Back Into the Wild: Feces DNA Analyses Track the Return of a Beluga Whale Back to Its Natural Environment

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Abstract

Following the sudden appearance, and subsequent rehabilitation of an assumed previously captive beluga whale off the coast of Norway, we investigate the animal's ability to readapt to life in the wild. Using DNA analysis, a survey was set up to assess diet throughout the rehabilitation process, and during a return to wild foraging when rehabilitative feeding had ceased. Metabarcoding of collected feces, confirmed the diversification of the beluga whale's diet to local prey. These findings are indicative of improved foraging behavior, and the ability of this individual to readapt to life in the wild following a period of rehabilitation. Beyond the case study presented here, we demonstrate the power of DNA analysis as a non-intrusive tool to assess the diet of large mammals, and assess progress adapting to life in the wild following release from captivity and rehabilitation programs.

Introduction

A male beluga whale (*Delphinapterus leucas*), with a harness fitted to his body, was first sighted in Tufjord, northern Norway on 26 April 2019. The harness had an equipment mount attachment, labelled "Equipment of Saint Petersburg". Based on these observations and geographic considerations, it was hypothesized that the animal had previously been trained and used in the Russian Navy, though this was never confirmed. On 30th April 2019, the beluga whale appeared in the harbor of Hammerfest where he remained (unrestricted) until mid-July of the same year. While here, the whale quickly engaged with people and triggered substantial interest on social media, rapidly becoming a celebrity in Norway nicknamed 'Hvaldimir' (a combination of 'Hval' for 'whale' in Norwegian and '-dimir' in reference to Russian President Vladimir Putin; Fig. 1A). It was feared that the whale was not able to feed himself, and so the non-profit organization, Norwegian Orca Survey (NOS), arrived onsite to assess the whale's condition, which, on first examination, was determined as 'poor'. Following this, several days of observation and multi-sensor tagging (E.J., unpublished data) failed to reveal any feeding activity (Fig. 1B). An official permit from the Norwegian Directorate of Fisheries was subsequently acquired, and NOS initiated a feeding program to rehabilitate Hvaldimir, supported by both local and international expertise and sponsorship (Fig. 1C).

The immediate goal of implementing the rehabilitative feeding program was to improve Hvaldimir's condition to promote his welfare and subsequent survival. After a few weeks of effort, Hvaldimir's body condition appeared more robust, and he displayed higher levels of energy (though neither of these were quantified). Continued monitoring using animal-borne videography (GoPro Hero 4 Black edition attached with suction-cups) further revealed that the whale was spending more time exploring his habitat and showed an interest in live fish. On 19 July 2019, after 2.5 months of routine feeding, Hvaldimir left the Hammerfest Harbour and travelled along the Norwegian coast, stopping in many different places where he stayed for various periods of time (E.J, L.R, R.K unpublished data, based on observations from local residents). Limited logistics to reach mostly remote places only allowed for short, intermittent periods of monitoring for the rest of 2019. Thus, innovative means had to be found to assess Hvaldimir's feeding activity during opportunistic, rare encounters. During the initial period of rehabilitation in Hammerfest

Harbour and subsequent opportunistic encounters, feces were collected with the objective to use non-invasive techniques based on DNA extraction to infer diet evolution, and possible diversification from herring, i.e. a clear signature of active and successful wild foraging behavior following the feeding program previously undertaken while Hvaldimir resided in Hammerfest Harbour. Metabarcoding combining sequence-based identification with high-throughput sequencing technology (HTS; Pompanon et al., 2012), has developed over the past few years as a powerful, and nonintrusive (when applied to feces rather than stomach contents ²) method to follow diet. Its use has recently been extended to marine mammals like sea lions ³ and walruses ⁴.

The rehabilitation or rewilding of marine mammals is a complex, broad, and highly discussed topic ^{5,6}. In general, to release animals into the wild, that were once under human care or captive, it must be clear that the animal is both healthy and has the skills essential for survival. Self-feeding is an obvious necessity. Here, we describe produced metabarcodes of the DNA extracted from feces used to assess Hvaldimir's feeding activity and diet composition at various stages of his rehabilitation and return to the wild between June and September 2019.

Methods

Sampling and DNA extraction

Hvaldimir's initially feeding comprised ~13-20 kg of dead (frozen and thawed) Atlantic herring (*Clupea harengus*) per day, distributed over four to six feeding sessions (EJ, LR unpublished data). After visible improvement in body condition, the amount was reduced, and a variable daily food provisioning program was initiated with the hope to stimulate natural foraging and Hvaldimir's autonomy in feeding. During this time, feces sampling commenced. Fecal samples were collected opportunistically in summer 2019. Sampling was carried out during land-based feeding or husbandry sessions as the animal spontaneously defecated. Specifically, fecal pellets were collected as they reached the sea surface and floated for some time. Six fecal samples could be collected between June and September 2019 (Table 1). Upon collection, all samples were stored at -20°C until transport (frozen) to the laboratory in France (Ifremer-MARBEC, Sète) for DNA extraction.

For each sample, DNA extraction was performed on approximately 3-5 g of feces using the PowerMax Soil DNA Isolation Kit (Qiagen, Hilden, Germany) to maximize the amount of starting material. Due to the potential protein content of such samples compared to soil ones, a first lyses step was included using 750 µl bead solution, 65 µl of solution C1 and 20 µl of proteinase K incubated for 30 min 65°C. All other steps were performed following the manufacturer's instructions. Two controls, one positive (herring flesh) and one negative (an empty column), were added, following the exact same steps during the same extraction batch.

PCR was performed on all DNA samples and controls, separately for two barcoding gene regions: COI (Metazoa) using mlCOIintF GGWACWGGWTGAACWGTWTAYCCYCC ²⁰, jgHCO2198

TANACYTCNGGRTGNCCRAARAAYCA²¹ for an approximate fragment length of 313bp, and 18S-V1V2 (Metazoa) with SSUF04 GCTTGTCTCAAAGATTAAGCC²², SSURmod CCTGCTGCCTTCCTTRGA²³, for a variable length of this ribosomal loop of 300 to 500bp. Compared to these original primer sequences, Inosine (I) were changed with "wobbles" (N) to create degenerate primers compatible with the High-Fidelity Phusion Taq Polymerase used (which does not recognize Inosine). All primers were synthesized with standardized Illumina adapters forward

TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGMKGGWACW; reverse

GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGMK and later combined with Illumina barcodes to allow multiplexing. Each 30 µl amplification reaction contained 2 µl of DNA template, each primer at 0.7 pM, 15 µl of Phusion® High-Fidelity PCR 2X Master Mix with GC buffer (New England Biolabs, Ipswich, MA US), and molecular water to the final volume. Additionally, all COI reactions included an additional 1 mM MgCl₂ (1.2 ul/25 mM), which together with the 1.5 mM MgCl₂ contained in the Mix-Phusion resulted in 2.5 mM MgCl₂. The PCR cycling conditions were as follows: 98°C for 30 s, followed by a marker specific number of cycles (35 for 18S and 40 for COI) of 98°C for 10 s, annealing (57°C for 18S-V1 and 48°C for COI) for 45 s, and 72°C for 60 s, with a final elongation at 72°C for 10 min. All PCR products were examined via gel electrophoreses and quantified using a Qubit 3.0 fluorometer (Invitrogen, Denmark). Sequencing and library preparation were performed by the Labex platform GenSeq (Montpellier University, France). The Nextera XT Index Kit (Illumina, Hayward, CA, USA) was used separately for each gene region for Library prep (PCR based), including PhiX for standardization. Sequencing was performed on an Illumina MiSeq instrument with the corresponding reagent kit (2x250 bp).

Bioinformatics

Data were analyzed following the bioinformatic pipeline described by Brandt *et al.*²⁴. The FASTQ files were first processed using Cudadapt²⁵ to remove all primers and leftover adapters. The error correction algorithm implemented in the program DADA2²⁶ was then applied, after pre-filtering reads using a maximum expected error (MaxEE) of 5 and truncation length of 250 bp (with minimum truncQ=2). The parameters used for fragment size selection were an expected total length of 250-350 base pairs (bp) for COI and 300-500 bp for 18S-V1V2 assembled fragments, and a chimera removal step was included. A list of unique sequences referred to as amplicon sequence variants (ASVs) was obtained along with the number of their occurrences (reads) in each sample and control. To avoid confounding intraspecific diversity and species diversity, particularly for metazoans^{27,28}, processed ASVs were clustered into operational taxonomic units (OTUs) using the program swarm2²⁹ with an iterative local threshold d of 6 for COI and 4 for 18S-V1V2.

Taxonomic assignment was performed at the ASV level against the reference database Silva release 132³⁰ for 18S-V1V2 ribosomal sequences and Midori³¹ for COI. Assignments were performed using the RDP naive Bayesian classifier method³². Possible cross-contaminants introduced during extraction, PCR, and sequencing were removed using the *decontam*³³ R package (using the prevalence method with a

threshold of 0.5), using information on the identity and number of reads found in the samples and PCR-negative controls. For all gene regions, the final OTU counts were adjusted using an R-based script³⁴ to account for potential tag switches that can occur during library preparation³⁵. To limit the influence of the remaining spurious sequences and particularly possible nuclear-degenerated copies (*numts*), known to occur when working with mitochondrial DNA(e.g., Song *et al.*,³⁶), the program LULU³⁷ was applied to the COI data, with an identity of 0.84 and a co-occurrence of 0.9.

Results/discussion

Our results clearly illustrate the evolution of Hvaldimir's feeding behavior throughout the rehabilitation process and during a return to wild feeding. Whereas the very first feces contained mostly a nematode *Anisakis simple*, the last samples show an alternation of herring (*Clupea harengus*) and saithe(*Pollachius virens*), in a timeline fitting the feeding by NOS (Figure 2). The latter is a species native to the region, indicating active foraging and predation by Hvaldimir in line with the known predatory behavior of wild belugas, which feed on pelagic and benthic fishes in the open sea as well as in coastal and fjord areas ⁷.

Remarkably only saithe was identified on September the 2nd (<2h after Hvaldimir was fed with herring) and 4th (15h after he was fed with herring) with 94% and 99% of total reads. Contrastingly, on the day in-between, 7.5 hours after he was fed with herring, only herring was detected (100% of total reads). The lag between the feeding and the detection of prey species in the feces is an interesting observation for further studies, informing on the expected time frame of digestion and the feeding activity covered by a given sample collection.

Studies of diet composition from wild/dead beluga have typically relied on morphological, stable isotope, and fatty acid analyses ^{8,9}. These have shown that the beluga whale is an opportunistic predator, targeting both fish and invertebrates less than 30 cm in length. Changes in diet composition occur frequently by region, depth, and season^{8,9}. Results here, from the concomitant use of COI and ribosomal 18S in metabarcoding analyses build upon this, and suggest a broader and more diverse range of prey are consumed, including many small or soft-bodied species¹⁰. Taxa detections using 18S-V1 include Acanthocephala, Annelida (Polychaeta), Arthropoda (Malacostraca, Maxillopoda), Ctenophora, Cnidarian (Hydrozoa, Myxozoa), Echinodermata (Ophiuroidea, Holothuroidea, Echinoidea), Gastrotricha, Mollusca (Bivalvia, Gastropoda), Nematoda, Platyhelminthes (Cestoda, Monogenea, Trematoda), Porifera (Calcarea, Demospongiae), Rotifera (Monogononta), Tunicata (Appendicularia, Ascidiacea). Long-term time-series of stomach contents morphological inventories support these observations, with reports that include: Polychaeta, various Crustacea, Gastropods, Bivalves, and Cephalopods ⁸.

Relative read abundance can be linked to relative biomass for vertebrates as well as invertebrate feces/stomach content ^{2,10,11}. Here, the high relative abundance of mussels (*Mytilida* sp. with up 26%) and polychaete worms (Polycheta with 36%) may thus reflect active predation. However, based on the size and relative abundance of other taxa, most appear attributed to contamination from surrounding

water, or reflect secondary predation ². Nevertheless, it is interesting to point out a large relative abundance of reads for Cnidaria. In August, both gene regions led to detect mostly cnidarian DNA, *Obelia longissimi* from COI and a large cluster of Hydrozoa and Myxozoa from 18S. In this particular area, *Obelia* sp. is found in the saithe diet ¹², and this observation may thus either reflect opportunistic “grazing” or earlier undetected diversification through active hunting of saithe.

Among invertebrates, several parasites were detected. The occurrence of one member of the group Acanthocephala, identified as *Echinorhynchus gadi* which is a known parasite for saithe, cod, herring, and others ^{13,14}, correlates with the detection of saithe or herring in Hvaldimir’s feces. Some Platyhelminthes from the parasitic groups Trematoda, Cestoda, and Nematods, such as *Anisakis simplex*, were also detected, and can also be considered as indirect evidence for the digestion of fish prey. The occurrences of nematodes in fish or marine mammals are to be expected, although high numbers can have health implications and in extreme cases lead to death ¹⁵. The parasitic nematode *Anisakis simplex*, also called ‘herring worm’, due to famous human infestation through the consumption of raw herring and other seafood^{16,17}, has a life cycle pervasive across marine food webs, from mammals to fishes and arthropods. It is thus a common parasite of belugas, and known to be associated with natural prey species including herring and capelin, which are common paratenic hosts ¹⁸. Depending on its prevalence, it may cause chronic ulcerative gastritis, and is thought to be implicated in the death of many stranded belugas in northwest Europe ¹⁹. The overwhelming relative abundance of *A. simplex* in the two first samples (90-100% reads for both gene regions), supports a dominance of parasitic nematodes within the feces at the time when the body condition of Hvaldimir’ was noted as poor and displaying serious signs of malnutrition. This may reflect an empty stomach with a persistence of the nematode after a herring consumption too old to be detected, and/or acute parasitosis. Relative abundance decreased over time from 100% in June to <1% in September (see Figure 2), supportive of Hvaldimir’ improved health overtime, further confirmed by visual observations of improved body condition and energy levels.

Conclusions

This short case study on the beluga whale ‘Hvaldimir’ demonstrates the power of eDNA based analyses of feces as a non-invasive tool to survey the diet and parasitic status of specimens, particularly in cases of rewilding once captive, or rehabilitated animals. In addition, results show a time-lag is determinant in the detection of prey and possibly associated parasites, which has important implications for the accuracy of dating when interpreting results from wild feces sampling.

Declarations

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Competing interests

None

Author contributions

S.A.H. and E.J. conceived the study; E.J., L.R. and R.K. collected the fecal samples; B.G. carried out the experiment; B.G. and S.A.H. analyzed the data and wrote the paper with input from all authors; all authors gave final approval for publication of the manuscript.

Data availability

The deposition of DNA sequences: raw sequences are available at the Sequence Read Archive (SRA): SRR15570209-SRR15570217, with BioSamples SAMN20927803-SAMN20927811, BioProject: PRJNA756855.

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Tables

Table 1: Information related to sampling of Hvaldimir’s fecals in June-September 2019 in northern Norway

sample ID	Stage	date	time	location	Notes
#1	Day 24	8 Jun	15:45	Hammerfest harbor	Time since last feed (5 kg herring) was ~ 4 h.
#2	N/A	Jun-Jul	N/A	Hammerfest harbor	No exact date; was collected in daytime between two herring feeds
#3	Day 92	15 Aug	evening	Altneset, Seiland	Collected during a six-week period when Hvaldimir was <u>not</u> provisioned with food
#4	Day 110	2 Sept	13:45	Hammerfest harbor	This sample was collected when Hvaldimir had returned to Hammerfest harbor (30 Aug) following six weeks spent on his own; time since last feed (5 kg herring) was ~ 1h40 min.
#5	Day 111	3 Sept	17:45	Hammerfest harbor	Time since last feed (4.5 kg herring) was 7.5 h
#6	Day 112	4 Sept	09:45	Hammerfest harbor	Time since last feed (4.4 kg herring) was 15 h (night before)
pc		-	-	-	Positive control: Atlantic herring (<i>Clupea harengus</i>)

Figures

Figure 1

Pictures of the beluga whale ‘Hvaldimir’ in summer 2019; A) in the harbor of Hammerfest, northern Norway, playful and seeking human contact; B) with the back-mounted, suction cup attached GoPro video camera used to record his foraging behavior; and C) being hand-fed herring during a routine feeding session.

Figure 2

Relative proportion of taxa encountered from COI and 18S Metabarcodes, excluding all non-metazoan and beluga clusters, separately for both gene regions with COI and 18S per sample. Time since last feed is also detailed in Table 1.