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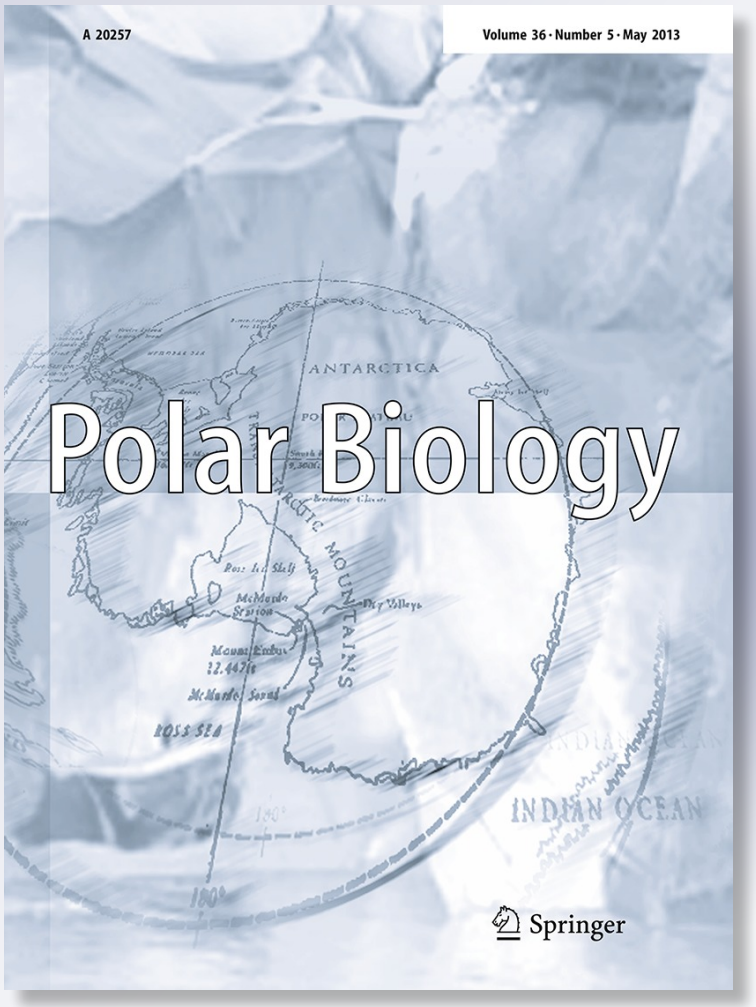

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SHORT NOTE

Faecal DNA amplification in Pacific walruses (*Odobenus rosmarus divergens*)

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Abstract Dietary information is critical for assessing the population status of seals, sea lions and walruses—and is determined for most species of pinnipeds using non-invasive methods. However, diets of walruses continue to be described from the stomach contents of dead individuals. Our goal was to assess whether DNA could be extracted from the faeces of Pacific walruses (*Odobenus rosmarus divergens*) collected at haulout sites, and whether potential prey species or taxa could be amplified from that DNA. We extracted DNA from 70 faecal samples collected from ice pans in the Bering Sea during the spring of 2008 and 2009 (with between 4.6 and 308.9 ng/μl of DNA in every sample). We also extracted DNA from 12 potential prey species or taxa collected by bottom-grabs in 2009 to identify positive controls for primers and to test the ability of previously published taxon-specific and species-specific primers to correctly identify the prey using conventional PCR. We tested primers that successfully amplified DNA from the tissue of at least one potential prey species or taxon on all 70 walrus faecal samples. We found that two sets of primers successfully amplified many of the potential prey species or taxa using DNA from their tissue, and that one of these primer sets produced positive amplification in 4 of the 70 faecal samples. The band size that was produced for prey organisms and in the faecal samples was consistent with expectations, although prey identities were not verified with sequencing. Our pilot study demonstrates that DNA can be successfully extracted and amplified from walrus faeces, providing a stepping stone towards describing the diets of walruses from faecal DNA.

Keywords Pacific walrus · PCR · Prey identification · Faecal DNA

Introduction

Methods of determining the diets of pinnipeds have evolved with time towards using less invasive methodologies for seals and sea lions, but continue to rely on killing individuals to identify the prey contained in the stomachs of walruses. Seal and sea lion diets were also once determined from the stomach contents of animals killed at sea or close to shore (Scheffer 1928; Frost and Lowry 1980; Prime and Hammond 1987; Sheffield and Grebmeier 2009), but are now regularly determined from the identifiable prey remains (bones and other hard parts) recovered from faecal matter (Merrick and Loughlin 1997; Sinclair and Zeppelin 2002; Tollit et al. 2007, 2009) or from the fatty acid signatures of prey assimilated in the blubber (Iverson et al. 1997, 2004; Tollit et al. 2006; Budge et al. 2007). However, problems with false positives, prey and predator-specific calibrations, and issues with rates of assimilation of different prey into predator tissues appear to limit the utility of fatty acid analysis (Tollit et al. 2007; Nordstrom et al. 2008; Rosen and Tollit 2012). Stable isotopes are also used to identify trophic-level information

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about prey consumption (Hobson et al. 1997), but there are many challenges to using them for species-specific diet composition (Newsome et al. 2009; Phillips 2012). More recently, molecular techniques have been developed and applied to identify prey DNA contained within the soft matrix of faecal samples collected from seals and sea lions (Jarman et al. 2002; Deagle et al. 2005a, b; Tollit et al. 2009; King et al. 2008). In general, DNA-based diet identification is considered to be a more robust measure of diet analysis than many of the other methods, although there are limitations based on differential prey digestion (King et al. 2008).

Unlike most species of pinnipeds, diets of walruses continue to be determined from the stomach contents of animals killed on or near sea ice and terrestrial haulouts (Sheffield et al. 2001) because hard parts are generally absent from walrus scats, and prey libraries have not been developed to enable fatty acid analysis. These stomach contents of hunted Pacific walruses have contained mostly benthic invertebrates (primarily bivalves and gastropods) consumed at depths of <100 m (Sheffield and Grebmeier 2009). However, many other species are consumed, some of which include small crustaceans, polychaete (Annelid) worms, molluscs, seabirds (Mallory et al. 2004; Lovvorn et al. 2010), and occasionally seals (Lowry and Fay 1984; Sheffield et al. 2001; Sheffield and Grebmeier 2009). Walruses feed by oral suction and consume primarily soft tissue (Sheffield and Grebmeier 2009), leaving little if any hard remains to pass into their scats. It is thus not possible to determine walrus diet comprehensively from hard part analyses. A more complete understanding of walrus diet might be determined using the DNA-based prey detection methods that have been tested on the faecal matter of other pinniped species (Deagle et al. 2009; Tollit et al. 2009; Bowles et al. 2011).

The goals of our study were to determine whether DNA from potential prey species, or species incidentally consumed, could be extracted and amplified from the faeces of Pacific walruses in the Bering Sea. We therefore collected walrus faeces and potential prey from the wild and amplified DNA from the faeces and tissue of potential prey species.

Methods

Faeces and prey collection and processing

We obtained 70 Pacific walrus faecal (scat) samples from sea ice haulouts in March of 2008 and 2009 south of St. Lawrence Island in the Bering Sea. The haulouts appeared as large dark brown patches on the snow-covered ice surfaces and were located using a helicopter launched from an icebreaker. We collected the frozen scats using hammers and chisels and kept them frozen in plastic bags until DNA was extracted. Each scat sample was considered to be from a different individual animal based on colour and texture of the samples, and the physical distance between faecal remains frozen into the ice.

In addition to collecting walrus scats, we obtained benthic species that have been previously found in walrus stomachs. These potential prey species were obtained using van Veen bottom-grabs taken from the icebreaker USCGC Healy in March 2009 at a depth of ~100 m within the upper 0.25 m of the soft ocean bottom. The bottom-grabs contained >30 families and >40 species. Potential walrus prey were selected and frozen from these samples (Sheffield et al. 2001; Sheffield and Grebmeier 2009) and were later thawed to extract DNA to test primers. Our selection of prey included the most common taxa present in the bottom-grabs and ensured that every major prey taxon possible was represented (i.e. molluscs, annelids, crustaceans and echinoderms). Using a suite of common benthic invertebrates from the general area where walruses were feeding was a starting point for primer testing, given our objective to determine whether DNA could be extracted from the walrus faeces and whether any species consumed could be amplified.

DNA analysis

We selected primers to amplify the walrus prey from the bottom-grabs from published studies according to whether: (1) the primers had successfully amplified a phylum, class, family, genus or species of the potential prey species contained in the bottom-grab sample and (2) we could replicate the published protocol using our laboratory equipment.

We extracted DNA from ~100 mg of faecal material scraped from each of the frozen walrus scats ($n = 70$) using the DNeasy stool mini kit (Qiagen) according to the 'Isolation of DNA from stool for human DNA analysis' protocol, as per previous faecal dietary studies (Deagle et al. 2005a; King et al. 2008; Bowles et al. 2011). Faeces were not homogenized because the samples were already considered to be well mixed. Walrus scat is very diffuse and is readily spread across the ice surface. Individually identifiable scats were chipped from the ice surface and placed into separate plastic bags, thus creating a mixed sample. For prey species, we extracted total DNA from tissue using the DNeasy blood and tissue kit (Qiagen) following the 'animal tissue' protocol. We chose 12 prey species or taxa for primer testing and did two independent DNA extractions from each prey species or taxon (Table 1). Concentrations of DNA were measured using a Nanodrop (ND-1000) spectrophotometer.

Table 1 Potential prey species of Pacific walruses from which DNA was extracted and primers were tested

Phylum	Class	Family	Genus + species	Common name	Sample #	Amplified (Y/N)
Mollusca	Bivalvia	Tellinidae	*Macoma moesta*	Flat macoma (clam)	1, 2	Y
		Nuculidae	*Ennucula tenuis*	Smooth nutclam	3, 4	Y
		Nuculanidae	*Nuculana radiata*	Rayed nutclam	5, 6	Y
	Gastropoda	Solariellidae	*Solariella obscura*	Obscure solarelle	23, 24	Y
Cephalorhyncha	Priapulida	Priapulidae	*Priapulus caudatus*	Cactus worm	7, 8	Y
Annelida	Polychaeta	Unidentified	Genus sp.	Marine polychaete	11,12	Y
		Maldanidae	Genus sp.	Bamboo worms	13, 14	Very faint
		Pectinariidae	*Pectinaria hyperborea*	Marine polychaete	9, 10	Y
Arthropoda, subphylum crustacea	Malacostraca	Gammaridae	Genus sp.	Amphipod	17, 18	Y
		Paguridae	*Pagurus* sp.	Hermit crab	21, 22	Very faint
Echinodermata	Ophiuroidea	Ophiuridae	*Ophiura sarsii*	Notched brittle star	15, 16	Y
	Holothuroidea	Myriotrochidae	*Myriotrochus rinkii*	Sea cucumber	19, 20	Y

Positive amplification (Y) indicates that that sample was successfully amplified using the 16S primers from Deagle et al. (2005b). Sample numbers correspond to the lane labels in Online Resource 1

We conducted PCRs according to the methodologies described in the studies in which they were published, and only modified protocols as needed to use the equipment available to us. We modified the protocol of Deagle et al. (2005b) to amplify prey using HotStar Taq polymerase (Qiagen) and accompanying buffer instead of AmpliTaq Gold, with the 16S primers, but all other reaction conditions remained the same. We used 2 µl of neat DNA in every reaction for prey tissue samples and 10 µl for faecal samples.

Results

Primers from Folmer et al. (1994) and Deagle et al. (2005b) successfully amplified DNA from many potential prey species or taxa, and one set of primers from Deagle et al. (2005b) successfully amplified DNA from the walrus faecal matter. These primers—16S1F F 5′ GGACGAGAAG ACCCT and 16S2R R 5′ CGCTGTTATCCCTATGGT AACT—were designed for the 16S mitochondrial gene and had an expected product size of 183–280 bp. Concentrations of extracted DNA ranged from 4.6 to 308.9 ng/µl from ~100 mg of faecal material, and from 3.0 to 115.5 ng/µl for DNA extracted from prey species or taxa.

All of the prey species or taxa that we tested amplified with the 16S primers (Table 1 and Online Resource 1). Although amplification was weak for two taxa (bamboo worms—Polychaeta Maldanidae and the hermit crab—*Pagurus* sp.), all of the products amplified at the expected product size (~183–280 base pairs). Thus, these primers should have amplified DNA from the prey remains contained in the walrus faeces if that species was present.

Four of the 70 walrus faecal samples amplified at ~250 base pairs (Online Resource 2), which was consistent with our expectations based on amplification of prey DNA (Online Resource 1). However, we do not know which prey these amplifications represented because many of the potential prey species or taxa amplified at similar base pair sizes (Online Resource 1). In fact, the amplifications could have been DNA from multiple prey species or taxa that amplified simultaneously.

Discussion

This is the first study to show amplification of DNA directly from walrus faeces that we are aware of. We showed that all 12 of the potential prey species or taxa that we tested from the bottom-grab can be amplified with a single primer set, and that the same primers successfully amplified DNA from four of the walrus faecal samples.

Amplification of DNA from the tissue of prey samples from the bottom-grab was generally consistent over the extraction duplicates (Online Resource 1). Most of the potential prey species or taxa showed multiple bands of amplification (Online Resource 1), which could indicate non-specific binding. Primers that bind non-specifically to prey DNA may cause competition for reagents and result in less amplification of the target DNA region. This may explain why we amplified DNA from just 4 of the 70 faecal samples that we tested. Optimization of the primers for each prey species or taxon should address this issue, which we did not do because it was beyond the scope of this pilot study. Although there may have been non-specific binding, amplification of DNA from the potential prey species or

taxa was robust across all species or taxa tested using the 16S primers (Table 1 and Online Resource 1) (Deagle et al. 2005b), making these primers a useful starting point for amplifying DNA from these species or taxa if they were present in the walrus faecal DNA.

It was surprising that only four faecal samples amplified (Online Resource 2) given that so many of the potential prey species or taxa from the bottom-grabs are seen in walrus stomachs (Sheffield and Grebmeier 2009; Sheffield et al. 2001), and the prey species or taxa could be amplified using the 16S primers (Table 1 and Online Resource 1). However, most of the faecal DNA is comprised of DNA from microorganisms inhabiting the predator's gut, followed by predator DNA and lesser amounts of prey DNA. This means that the amount of prey DNA is a very small portion of the total DNA contained in a faecal sample (Bowles et al. 2011). Thus, one possible explanation for so few visible amplifications in the faecal samples is that the small amount of prey DNA, in conjunction with non-specific binding of primers and competition for reagents, may have resulted in fewer amplifications of prey DNA in the faecal samples. An alternative explanation is that the amount of amplified product may have been too small to be visible on an agarose gel. Thus, a more sensitive form of PCR, such as Denaturing Gradient Gel Electrophoresis DGGE (Tollit et al. 2009) or real-time PCR (Deagle and Tollit 2007; Bowles et al. 2011), might have detected many more amplified samples.

Another possible explanation for the small number of faecal samples that amplified is that there was simply very little prey DNA in the walrus faeces. The prey items for which DNA amplified were physically small, ranging from about 1–2 cm in length, and may therefore have been incidentally consumed by the predator, rather than being the main meal. Thus, the overall contribution of the DNA from the prey that was passed through the walruses gut may have been small compared to other prey types. Again, this may have resulted in fewer amplifications and may mean that a more sensitive method of visualization is needed to see the PCR product.

Lastly, there is the possibility that the low amplification success rate was due to degraded DNA that was damaged either by UV exposure while on the sea ice, or by freeze–thaw cycles as it was transported from the location where it was collected to the location where we extracted the DNA. However, spectrophotometer measurements indicated that there was DNA of some sort in all of the faecal samples. Also, the DNA fragment size that we amplified was relatively small ($\sim$250 bp), such that some DNA degradation should not have had much effect on the amplification success. Nevertheless, DNA quality issues should also be considered as a possible explanation for our low amplification success rate.

Since all of the potential prey species we tested amplified at similar base pair sizes with the 16S primers (Table 1 and Online Resource 1 and Online Resource 2), it was not possible to determine which prey species were amplified in the four faecal samples simply by visualizing the PCR product on the agarose gel. However, it should be possible to identify exactly which prey are present by cloning and sequencing these samples, or by using a PCR technique that provides sequence-based resolution, such as DGGE (Deagle et al. 2005a).

Non-invasive diet analysis techniques are being used with increasing frequency for wildlife management (Waits and Paetkau 2005; King et al. 2008) and are contributing to understanding trophic relationships between competitors and predators and prey (Pimm 2002; Trites 2003). Faecal DNA analysis is becoming more common place and is particularly useful for describing the diets of marine mammal species that spend a significant amount of time in the water out of sight, provided that there is a means by which to collect their faeces (King et al. 2008; Tollit et al. 2009; Bowles et al. 2011; Deagle et al. 2010). Continued development and application of this technique could contribute considerably to assessing diets of walruses in a rapidly changing northern environment.

In summary, we successfully amplified DNA from a small subset of walrus faecal samples and believe that it is possible to identify prey species or taxa (e.g. cephalopods or polychaetes) consumed by walruses with further optimization and development of other species-specific or taxon-specific primers, and/or sequencing. Looking to the future, novel high-throughput assays could allow for sequencing of all prey species in a single sample (Deagle et al. 2009, 2010). These promising results lay a foundation for further work to identify most of the species consumed by walruses using DNA analyses.

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