed marine mammals. The remains (skin, fat, vertebrae and flesh with the dorsal fin) were not identifiable at species level by non-genetic methods. Portions of the mitochondrial DNA control region and of the gene coding for cytochrome *b* were therefore sequenced. Both the short-beaked common dolphin *Delphinus delphis* and possibly the striped dolphin *Stenella coeruleoalba* were identified. Shortfin makos are able to consume marine mammals almost as large as themselves. Well-preserved *D. delphis* were juveniles.

KEY WORDS: Shortfin mako · Predator · Short-beaked common dolphin · DNA analysis

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INTRODUCTION

Predation of marine mammals by sharks is often mentioned in the literature. However, direct observation of a successful attack has very rarely been reported; some failed attacks have been noted. For example, Long (1991) observed scars on the body of a pygmy sperm whale *Kogia breviceps*, probably inflicted by a white shark *Carcharodon carcharias*. A fresh wound and healed scars on a Cuvier's beaked whale *Ziphius cavirostris* were attributed to cook-cutter sharks *Isistius plutodus* (Pérez-Zayas et al. 2002). Most such observations are based on marine mammal remains found in shark stomachs (e.g. Crespi-Abril et al. 2003, McCord & Campana 2003, Lopez et al. 2010). In fact, the presence of marine mammal remains in shark stomachs does not neces-

sarily imply predation but may also be due to scavenging, i.e. consumption of already dead individuals. Such scavenging behaviour has been directly observed in *C. carcharias* (Domeier 2009).

The shortfin mako shark *Isurus oxyrinchus* is a pelagic species with a worldwide distribution (Compagno 2001, Moreno 2004). Every year, ~400 t of shortfin mako are landed at the fish market of Vigo, Spain (J. M. Portela Fernández, pers. comm.). Its diet mainly consists of fish and cephalopods (Vaske & Rincón-Filho 1998, Velasco Tarelo 2005, Maia et al. 2006, Gorni et al. 2012, Biton Porsmoguer et al. 2014). Evidence of the consumption of marine mammals is less well documented. *Tursiops truncatus* (Delphinidae) was anecdotally observed in shortfin makos in the central Pacific (Lopez et al. 2009). The remains were found in the stomach contents of a

female shortfin mako. A calf of the spotted dolphin *Stenella attenuata* was found in the stomach of a 210 cm total length shortfin mako in central Atlantic waters (Monteiro et al. 2006). Unidentified mammals, including delphinids, were observed in the diet of sharks in the central Pacific (Mucientes Sandoval & Saborido-Rey 2008) and in the northwestern Atlantic Ocean (Wood et al. 2009). Marine mammals were previously identified as prey consumed by shortfin mako in the north-eastern Atlantic and Pacific Oceans (Maia et al. 2006, Mucientes Sandoval & Saborido-Rey 2008). However, these marine mammal prey were not identified to species level, and no information is available concerning the trophic interactions between the shortfin mako and the Delphinidae species.

As part of a study on the diet of shortfin mako caught by Spanish and Portuguese longliners in the northeastern Atlantic, we observed remains of cetaceans, sometimes in poor condition. Although identifiable remains were found in some cases, e.g. vertebrae and dorsal fin, identification beyond the family level was not possible using basic morphological examination. The aim of this paper was to use molecular methods to pinpoint the dolphin species found in these stomachs. Genetic approaches have proven useful in studies of feeding ecology in marine top predators. DNA-based methods can complement visual identification of prey by refining taxonomic identifications and by revealing prey items that would not be detected visually (e.g. Barnett et al. 2010, Dunn et al. 2010, Méheust et al. 2015). Although it is beyond the scope of the present study, recent advances in metabarcoding involving next-generation sequencing methods have also improved prey identification (Pompanon et al. 2012) and can be combined with non-lethal sampling techniques such as stomach flushing.

MATERIALS AND METHODS

During 2012 and 2013, 149 shortfin mako stomachs were analyzed. All individuals were caught by Spanish and Portuguese longliners between the Azores Archipelago and the Iberian Peninsula (30 to 45° N, 8 to 35° W). Fig. 1 shows the

locations of sharks identified with marine mammal prey in their stomach contents.

We obtained stomachs of shortfin mako from the fish market of Vigo when sharks were landed. After removal, intact stomachs were preserved at -20°C and sent for analysis to the Mediterranean Institute of Oceanography (MIO) in Marseille (France).

Prey were identified, and a number of indices were calculated — %N: mean percentage of prey by number; %O: mean percentage frequency of occurrence (whatever the number of prey) in non-empty stomachs; %M: mean percentage by mass based on digested non-reconstituted prey; and %IRI: mean index of relative importance in non-empty stomachs (Pinkas et al. 1971).

A high percentage (94 %) of the 149 shortfin makos sampled were juveniles and measured <195 cm for males and <280 cm for females (Moreno 2004) (Fig. 2). The marine mammal remains were only found in stomach contents of 6 female individuals that measured between 115 and 210 cm (Table 1). Mammal remains consisted of 2 dorsal fins (Fig. 3), parts of the vertebral column, isolated vertebrae, skin and pieces of fat (Table 1).

Vertebrae were extracted and compared with a reference collection of the Muséum National d'Histoire Naturelle (MNHN) in Paris (France). However, visual comparison of the marine mammal vertebrae found in stomach contents and the vertebrae in the MNHN collection could not provide reliable species identification. High variability in bone structure can occur in

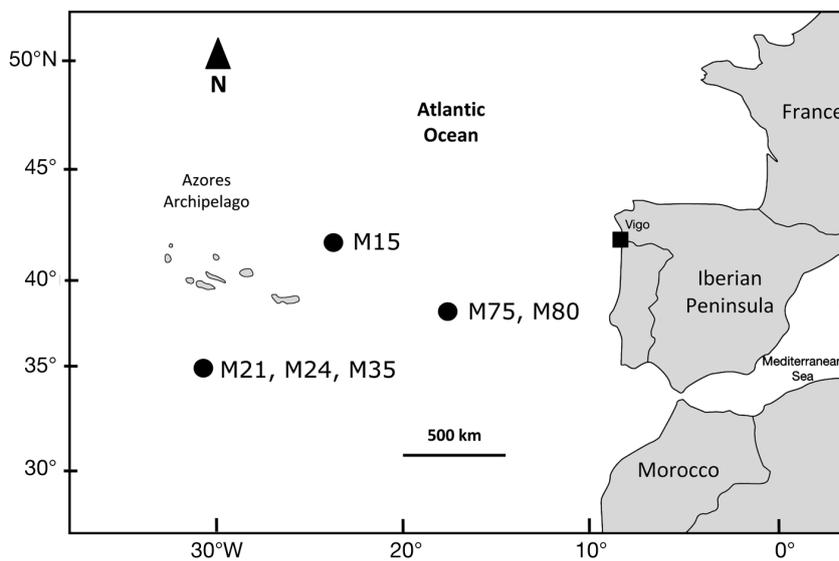


Fig. 1. Sampling area in the northeastern Atlantic. Identification numbers of shortfin makos *Isurus oxyrinchus* with marine mammal prey in their stomach contents are indicated next to each sampling point

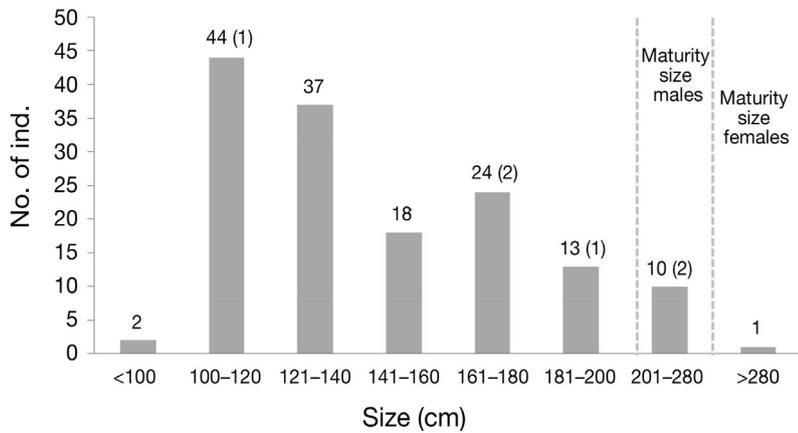


Fig. 2. Size distribution and number of individuals by size class for all sampled shortfin makos *Isurus oxyrinchus*. Sizes at maturity for males and females are marked by broken lines. The numbers of individuals with mammal prey remains in stomach contents are indicated in parentheses for each size class

marine mammals, and examination of all the vertebrae for each individual is necessary to distinguish the species (Perrin et al. 1987, Jefferson et al. 1993). DNA analysis was therefore the only solution to identify marine mammal prey (Table 1).

Fragments of tissues and fat were isolated a short time after stomachs were thawed. Each tissue sample was extracted inside the muscle to exclude all possible contact with stomach fluid and possible contamination with other prey. Each sample was cleaned with distilled water. Physical remains were analyzed for all sharks to identify species but not stomach fluids, because the prey consumed by the shark's prey may also have

Table 1. Marine mammal prey. Part of the body found in stomachs of shortfin makos *Isurus oxyrinchus*, and methods of analysis for identification. See Fig. 1 for localization of the catch

Shark ID	Total length of the shark (cm)	Date of capture	Part of body in stomach	Methods for prey identification	Success (S) or failure (F) of DNA analysis
M15	180	Mar 2012	Dorsal fin, part of vertebral column	DNA, comparison with a collection of vertebrae	S
M21	115	Mar 2012	Dorsal fin, part of vertebral column	DNA, comparison with a collection of vertebrae	S
M24	200	Mar 2012	Fat and skin	DNA	F
M35	175	Mar 2012	Fat and skin	DNA	F
M75	210	Oct 2012	Vertebrae	Comparison with a collection of vertebrae	Not attempted
M80	181	Oct 2012	Fat, skin and vertebrae	DNA, comparison with a collection of vertebrae	S



Fig. 3. Dorsal fins of marine mammals in the shortfin makos M15 (left; scale: 25 cm) and M21 (right; scale: 30 cm). Photographs: Sebastián Biton Porsmoguer

been found there mixed with the stomach contents. Tissues were preserved in 90% ethanol. DNA was isolated from approximately 15 mg of tissue using the NucleoSpin[®] tissue kit (Macherey-Nagel EURL) following the manufacturer's protocol. The quality of the isolated DNA was visualized on a 1% agarose gel stained with ethidium bromide, and DNA concentration was determined using a Nanodrop[™] 2000 (Thermo Scientific). Species identification was performed using 2 portions of mitochondrial DNA: a portion of the gene coding for cytochrome *b* and a portion of the control region. These 2 markers display different levels of variability and are the most widely used for cetacean molecular identification, allowing comparison with reference databases (Ross et al. 2003).

Approximately 460 base pairs (bp) of the cytochrome *b* gene were amplified using primers designed for vertebrates: L14724 (5'-TGA CTT GAA RAA CCA YCG TTG-3'; Palumbi et al. 1991) and H15149 (5'-CAG AAT GAT ATT TGT CCT CA-3'; Kocher et al. 1989). The 5'-end of the control region and a portion of the flanking proline tRNA were amplified using cetacean-specific primers L15824 (5'-CCT CAC TCC TCC CTA AGA CT-3'; Rosel et al. 1999) and H16498 (5'-CCT GAA GTA AGA ACC AGA TG-3'; Rosel et al. 1994). PCR conditions were as described in Viricel et al. (2014). No-template negative controls were included in the DNA extraction and PCR reactions.

PCR profiles were as given in Viricel & Rosel (2012) for primers L14724/H15149 and as given in Vollmer et al. (2011) for primers L15824/H16498. Purification of PCR products and Sanger sequencing were conducted by Genoscreen. Sequences were edited manually using Sequencher[®] v.4.7 (Gene Codes Corp.). Species identification was achieved using 2 online reference databases: (1) GenBank using the BLAST function with default parameters (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and (2) DNA Surveillance (Witness for the Whales: <http://dna-surveillance.fos.auckland.ac.nz:23060/page/whales/title>) with the search settings Cluster (Advanced), performing 1000 bootstrap replicates to assess statistical support for the clade where our query sequence fell, and using the reference database 'Delphinidae+Stenoninae Vs3.1'. DNA Surveillance provides pairwise F84 genetic distances between the query sequence and sequences in the reference database, as well as a neighbor-joining tree to visualize the placement of the query sequence (Ross et al. 2003).

RESULTS AND DISCUSSION

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Results of DNA analysis

Sequences of good quality (424 to 444 bp) for both genes were obtained for 3 samples (Table 2; GenBank Accession Numbers: KR611984 to KR611989). Results from both online identification tools revealed that these sequences were genetically close (99% identity) or identical to sequences from species in the Delphininae subfamily. Examination of the best match for each mitochondrial DNA portion indicated that the tissue found in 2 shortfin mako stomachs most likely corresponded to short-beaked common dolphins *Delphinus delphis*, while the tissue from a third shortfin mako belonged to either a short-beaked common dolphin, or a striped dolphin *Stenella coeruleoalba* (Table 2).

These 2 species belong to the *Stenella-Tursiops-Delphinus* complex, which is composed of closely related species that are notoriously difficult to distinguish by DNA analysis (Amaral et al. 2007, Kingston et al. 2009, Viricel & Rosel 2012). Thus, bootstrap support for the clades in which our sequences fell

Table 2. Results of DNA analysis. Molecular species identification results for tissue samples from 3 shortfin mako *Isurus oxyrinchus* stomachs. Results from 2 online identification tools are shown for each mitochondrial DNA portion sequenced. For GenBank, results correspond to the percent identity and the species of the best match. For DNA Surveillance, the species in the reference database with the shortest genetic distance to our query sequence is shown, and bootstrap support for the clade is reported in parentheses when >50%. The last column indicates the most likely cetacean species for each sample

Shark ID	Cytochrome <i>b</i>		Control region		Cetacean species
	GenBank	DNA Surveillance	GenBank	DNA Surveillance	
M15	99% <i>Delphinus delphis</i>	<i>D. delphis</i> (84% <i>Delphinus</i> sp.)	100% <i>D. delphis</i>	<i>D. delphis</i>	<i>D. delphis</i>
M21	99% <i>D. delphis</i> / <i>Stenella coeruleoalba</i> / <i>Stenella clymene</i>	<i>D. delphis</i> (86% <i>Delphinus</i> sp.)	99% <i>D. delphis</i>	<i>D. delphis</i> (78% <i>D. delphis</i>)	<i>D. delphis</i>
M80	99% <i>S. coeruleoalba</i>	<i>S. coeruleoalba</i> (64% <i>Stenella coeruleoalba</i>)	99% <i>D. delphis</i>	<i>D. delphis</i>	<i>D. delphis</i> or <i>S. coeruleoalba</i>

was >50% in only 4 out of 6 tree reconstructions in DNA Surveillance (Table 2). Identification results were consistent with the species range of these 2 dolphin species. Both species are commonly observed in waters of the study area (Ferreira Vasco 2012, Silva et al. 2014) and are classified as 'least concern' in the Red List of the IUCN (International Union for the Conservation of Nature) (Hammond et al. 2008). Specimens consumed were newborns, and the sampling area, near the Azores archipelago, could represent a nursing area, which would confirm the observations of Silva & Sequeira (2003). Marine mammal remains were found only in female individuals. The limited number of sharks with mammal remains in stomach contents and the fact that females were dominant in the samples (57%, 85 females for 64 males) could be the main reasons. The sex ratio (defined as the percentage of females in the samples) of the non-dolphin eaters is 0.55. The body parts found in stomachs were little digested and, therefore, had been recently eaten, which indicates that prey capture probably occurred in open ocean waters rather than in coastal areas. Shortfin mako is a top predator with an opportunist strategy (Velasco Tarelo 2005). The digestion process is very quick for sharks, and they may spend several days without feeding (Karpevitch & Bakoff 1937, Medved 1985). Many stomachs were empty (vacuity percentage = 35.6%). In each stomach containing dolphin tissue, we only found remains from a single prey corresponding to a marine mammal, so a mix of different species was not possible. DNA analysis is a valuable method for identifying the remains of cetacean species within shark stomachs, except when the digested tissue is mostly composed of fat; thus, some of our identification attempts were unsuccessful using standard extraction methods (Table 1). More laborious organic extraction methods might be more effective for DNA isolation in cases where large amounts of fatty tissue are present (e.g. Biase et al. 2002).

Diet and behaviour

Marine mammal prey represented about 5% (in number and mass) of the diet of shortfin makos (Table 3). Vertebrae of all teleost prey were found in shark stomachs. Using the method of Béarez (1996), we were able to identify all the teleost prey by their vertebrae. DNA analysis was only used for marine mammals, since identification by vertebrae was not possible in this case. The diet of the individuals that did not consume dolphins was mainly dominated by

Table 3. Prey found in the stomach of shortfin makos *Isurus oxyrinchus* in 2012 (March and October) and 2013 (March and October). N: number of prey; %N: mean percent number; %O: mean percent occurrence; %M: mean percent mass (not reconstituted); %IRI: mean percent index of relative importance

Prey	N	%N	%O	%M	%IRI
Crustaceans	6	1.9	0.1	1.04	1.2
Cephalopods	70	26.0	7.7	27.0	25.3
Teleosts	248	66.2	17.8	65.7	68.1
Sea turtles	1	0.2	0.01	1.04	0.1
Cetaceans	6	5.7	0.17	5.2	5.3
Total prey	331	100	25.8	100	100

teleosts (~66% of the total number of individual prey) and especially by Atlantic saury (*Scorpaenopsis scorpaenoides*) (Biton Porsmoguer et al. 2013, 2014). The other prey items were crustaceans, cephalopods and sea turtles. The diets of sharks consuming or not consuming marine mammals were generally similar within each size class.

It was possible to perform morphological identification, as well as sex and size estimations, of mammal prey on the basis of the height and length of their dorsal fin. The heights of the 2 dorsal fins of *D. delphis* were 8 and 11 cm, respectively, in the stomachs of Sharks M15 and M21 (Fig. 3). However, dorsal fin length and sex (female or male), respectively, could not be measured or determined because the dorsal fins were incomplete, so that only a rough calculation of the total body length was possible: approximately 1 m. Therefore, the consumed dolphins probably belonged to the 0 to 1 age class (Murphy & Rogan 2006).

The discovery of dolphin dorsal fins in shortfin mako stomachs was unexpected. Several hypotheses can account for this feature. (1) Their presence could represent a typical pattern of attack (perhaps shortfin mako prefer this part of the body). (2) The bait used by longliners — Atlantic mackerel *Scorpaenopsis scorpaenoides* or longfin inshore squid *Loligo pealeii* — may be attractive for marine mammals, resulting in their unwanted capture and secondary consumption by sharks; the middle part of the body would be the easiest to bite. Fishermen sometimes catch swordfish *Xiphias gladius* with a part of their body missing; victims of shark attacks after capture on a longline. Occasional bycatches of dolphins by longliners have also been documented (Hernandez-Millan et al. 2008, Hamer et al. 2012). (3) In open water sharks may consume dolphin juveniles that are already dead, and the middle part of the body, with the dorsal fin, would be the easiest to bite.

The 6 shortfin mako individuals with marine mammals in their stomachs were all juveniles. This does not mean that adult sharks do not consume marine mammals, but probably reflects the fact that juveniles represent the majority of sharks inhabiting the region; the high concentration of juveniles in the area is known from the reports of longliners (Maia et al. 2006, Biton Porsmoguer et al. 2014). It is especially interesting that shortfin makos are able to consume marine mammals of a similar or slightly smaller size than themselves. However, the smallest of these sharks (M15 and M21) engulfed only part of the prey, which was sufficient to fill their stomachs (1.91 and 4.24 kg wet mass, respectively).

Conclusions

Six out of 93 juvenile shortfin mako with non-empty stomachs had consumed marine mammal prey, including at least the short-beaked common dolphin *D. delphis* and, perhaps, the striped dolphin *S. coeruleoalba*, as evidenced by DNA analysis. These juvenile sharks were able to consume marine mammals almost as large as themselves. We determined from the partially digested and well-preserved dorsal fins of common dolphins that these prey were probably newborns. In the northeastern Atlantic, the short-beaked common dolphin has a similar diet to that of the shortfin mako (Pusineri et al. 2007). Both the shortfin mako and dolphin prey are predators at the top of the food chain, and are possibly competitors. Shortfin mako may be either predators or scavengers and may consume the identified dolphins, dead or alive.

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