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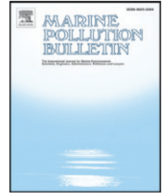

The significance of cephalopod beaks in marine ecology studies: Can we use beaks for DNA analyses and mercury contamination assessment?☆

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ABSTRACT

Cephalopod beaks found in the diet of predators have been a major source of scientific information. In this study, we evaluated the usefulness of DNA and contaminants analysis (total mercury — T-Hg) in cephalopod beaks in order to assess their applicability as tools in marine ecology studies. We concluded that, when applying DNA techniques to cephalopod beaks from Antarctic squid species, when using flesh attached to those beaks, it was possible to obtain DNA and to successfully identify cephalopod species; DNA was not found on the beaks themselves. This study also showed that it is possible to obtain information on T-Hg concentrations in beaks: the T-Hg concentrations found in the beaks were 6 to 46 times lower than in the flesh of the same cephalopod species. More research on the relationships of mercury concentrations in cephalopod beaks (and other tissues), intra- and inter-specifically, are needed in the future.

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1. Introduction

Cephalopods (Mollusca: Cephalopoda) are widely recognized as playing a pivotal role in many marine ecosystems, being consumed by a wide range of predators (Boyle and Rodhouse, 2005; Clarke, 1996b; Hoving et al., 2014; Xavier et al., 2015; Xavier and Cherel, 2009). Their beaks are well known to resist digestion and can stay in predator stomachs for days, weeks or even months (Ashmole and Ashmole, 1967; Duffy and Jackson, 1986; Furness et al., 1984; Gales and Cheal, 1992; Jackson and Ryan, 1986; Votier et al., 2003; Xavier et al., 2005). More than 28 000 beaks have been found in the stomach of a single sperm whale (Akimushkin, 1955; Clarke, 1977).

In 1962, Malcolm Clarke showed the importance of cephalopod beaks for marine ecology (Clarke, 1962), as cephalopod soft bodies are rarely found in the stomach of their predators (Clarke, 1977, 1980b). Back then, little was known about interactions of cephalopods with top predators, in particular the relevance of each cephalopod species in the diet of top predators. Consequently, the construction of reliable food webs including cephalopods then was difficult if not impossible. The efforts of Malcolm Clarke and colleagues catapulted our ability to understand diet composition of predators that feed on cephalopods by using their beaks (Cherel and Klages, 1998; Clarke, 1986, 1996a, 1996b; Croxall and Prince, 1996; Klages, 1996; Smale, 1996).

Cephalopod beaks in the diet of top predators have been acknowledged as good tools for a variety of studies on marine ecology. They can provide information on size, frequency of occurrence and mass of cephalopods that are part of a top predator's diet (Clarke, 1980b; Xavier et al., 2005). Beak data analyses have been used to monitor seasonal and annual changes in availability (Xavier et al., 2003, 2007b, 2013), to aid fisheries assessment and management (Xavier et al., 2007b), to assess potential competition between predators (Xavier and Croxall, 2007), to evaluate the amount of potential scavenging both by a predator (Croxall and Prince, 1994), or to recognize a new species in a given area (Clarke et al., 2002). Information regarding age (Clarke, 1965; Perales-Raya et al., 2010, 2014), growth, reproduction

☆ Capsule abstract: DNA and contaminants analyses for the first time in cephalopods beaks showed that flesh attached to beaks allows DNA species ID and beaks had 6–46 times less total mercury than flesh.

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(Clarke, 1980b, 1993; Hernández-Garcia et al., 1998; Jarre et al., 1991), distribution (Clarke, 1980b, 2002; Liu et al., 2015; Xavier et al., 2002a, 2002b, 2006, 2014), paleontology (Clarke and Maddock, 1988), feeding ecology, behavior (Castro and Hernández-Garcia, 1995; Franco-Santos and Vidal, 2014), spawning areas (Cherel and Weimerskirch, 1999), post-spawning mortality (Xavier and Croxall, 2007), sexual dimorphism (Bolstad, 2006; Cherel et al., 2009a; Jackson, 1995), biomass estimations, cephalopod consumption (Clarke, 1983, 1987; Clarke et al., 2002; Santos et al., 2001; Xavier et al., 2007b) and predator migrations (Clarke and Stevens, 1974) can also be provided by studying cephalopod beaks. Recent stable isotope analyses of beaks enabled the determination of habitat preferences and trophic levels for a wide range of cephalopods (Cherel et al., 2009b, 2011; Cherel and Hobson, 2005; Guerra et al., 2010). Also, cephalopod beaks exhibit unique characteristics with mechanical properties that can be applied to engineering and biomaterial research (Dilly and Nixon, 1976; Miserez et al., 2007, 2008; Uyeno and Kier, 2005).

Despite the countless applications of cephalopod beaks in marine ecology studies, DNA-based identification and chemical contamination assessments have not yet been evaluated. DNA has been used as an important tool to identify and discover new cephalopod species as well as gain insights into their ecology and evolution (Allcock et al., 2014; Strugnell et al., 2009; Strugnell and Lindgren, 2007; Xavier et al., 2015). Studies using DNA for the identification of cephalopods in stomach contents have also been conducted (Strugnell and Lindgren, 2007), relying on DNA extraction from tissues of recently consumed cephalopods (Strugnell et al., 2005).

Another application not commonly applied to beaks is contaminants assessment. Mercury is listed as one the most hazardous substances, with all chemical forms (elemental, inorganic and organic) exhibiting toxicological characteristics, and thus increasingly raising environmental concerns. Once mercury enters the marine ecosystems it can be easily methylated by bacteria, which accelerates bioaccumulation and biomagnification along food webs, ultimately concentrating in top predators (Wiener et al., 2007). The methylation process increases toxicity with methylmercury being the most toxic form. Mercury uptake occurs mainly through diet (Mieiro et al., 2012) and it is accumulated in specific tissues (e.g. muscle tissue stores most as methylmercury (Bustamante et al., 2006; Mieiro et al., 2011)). To our knowledge, no studies so far explored the possibility of using beaks to assess environmental and ecological relevant mercury concentrations.

Our study aims to use cephalopod beaks from squid that occur in the Southern Ocean (here defined as south of the subtropical front) in order to: (1) apply DNA barcoding to both beaks and muscle tissue attached to the beaks to assess its feasibility for cephalopod identification; (2) assess the utility of beaks to evaluate total mercury accumulation in cephalopods by comparing concentrations in beaks and muscle; (3) discuss the future applicability of DNA barcoding and mercury analysis in ecological studies of cephalopods.

2. Material and methods

2.1. DNA analyses

Cephalopod lower beaks of two of the most common species in top predators diets (i.e. *Kondakovia longimana* and *Moroteuthis knipovitchi*; see Xavier and Cherel, 2009) were collected from stomach contents of gray headed *Thalassarche chrysostoma* and black-browed *Thalassarche melanophrys* albatrosses breeding at Bird Island, South Georgia, following Xavier et al. (2003), Guerreiro et al. (2015) and Alvito et al. (2015). Lower beaks samples from adult Southern Ocean squid were fixed in ethanol (70–90%) and stored at −20 °C until DNA extractions were carried out. At the laboratory, the beaks were then macerated and proteinase K (20 μg/mL) was added overnight. DNA extraction was performed using the JETFLEX Genomic DNA Purification Kit (Genomed, Germany). DNA yield was quantified using NanoDrop equipment (Thermo Scientific, USA).

For DNA analyses of tissue samples that were attached to cephalopod beaks (i.e. from buccal mass), from more squid species common in the diet of top predators (*Galiteuthis glacialis*, *Psychroteuthis glacialis*, *Gonatus antarcticus* and *Alluroteuthis antarcticus*). DNA extraction was done by using a Glass Fiber Plate DNA Extraction method (Ivanova et al., 2006).

The primer pair LCO1490_t1 and HCO2198_t1 was used to amplify a 658 bp fragment of the COI gene. Samples which did not amplify successfully were re-run using a combination of overlapping primer sets: C_LepFolF,MLepR2 and MLepF1,C_LepFolR. The PCR thermal regime for all primer sets was: initial denaturing at 94 °C for 1 min; five cycles at 94 °C for 1 min, 45 °C for 1.5 min and 72 °C for 1.5 min; 35 cycles of 94 °C for 1 min, 50 °C for 1.5 min and 72 °C for 1 min followed by a final cycle at 72 °C for 5 min. Each PCR product was cleaned by Sephadex. Prior to sequencing, the clean PCR product was diluted 1:10 with sterile water and 2–5 μL of it was sequenced in both directions using ABI 3730xl automated DNA sequencers. All sequences and supporting information have been deposited in the Barcode of Life Datasystems (BOLD) database (Ratnasingham and Hebert, 2007) in the project DIETA, and were submitted to GenBank (accession numbers are given in Table 1).

2.2. Mercury analyses

Cephalopod lower beaks of some of the most important cephalopod species in top predator diets (*G. glacialis*, *G. antarcticus*, *K. longimana*, *M. knipovitchi* and *P. glacialis*; see Xavier and Cherel, 2009) were collected from stomach contents of albatrosses breeding at Bird Island, South Georgia as well as Patagonian toothfish *Dissostichus eleginoides* from the South Sandwich Islands, following Xavier et al. (2002b, 2003) and Seco et al. (2015). At the laboratory, all beaks were ground to a fine powder using liquid nitrogen for further analyses of mercury concentrations. Total mercury (T-Hg) was determined by atomic absorption spectrometry (AAS) with thermal decomposition and gold amalgamation, using an Advanced Mercury Analyzer (AMA) LECO 254 (Costley et al., 2000). This method does not require previous sample treatment, and also allows for a small sample mass to be used. In this case, an average of 36 mg per beak replicate was used for Hg determinations. The limit of detection of the AMA–LECO 254 analyzer is 0.01 ng of mercury. Accuracy and precision of the analytical methodology for T-Hg determinations were assessed by daily replicate analysis of certified reference materials (CRM), namely Tort-2 (lobster hepatopancreas). Precision of the method was always better than 9% (n = 9), with a recovery efficiency of 105 ± 7% (n = 27).

Table 1
Taxa of squid known to inhabit in Southern Ocean waters, following Rodhouse et al. (2014; 19 species), that already have their respective COI accession number (* = species that were studied in this study).

Species name	Accession number
*Alluroteuthis antarcticus**	AF131871
Bathyteuthis abyssicola	AF000030
Batoteuthis skolops	AY557527
Chiroteuthis veranyi	AF000032
Galiteuthis sp.*	KF309247
*Gonatus antarcticus**	AY681064
Kondakovia sp.*	EU735403
Martialia hyadesi	AB270940
Mastigoteuthis psychrophila	KC860979
Mesonychoteuthis hamiltoni	EU735397
Moroteuthis ingens	AB264119
*Moroteuthis knipovitchi**	AF131875
Moroteuthis robsoni	AB264117
*Psychroteuthis glacialis**	AF131876
Todarodes filippovae	AB270935

2.3. Statistical analyses

For cephalopod beaks that could be identified to species level we used allometric equations to convert lower beak size to mantle length (ML) and body mass (g), in Xavier and Cherel (2009). After assessing the normality of the data, non-parametric tests were used to assess relationships between T-Hg and ML/body mass. Values on statistics are given as means ± standard deviation unless if stated.

3. Results

3.1. DNA extraction and sequencing analysis

A total of 20 clean cephalopod lower beaks, with no visible tissue, were used for DNA extraction, with 10 beaks belonging to *K. longimana* (10.9 ± 0.9 mm Lower Rostral Length (LRL); range: 8.9–12.0 mm LRL) and 10 beaks belonging to *M. knipovitchi* (4.6 ± 0.5 mm LRL; range: 3.9–5.4 mm LRL). With the methods applied, it was not possible to retrieve any DNA. Another set of cephalopod beaks with visible flesh attached (i.e. buccal mass), were used to retrieve DNA for COI gene amplification. The buccal mass flesh used was identified as *K. longimana* (n = 10), *G. glacialis* (n = 1), *M. knipovitchi* (n = 6), *P. glacialis* (n = 1), *G. antarcticus* (n = 1) and *A. antarcticus* (n = 2). This DNA barcoding confirmed the identification of all species by beak morphology (Xavier and Cherel, 2009).

3.2. Mercury concentrations

The total mercury (T-Hg) levels of lower beaks from five squid species of the Southern Ocean were obtained (Table 2, Fig. 1). Concentrations ranged from 0.004 (*K. longimana* and *G. glacialis*) to 0.047 mg kg^{-1} dry weight (*M. knipovitchi*), indicating low mercury concentrations in beaks. There were significant interspecific differences in T-Hg concentrations (Kruskall–Wallis H = 14.56, $p < 0.01$) between *P. glacialis* and *K. longimana* (Dunn's test Q = 3.11 $p < 0.05$). The average T-Hg concentration found in species with larger beaks (*K. longimana*) was similar to species with smaller beaks (*G. glacialis*; Table 2, Fig. 1) but with the higher estimated ML (Table 3). *M. knipovitchi*, *P. glacialis* and *G. glacialis* showed a higher intra-species variability while T-Hg levels in beaks of individuals of *G. antarcticus* were more consistent (Table 2, Fig. 1). No correlation was found between T-Hg concentration and the lower rostral length (Spearman correlation $\rho = 0.06$ $p = 0.77$) or with the body mass (Spearman correlation $\rho = 0.009$ $p = 0.96$) of the studied species. However, there was a negative correlation between T-Hg concentration and the mantle length (Spearman correlation $\rho = -0.487$ $p = 0.02$). When comparing the T-Hg concentration of lower beaks (present data) with those in flesh/muscle (Anderson et al., 2009) for the same cephalopod species from the same region of the Southern Ocean (Atlantic sector, around South Georgia), the levels found in the beaks were significantly lower than those found in flesh/muscle (Mann–Whitney U = 0.00; $p < 0.01$). The species showing least variability in T-Hg concentration in both studies were *G. antarcticus* and *K. longimana*.

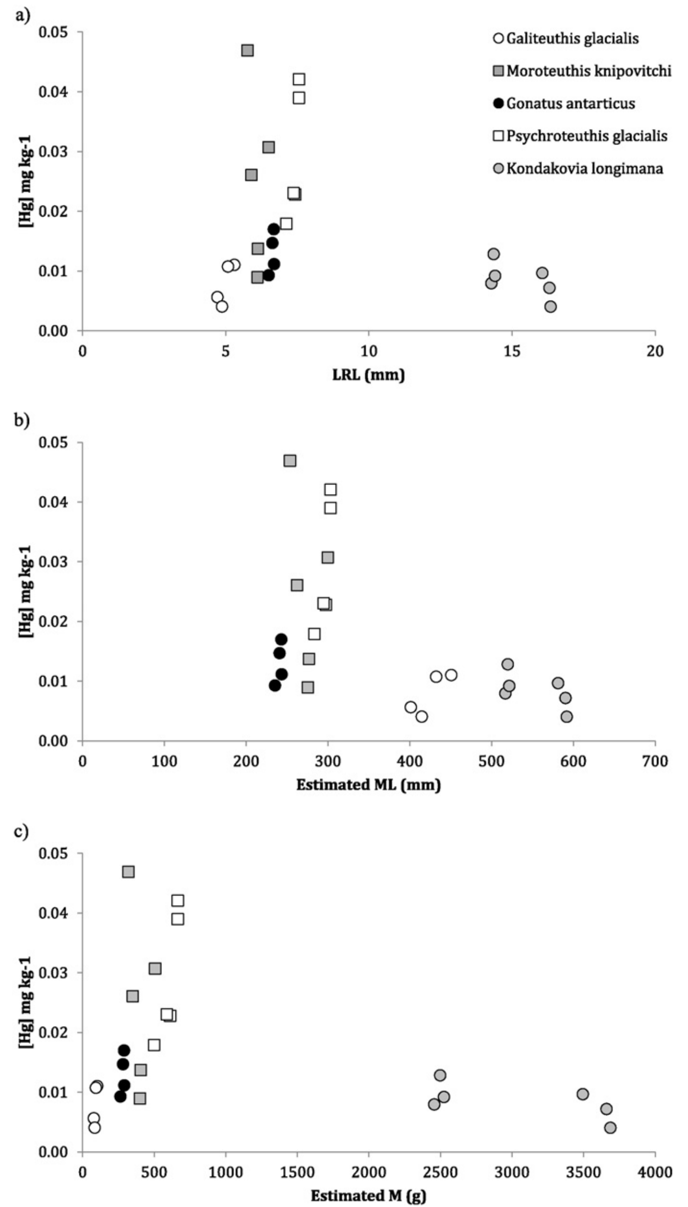

Fig. 1. Mercury concentration ([T-Hg]/mg kg^{-1}, dry weight) obtained from beaks according to size dimensions: (a) lower rostral length (LRL/mm), (b) estimated mantle length (ML/mm) and (c) estimated body mass (M/g) of five southern ocean cephalopod species. This figure is for visual comparison rather than for determining trends as these different species have different morphologies, physiology, life histories and growth rates.

4. Discussion

Given the difficulty to capture cephalopods, the use of recovered beaks from stomach contents from cephalopod predators has been

Table 2
Total mercury concentration (mg kg^{-1}, dry weight; mean values, standard deviation (SD), range and variation coefficient (%)) in cephalopod–beaks (present study) and muscle (Anderson et al., 2009).

	Beaks					Muscle				
	n	[Hg]	SD	Range	CV %	n	[Hg]	SD	Range	CV%
Galiteuthis glacialis	4	0.008	0.004	0.004–0.011	45	3	0.23	0.07	0.18–0.31	30
Moroteuthis knipovitchi	5	0.025	0.015	0.009–0.047	59	4	0.16	0.09	0.07–0.29	58
Gonatus antarcticus	4	0.013	0.003	0.009–0.017	27	2	0.6	0.02	0.58–0.61	4
Psychroteuthis glacialis	5	0.029	0.011	0.018–0.042	37	2	0.18	0.11	0.10–0.25	61
Kondakovia longimana	6	0.008	0.003	0.004–0.013	34	2	0.1	0.02	0.08–0.11	22

Table 3
Total mercury concentration in cephalopod tissues from different sampling areas. N — sampling size; ML — mantle length (mean ± SD or range (min-max)/mm), Hg tissue T-Hg concentration (mean ± SD (range)/mg kg^{-1}, dry weight). See exceptions (a–d) below.

Species	Sampling area	N	ML	Hg beaks		Hg flesh		Hg digestive gland		References
Onychoteuthidae										
Kondakovia longimana	Southern Ocean	6	554 ± 37.7	0.008 ± 0.003	(0.007–0.013)	–	–	–	–	Present study
	Southern Ocean	2	–	–	–	0.1 ± 0.02	(0.08–0.11)	–	–	Anderson et al. (2009)
Moroteuthis knipovitchi	Southern Ocean	5	274 ± 17.5	0.025 ± 0.015	(0.009–0.047)	–	–	–	–	Present study
	Southern Ocean	4	–	–	–	0.16 ± 0.09	(0.07–0.29)	–	–	Anderson et al. (2009)
Moroteuthis ingens	Southern Ocean	15	243–364	–	–	0.086 ± 0.017	(0.06–0.13)	–	–	McArthur et al. (2003)
Gonatidae										
Gonatus antarcticus	Southern Ocean	4	241 ± 3.75	0.013 ± 0.003	(0.009–0.017)	–	–	–	–	Present study
	Southern Ocean	2	–	–	–	0.6 ± 0.02	(0.58–0.61)	–	–	Anderson et al. (2009)
Psychroteuthidae										
Psychroteuthis glacialis	Southern Ocean	5	296 ± 8.15	0.029 ± 0.011	(0.018–0.042)	–	–	–	–	Present study
	Southern Ocean	2	–	–	–	0.18 ± 0.11	(0.10–0.25)	–	–	Anderson et al. (2009)
Cranchiidae										
Galiteuthis armata	NE Atlantic	3	252 ± 91	–	–	0.252 ± 0.041	(0.206–0.284)	–	–	Chouvelon et al. (2012)
Galiteuthis glacialis	Southern Ocean	4	425 ± 21.5	0.008 ± 0.004	(0.04–0.11)	–	–	–	–	Present study
	Southern Ocean	3	–	–	–	0.23 ± 0.07	(0.18–0.31)	–	–	Anderson et al. (2009)
Teuthowenia megalops	NE Atlantic	4	134 ± 12	–	–	0.150 ± 0.033	(0.111–0.192)	–	–	Chouvelon et al. (2012)
	NE Atlantic	1	180	–	–	–	0.205	–	0.172	Bustamante et al. (2006)
Ommastrephidae										
Illex coindetii	NE Atlantic	22	130 ± 54	–	–	0.193 ± 0.078	(0.061–0.331)	0.192 ± 0.076	(0.081–0.357)	Bustamante et al. (2006)
Todaropsis eblanae	NE Atlantic	9	101 ± 43	–	–	0.281 ± 0.129	(130–500)	0.217 ± 0.108	(0.120–0.463)	Bustamante et al. (2006)
	NE Atlantic	23	100 ± 41	–	–	0.206 ± 0.201	–	0.128 ± 0.099	–	Pierce et al. (2008)
Todarodes sagittatus	NE Atlantic	22	260 ± 42	–	–	0.324 ± 0.380	(0.139–1.998)	–	–	Chouvelon et al. (2012)
	NE Atlantic	5	98 ± 34	–	–	0.188 ± 0.089	(0.073–0.289)	0.168 ± 0.052	(0.112–0.231)	Bustamante et al. (2006)
	NE Atlantic	12	343 ± 100	–	–	0.425 ± 0.194	–	0.280 ± 0.105	–	Pierce et al. (2008)
	Adriatic Sea	14	–	–	–	0.25 ± 0.03[d]	(0.02–0.62)	–	–	Perugini et al. (2009)
Histioteuthidae										
Histioteuthis reversa	NE Atlantic	7	54 ± 22	–	–	0.219 ± 0.087	(0.132–0.320)	–	–	Chouvelon et al. (2012)
	NE Atlantic	6	38 ± 22	–	–	0.102 ± 0.031	(0.065–0.147)	0.088 ± 0.044	(0.031–0.137)	Bustamante et al. (2006)
Loliginidae										
Alloteuthis sp.	NE Atlantic	20	67 ± 15	–	–	0.098 ± 0.011	–	0.072 ± 0.011	–	Pierce et al. (2008)
Alloteuthis subulata	NE Atlantic	15	152 ± 32	–	–	0.196 ± 0.040	(0.121–0.262)	–	–	Bustamante et al. (2006)
Loligo vulgaris	NE Atlantic	36	179 ± 56	–	–	0.149 ± 0.032	(0.072–0.200)	–	–	Chouvelon et al. (2012)
	NE Atlantic	21	151 ± 47	–	–	0.264 ± 0.086	(0.113–0.398)	0.406 ± 0.171	(0.113–0.681)	Bustamante et al. (2006)
	NE Atlantic	10	130–420	–	–	0.05 ± 0.02[d]	(0.02–0.08)	–	–	Lourenço et al. (2009)
	Mediterranean Sea	95	120–256	–	–	0.072[c,d]	(0.030–0.95)	–	–	Rjeibi et al. (2015)
Loligo forbesi	NE Atlantic	38	290 ± 99	–	–	0.260 ± 0.119	(0.099–0.547)	–	–	Chouvelon et al. (2012)
	NE Atlantic	12	119 ± 48	–	–	0.179 ± 0.053	(0.091–0.645)	0.235 ± 0.104	(0.165–0.512)	Bustamante et al. (2006)
	NE Atlantic	101	129 ± 78	–	–	0.153 ± 0.081	–	0.216 ± 0.176	–	Pierce et al. (2008)
Loligo duvaucelii	Peninsular Malasya	10	160-530[a]	–	–	0.199 ± 0.162[b]	(0.150–0.406)	–	–	Ahmad et al. (2015)
Loligo uyii	Peninsular Malasya	4	240-384[a]	–	–	0.249[b]	(0.099–0.324)	–	–	Ahmad et al. (2015)
Loligo chinensis	Peninsular Malasya	7	306-600[a]	–	–	0.275 ± 0.122[b]	(0.158–0.309)	–	–	Ahmad et al. (2015)
Loligo sibogae	Peninsular Malasya	6	217-612[a]	–	–	0.364 ± 0.507[b]	(0.194–1.506)	–	–	Ahmad et al. (2015)
Loligo edulis	Peninsular Malasya	9	120-276[a]	–	–	0.267 ± 0.156[b]	(0.099–2.715)	–	–	Ahmad et al. (2015)

[a] Possibly refers to the mantle length, but can also refers to total length (see Ahmad et al., 2015).
[b] Median ± IQR.
[c] Median.
[d] T-Hg in mg kg^{-1} wet weight; mean moisture content is indicated to be 78% in literature (Lourenço et al. 2009; Rjeibi et al., 2015).

widely used in ecological studies, particularly for the purpose of species identification (Clarke, 1980a, 1986; Xavier and Cherel, 2009). However, there are only a few experts in the world trained to do this kind of identification (Clarke, 1986; Xavier et al., 2007a). In this study, we assessed the utility of a molecular approach, using DNA recovered from tissues attached to the beaks. We also assessed the utility of beaks to obtain information on mercury concentration in cephalopods.

4.1. DNA extraction and sequencing analyses

This study showed that it was possible to extract DNA directly from flesh attached to the beaks (i.e. from buccal mass), but not from the beaks themselves. The reason for the latter is likely caused by the beak's composition. They do not contain living cells (Miserez et al., 2010), and any residue tissue on their surface will be digested after a longer time in a predator's stomach. Larger buccal mass tissue bits attached to the beak contain enough DNA for further analysis and may allow using DNA barcoding to determine the species. We chose only species whose beaks could also be identified using beak morphology (Xavier and Cherel, 2009) in order to test if there is correspondence between both methods. DNA barcoding confirmed the identification of species by beak morphology, which is a promising result as it provides researchers with two methods to choose from depending on the needs of their study. Surveys on the feeding ecology of cephalopod predators usually start with samples that contain clean beaks as well as beaks with flesh attached to them. A fair number of squid species found in the Southern Ocean (Rodhouse et al., 2014) have already been barcoded, and these sequences are publicly available through GenBank (Table 1) or BOLD. However, there are numerous species living in the Southern Ocean that are unknown to science, without a barcode sequence (Xavier et al., 2014, 2015; Xavier and Cherel, 2009).

4.2. Mercury concentrations

Our study showed that it is possible to measure total mercury (T-Hg) concentration in cephalopod (lower) beaks, using a simple and easily accessible laboratory methodology. The total mercury concentrations found on the lower beaks of the studied cephalopod species were 6 to 46 times lower than those reported from muscle tissue of the same species in the Southern Ocean (see Table 2). Such results might be due to mercury organotropism (Bustamante et al., 2006; Jackson et al., 2007), since mercury accumulation is tissue-specific and muscle is known to harbor significant levels of mercury, mainly in organic form (Bustamante et al., 2006). Preferential accumulation of mercury in muscle tissue has also been reported for fish and is a protection mechanism that prevents mercury accumulation in other vital organs (e.g. brain) (Mieiro et al., 2011). Despite the proteinaceous nature of cephalopod beaks (beaks can have a protein content varying from 5% to 60% wet weight according to the pigmentation gradient (Miserez et al., 2008)), their slow growth rate (they are usually not replaced throughout a cephalopods relative short life), and mercury affinity for proteins, it seems that beaks are not a structure with high accumulation potential (as the mercury values were very low; see results). In addition, the permanency of the beaks in the acidic contents of their predators' stomachs may induce the release of Hg due to the chelating action (i.e. chemical broke down activity) of acids, which may disrupt the Hg bonds to proteins (Hajeb and Jinap, 2009), and reduce Hg concentration in beaks.

Mercury concentrations in cephalopods depend on both biological and environmental factors such as size, lifestyle, food availability, growth rate and geographical origin (Bustamante et al., 2006; Pereira et al., 2009; Villanueva et al., 2002). With respect to size, this study did not show any relation between the T-Hg concentration in beaks and the lower rostral length (see results). In fact, larger beaks of *K. longimana*, showed similar T-Hg values when compared with species with smaller beaks, such as *G. glacialis*. This suggests that bioaccumulation of mercury in beaks does not seem to be dependent on body size of cephalopods, which is in agreement with previous studies on other tissues by Raimundo et al. (2009) who found comparable Hg concentrations (based on octopod digestible gland samples) among individuals of different age/size. The same result was obtained for the relationship between estimated body mass and T-Hg in squid beaks in our study.

In terms of assessing T-Hg and ML relationships, *K. longimana* can reach more than 1000 mm of mantle length (ML), whereas the other studied species generally have ML lower than 500 mm (Gröger et al., 2000; Lu and Williams, 1994; Lynnes and Rodhouse, 2002). For our study, the estimated ML of the specimens of *G. glacialis* and *K. longimana* were in the same range and had the highest ML registered, which may explain the similarity between the T-Hg concentrations found between these species. Both species showed the lower T-Hg burdens found in this study, possibly due to a somatic growth dilution of the metal, which can be corroborated by the negative correlation found between ML and T-Hg; It has been shown that rapid growth can greatly reduce the mercury concentration in aquatic organisms by causing a greater than proportional gain in biomass relative to the metal concentration (Karimi et al., 2007).

Beaks from *M. knipovitchi* and *P. glacialis* showed T-Hg concentrations 3 times higher than *G. glacialis* and *K. longimana*, despite that their ML were lower, which is in line with the previous assumption that small species (with slower growth rate) may accumulate more mercury. *G. antarcticus* showed similar ML with *M. knipovitchi* and *P. glacialis*, but half of their T-Hg burden. This may be explained by the different feeding habits, different growth rates and distribution of these different species (Cherel et al., 2009a; Collins and Rodhouse, 2006; Pierce et al., 2008; Xavier et al., in press). In summary, there are no clear relationships between T-Hg with beak size and body mass, but there is a relationship between T-Hg and ML, emphasizing that this issue must be further investigated.

Finally, our results show intra-species variations of T-Hg concentrations, being particularly higher in *M. knipovitchi*, *G. glacialis* and *P. glacialis* (see Results; Fig. 1). Further studies will be needed to assess why such variations occur. They may be caused by various parameters related to the ecology of Southern Ocean cephalopods, such as biological (e.g. growth rate, size, sex, metabolic rate), ecological (e.g. feeding and habitat use) and environmental (mercury availability, primary productivity) factors (Chouvelon et al., 2012; Harmelin-Vivien et al., 2009).

The Antarctic seabed has been characterized as cold and thermally stable, without relevant changes in spatial or seasonal temperature (Xavier and Peck, 2015). As previously stated, mercury accumulation depends on a wide range of factors, namely abiotic factors, such as temperature, which not only affect the mercury cycle but also organism individual growth. Could mercury concentrations in Southern Ocean cephalopods be different from elsewhere? Using T-Hg in muscle tissue as a measure, there are no major differences between mercury concentrations in squid species from the Southern Ocean (Anderson et al., 2009; McArthur et al., 2003) compared to taxonomically close ones from the North Eastern Atlantic (Anderson et al., 2009; Chouvelon et al., 2012), Adriatic and Mediterranean Sea (Perugini et al., 2009; Rjeibi et al., 2015) and adjacent waters to Peninsular Malaysia (Ahmad et al., 2015) (Table 3), which suggests comparable mercury levels in the aquatic environments of both areas. This evidence reinforces mercury persistency and its global distribution.

In conclusion, when using DNA analyses, we can assess the identification of cephalopods only when there is flesh attached to beaks, as it was not possible to obtain DNA directly from the beaks using our methodology. However the success of DNA barcoding in cases where tissue remnants were still attached to beaks provides researchers with two tools that could be used in a complementary fashion to determine species identities in the stomach content of cephalopod predators (i.e. in some studies you can only be able to use DNA (only flesh available) while in other studies only beaks are available). It is possible to assess the mercury concentrations of cephalopod beaks and despite the fact that T-Hg in beaks was lower than usually found in muscle tissue,

beaks could be a tool to assess marine contamination in a wide range of cephalopod species (particularly oceanic squid species) that are more difficult to catch using traditional means (nets) (Clarke, 1977; Xavier et al., 2007a, 2015). Future studies in order to suggest some relationship with cephalopod measurements (like the inverse relationship with ML), studies should focus in testing the Hg concentrations with real measurements obtained from different size/sex cephalopods (rather than estimations from allometric equations).

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