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Source: Waterbirds, 45(2) : 159-166

Published By: The Waterbird Society

URL: https://doi.org/10.1675/063.045.0206

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eDNA Metabarcoding Analyses of Diet in Yellow-Billed Loons of Northern Alaska

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Abstract.—Environmental DNA is a burgeoning tool used to address wide-ranging scientific questions, including determining diets of difficult-to-sample predators. Loons are large piscivorous diving birds that capture and consume prey underwater, making it nearly impossible to visually determine their diet via observation alone. Identifying species' diets is important for understanding basic life history traits, and revealing key prey species can clarify species' roles in complex trophic webs, aid in understanding population and community dynamics, and help identify critical habitat for protection. Current information about loon diet is largely anecdotal, and traditional non-observational methods for quantifying loon diet have limitations. Analysis of eDNA from loon feces may provide biologists with a non-invasive technique for determining diet without negative sampling effects, and with increased resolution as compared to other techniques. We surveyed lakes in two areas of northern Alaska for Yellow-billed Loons (*Gavia adamsii*). Loon fecal samples were collected opportunistically from latrine sites without disturbing any animals and analyzed using novel marker sets to determine loon species and diet. Fish species were detected in all fecal samples, the most common being Alaska blackfish (*Dallia pectoralis*), and ninespine stickleback (*Pungitius pungitius*). This research demonstrates that eDNA metabarcoding analyses of loon fecal samples can determine the specific loon species that deposited the feces and characterize the piscine portion of their diet with limited disturbance to the animals. *Received 22 Apr 2021, accepted 9 Sept 2022.*

Key words.— Arctic Coastal Plain, Breeding, Environmental DNA, *Gavia adamsii*, Loon

Waterbirds 45(2): 159-166, 2022

As upper trophic level predators, seabirds are considered indicators of marine ecosystem health (Furness and Camphuysen 1997; Iverson *et al.* 2007), and as such, greater understanding of seabirds' role in trophic food webs, including identification of the prey they consume across time and space, is critical. Knowing whether consumer-driven (top-down) or resource-driven (bottom-up) forces dominate an ecosystem is crucial for tracking community and population dynamics, understanding the flow of energy and the bioaccumulation of contaminants, and predicting the reaction of ecosystems to small- and large-scale environmental changes. Traditional seabird diet assessments have typically relied on analyses of stomach contents - often by examining food regurgitated by chicks - as well as direct observation of food intake or invasive tissue collection (Duffy and Jackson 1986; Barrett *et al.* 2007). Limitations inherent in such diet determination methods include low resolution of prey species identification, requirement for lethal sampling, restriction of the temporal range of inference, inference of adult diet from chick diet and other biases in detection of certain prey types, such as underrepresentation of soft-bodied prey species (Barrett *et al.* 2007). Analysis of environmental DNA (eDNA) from feces of predators such as seabirds is a promising tool for determining diet (Symondson 2002; Deagle *et al.* 2010; Bowser *et al.* 2013; Oehm *et al.* 2017; Kleinschmidt *et al.* 2019) that may present biologists with a non-invasive method with fewer negative effects and increased resolution compared to other diet determination methods (Barrett *et al.* 2007; Deagle *et al.* 2007).

Loons (Family Gaviidae) are large piscivorous diving birds that capture and consume most of their prey underwater in marine systems during the non-breeding season and in freshwater systems during the nesting season; therefore, direct visual observation of their diet is nearly impossible. Currently, information about diet for most loon species is largely anecdotal (Rizzolo *et al.* 2020; Evers *et*

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al. 2020; Russell 2020); the only quantitative diet analyses for loons, which used stable isotope and fatty acid methods, may have low resolution at identifying prey types (Haynes *et al.* 2015; Rizzolo 2017). Disparate prey groups can have similar isotopic signatures; therefore, stable isotopes are generally only used to estimate trophic level and not to determine specific diet composition (Hobson *et al.* 1994; Käkelä *et al.* 2007). No loon-specific trophic enrichment factors (TEFs) or calibration coefficients have been calculated for these diet determination methods; therefore, researchers have applied TEF and calibration coefficients from other seabird species when attempting to quantify loon diet (Haynes *et al.* 2015; Rizzolo 2017). The turnover time of adipose tissue is also unknown for loons; therefore, results from fatty acid analyses of breeding loons may reflect the residual signatures from marine fish consumed prior to migration to freshwater lake breeding territories (Haynes *et al.* 2015). There also may be biases or negative effects associated with many diet determination techniques. Previous methods for quantifying diet of loons required capture and handling of individuals and included invasive blood and lipid sampling (Haynes *et al.* 2015; Rizzolo 2017). Loons are sensitive to disturbance and capturing adult loons can have negative effects on productivity (Uher-Koch *et al.* 2015). Accurate, non-invasive diet determination methods, such as the use of eDNA analyses of feces, are needed for loons given their sensitivity to disturbance and conservation concerns.

eDNA analyses of loon diets require a regional-level reference library of sequence data from prey items likely to be included in a loon's diet. Yellow-billed Loons (*Gavia adamsii*) are obligate piscivores that forage variously in marine and freshwater systems throughout the annual cycle. This rare species has a small global population estimated at approximately 16,000 - 32,000 (Uher-Koch *et al.* 2020b). The life history characteristics of Yellow-billed Loons, small global population size, low reproductive success, and restricted distribution led the U.S. Fish and Wildlife Service (USFWS) to evaluate this species as a candidate for protection under the Endangered Species Act (Federal Register 2014). Marine and estuarine fish species' distributions are likely important drivers of migratory pathways of Yellow-billed Loons during the non-breeding season, and freshwater fish species' abundance and distributions are an important determinant in nest-site selection during the nesting season (Earnst *et al.* 2006; Uher-Koch *et al.* 2020b).

In Alaska, expansive Arctic wetlands provide essential nesting habitat for the entire U.S. population of Yellow-billed Loons, and approximately 5–10% of the global population nests in Bering Land Bridge National Preserve (2.8 million acres), Cape Krusenstern National Monument (540,000 acres), and the National Petroleum Reserve-Alaska (23 million acres). These remote areas are very difficult to access (Haynes *et al.* 2015), rendering assessment of fish communities required by Yellow-billed Loons and other loon species difficult. To address this difficulty, we leveraged eDNA sampling techniques, utilized eDNA metabarcoding markers that can detect up to 37 species of freshwater fish occupying waterbodies in mainland Alaska, including the Arctic Coastal Plain (Menning *et al.* 2020), and developed novel metabarcoding markers that differentiate all five loon species (Red-throated Loon [*Gavia stellata*], Arctic Loon [*G. arctica*], Pacific Loon [*G. pacifica*], Common Loon [*G. immer* and], and Yellow-billed Loon). In this study we demonstrate that sample collection is rapid and easily accomplished, requires relatively minimal field effort, and leverages inexpensive and simple equipment. This approach provides several advantages over traditional methods of sampling fish communities including: (1) it greatly reduces bias towards the detection of particular species compared to any single traditional sampling method; (2) it reduces the impact of fisheries surveys on aquatic ecosystems and fish populations; and (3) it eliminates the need to handle or disturb loons at their nesting sites. This novel metabarcoding approach is also sufficiently flexible that additional target freshwater fish species, as well as loon species, could be added in future studies, including studies

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that combined eDNA analyses of lakes inhabited by loons and diet analyses based on eDNA extracted from loon feces sampled at lake shoreline loon latrines.

The aim of this pilot study is to use fecal eDNA analysis as a non-invasive technique to investigate fish prey of Yellow-billed Loons during the breeding season in northern Alaska in a manner similar to the study on Red-throated Loons by Kleinschmidt *et al.* (2019), clarifying our understanding of habitat preferences for Yellow-billed Loons and their important prey species. We modified and augmented eDNA metabarcoding markers (Menning *et al.* 2020) that determine fish species occupying waterbodies in Arctic Alaska—including those known to be occupied by Yellow-billed Loons—to include markers that can differentiate among the five species of loons nesting in waterbodies in Arctic wetlands in Alaska. We successfully tested these markers on eDNA extracted from loon feces and determined specific loon species corresponding to each fecal sample and the fish species comprising their diet. Similar to the findings of Kleinschmidt *et al.* (2019), we found that loons are opportunistic hunters preying primarily on fish species found in nearby lakes.

Methods

Study Area

We surveyed lakes at two study areas in northern Alaska during 2011–2014 (Fig. 1) for Yellow-billed Loon occupancy as part of studies on loon breeding ecology and for which fish communities had been investigated using traditional methods (Laske *et al.* 2016; Uher-Koch *et al.* 2020a). Yellow-billed Loons defecate on shorelines of breeding territories (Uher-Koch *et al.* 2020b), and 13 fecal samples were collected opportunistically from these latrine sites. We followed standard practices for sample collection, storage, and preservation (King *et al.* 2008). We collected only fresh (bird was seen defecating) or wet (bird was not seen defecating) fecal samples, which were then stored in ethanol (95%) at -20°C until laboratory processing.

For each fecal sample, approximately 1 g of fecal material was transferred to a 15-mL conical tube containing 5 mL of Longmire Buffer (LMB; Longmire *et al.* 1997). Each sample was gently vortexed until homogeneous. DNA was extracted using a 400-µL subsample of the homogenized LMB-preserved fecal sample with a Qiagen DNeasy Blood and Tissue kit (Qiagen Inc., Valencia, CA, USA) following manufacturers suggested protocols, with the exception that the manufacturer's suggested reagent volumes were doubled to account for the increased sample volume.

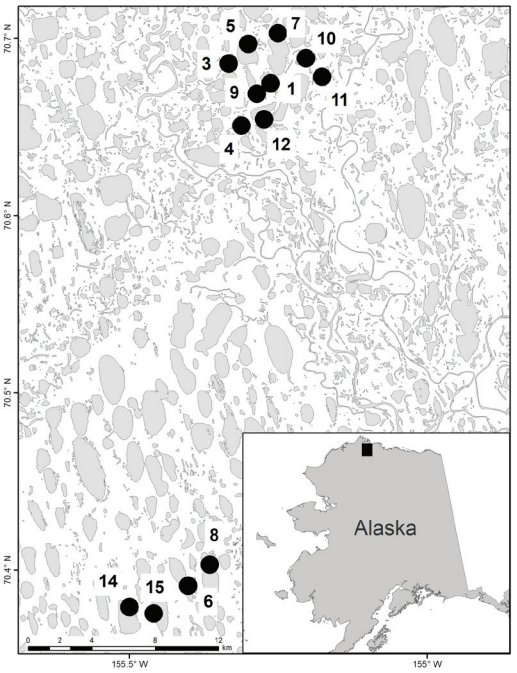


Figure 1. Location of latrine sites where *Gavia* sp. scat samples were collected for eDNA analyses on Arctic Coastal Plain of northern Alaska, USA. Numbers represent individual lake locations.

Loon scat eDNA libraries were prepared using a two-step polymerase chain reaction (PCR) protocol and sequenced using an Illumina MiSeq. PCR primers were designed following Menning and Talbot (2018) to target multiple loci of multiple fish species found in Alaska as well as all five loon species (Table 1). Two first step PCRs were conducted using equimolar concentrations of all fish and *Gavia* sp. primers respectively. These reactions consisted of 12.5-µL 2X Type-it Multiplex PCR Master Mix (Qiagen Inc., Valencia, CA, USA), 2.5-µL 10X primer mix (2 µM of each primer), and 10 µL of template DNA and were conducted under the following conditions: 95°C for 5 minutes, followed by 32 cycles of 95°C for 30 seconds, 60°C for 3 minutes, and 72°C for 30 seconds, with a final extension step at 60°C for 30 minutes. Following the first PCRs, excess primers and dNTPs (deoxynucleotide triphosphates) were removed using ExoSap (Affymetrix, Santa Clara, CA, USA), all loci were quantified by fluorometry using a Quant-IT Broad Range kit (Thermo Fisher Scientific, Waltham, MA, USA), normalized to equal concentrations, and pooled by sample.

To produce barcoded amplicons, a second step PCR was performed on each locus-pooled sample consisting of 2.0 µL of molecular grade water, 5.0 µL of Phusion High-Fidelity PCR Master Mix with HF Buffer

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Table 1. All *Gavia* sp. and piscine polymerase chain reaction primers used in this study.

Primer name	Target locus	Target	Forward primer	Reverse primer
AK12S	12S	General fish	CTCGTGCCAGCCACCGCG	GTATCTAATCCCRGTTTG
AKCorCytb1	Cytochrome b	*Corgonus* sp.	CGAGGDCTTTAYTAYGG	ATYCAYTGYACDAGGGC
AKCorCytb2	Cytochrome b	*Corgonus* sp.	CTRTAYAARGARACCTG	ATYCAYTGYACDAGGGC
AKCotCytb	Cytochrome b	*Cottus* sp.	CTMACVCGMTTCTTTGC	AARTTRTCWGGGTCTCC
AKOncCytb	Cytochrome b	*Oncorhynchus* sp.	CTTCTCACTATAATRAC	GTGAARGYCGAAAAATCG
AKSalvCOI	Cytochrome oxidase I	*Salvelinus* sp.	TTCTCYCTTCATTTAGC	AAYATAGTAATGCCTGC
AKOsmCytb	Cytochrome b	*Osmeridae*	TTYCTWCTYCTBTTAGC	AGGAGRAGAAGRACGGC
GaviaCOI	Cytochrome oxidase I	All 5 *Gavia* sp.	CTCYCTYCACCTAGCAGG	TGTRGTRTTTAGGTTTCG
GaviaCytb	Cytochrome b	All 5 *Gavia* sp.	ACYGGCCTACTACTAGC	CCRTARTARAGTCCTCG
GaviaND1	ND1	*Gavia adamsii, G. immer*	ACATTGTAGGCCCCTTCG	GCCTAGGTTTAGGTCGGC
GaviaND2	ND2	*Gavia adamsii* and *G. immer*	TGAACCCATATGCAAAGC	TAATGCGCATGCGGTTGC
GaviaND5	ND5	All 5 *Gavia* sp.	TTCGGAYTACTRCAACC	GTTTGARGCYCAGCCTG

(New England BioLabs, Ipswich, MA, USA), 1.0 µL of each I5 and I7 Nextera XT index primer, and 1.0 µL of pooled PCR product under the following conditions: 98°C for 30 seconds, followed by 20 cycles of 98°C for 10 seconds, 60°C for 30 seconds, and 72°C for 40 seconds, with a final 5-minute elongation step at 72°C. All two-step PCR products were quantified by fluorometry using a High Sensitivity Quant-IT kit (Thermo Fisher Scientific, Waltham, MA, USA), normalized to equal concentrations, and pooled. Quantified pooled PCR products were electrophoresed on a 1% agarose gel to estimate PCR fragment sizes and gel purified using Qiagen Gel purification kit (Qiagen Inc., Valencia, CA, USA). Gel purified PCR products were quantified by fluorometry using a Quant-IT High Sensitivity kit (Thermo Fisher Scientific, Waltham, MA, USA), diluted to 4 nM concentrations following Illumina guidelines (Illumina Document # 15039740 v01), re-quantified by fluorometry using a Quant-IT High Sensitivity kit, and further diluted to 20 pM following the Illumina NextSeq Protocol A (Illumina Document #15048776 v02) for library dilution. All remaining steps for library preparation followed Illumina MiSeq protocols (Illumina Part #15034097 Rev. B). The DNA library and PhiX were further diluted to 15 pM. Sequencing was performed using an Illumina MiSeq 300 cycle v2 reagent kit (2 X 151 paired-end cycle runs; Illumina Part #MS-102-2002) on an Illumina MiSeq with a 10% PhiX spike.

All de-multiplexed data were retrieved and analyzed in the same manner as Menning *et al.* (2020) with the exception that the default BLAST+ parameters reward/penalty were changed to 1/-3 respectively and the gapopen/gapextend parameters were set at 1/1 to ensure at least a 99% match to a reference database (Supporting Information). The reference database for fish and loons was developed following Menning and Talbot (2018) targeting all five *Gavia* sp. and all piscine species expected to be found at the study sites per previous traditional sampling methods in our study areas (Haynes *et al.* 2013; Haynes *et al.* 2014; Laske *et al.* 2016; Uher-Koch *et al.* 2020a). We did not include two fish species, rainbow smelt (*Osmerus mordax*) and threespine stickleback (*Gasterosteus aculeatus*), that were only found in three total lakes out of 102 sampled in the region and almost certainly do not make up a significant portion of Yellow-billed Loon diet in our study areas as based on inferred low occupancy of the lakes in the region. Quality-filtering to remove sequencing errors was conducted by only including match count information that exceeded 0.01% of the total number of reads passing filter per sample in the MiSeq run (Bokulich *et al.* 2013).

RESULTS

Using our novel *Gavia* sp. specific markers we were able to differentiate among loon species. One fecal sample from Lake 6, thought to be from a Yellow-billed Loon was identified as a Pacific Loon via Cytochrome

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Table 2. Species, common names, and loci inferred to be present in loon fecal samples when comparing metabarcoding sequencing reads to our reference database (Supporting Information).

Species (scientific)	Species (common)	Locus hit	Lake 1	Lake 3	Lake 4	Lake 5	Lake 6	Lake 7	Lake 8	Lake 9	Lake 10	Lake 11	Lake 12	Lake 14	Lake 15
Gavia adamsii	Yellow-billed Loon	CytB	X	X	X	X		X	X	X	X	X	X	X	X
		ND5	X	X	X	X		X	*	X	X	X	X	X	
Gavia pacifica	Pacific Loon	CytB					X								
		ND5					*								
Coregonus nasus	broad whitefish	12S, CytB1						X	X						
Coregonus pidschian	humpback whitefish	CytB1, CytB2						X	X						
Coregonus sardinella	least/sardine cisco	12S, CytB1, CytB2						X	X						X
Dallia pectoralis	Alaska blackfish	12S	X	X	X	X		*		X	*	X		X	X
Pungitius pungitius	ninespine stickleback	12S	X		X		X				X		*	X	
Salvelinus alpinus	Arctic char	12S, CytB2						X							
Salvelinus namaycush	lake trout	12S, CytB2						X							

*identified in data but the number of positive identifications did not meet the 0.01% of total reads per sample passing filter threshold

b and ND5 markers (Table 2). We were also able to identify loon fish prey species because prey fish DNA was detected in all of our loon fecal samples (Table 2). We identified 7 prey fish species utilized by Yellow-billed Loons with a mean of 1.92 (SD ± 1.38) fish species detected per sample. Across all sample locations, Alaska blackfish (*Dallia pectoralis*) and ninespine stickleback (*Pungitius pungitius*) were positively identified at 61.5% and 38.5% of the sites sampled, respectively, and combined represented 59% of all identified species. The remaining species positively identified included least cisco (*Coregonus sardinella*) found at three locations (Lakes 7, 8, & 15), broad and humpback whitefish (*Coregonus nasus* and *C. pidschian*, respectively) found at the same two locations (Lakes 7 & 8), and Arctic char and lake trout (*Salvelinus alpinus* and *S. namaycush*, respectively) found at the same location (Lake 7). The fish prey species most commonly detected in the 13 samples from Yellow-billed Loons were Alaska blackfish (8/13 samples), ninespine stickleback (5/13 samples), and least cisco (3/13 samples). The only fish species detected in the Pacific Loon feces sample (Lake 6) was ninespine stickleback. Seven potential fish prey species included in the reference database were not identified in any fecal samples (Supporting Information). All Illumina MiSeq data were accessioned at NCBI BioProject PRJNA638941; sample data can be found at Menning *et al.* (2022).

Discussion

Our data show the ability of eDNA analysis of fecal material to provide information on fish prey species consumed by loons occupying lakes in a vast, remote, roadless region of Alaska. Our results determined both the species identity of the loon leaving the feces and the piscine species composition of their diet via eDNA analyses of those feces, indicating that this method is a promising tool for determining diet of predators, and may provide higher taxonomic resolution than other diet determination methods such as stable isotopes (Hobson *et al.* 1994; Käkelä

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et al. 2007). eDNA metabarcoding analyses can serve to inform studies of prey species and predator habitat use and behavior, potentially as a stand-alone method or in conjunction with other methods such as stable isotope or fatty acid techniques (Sonsthagen *et al.* 2020). Based on our study, and other independent research evaluating the diet of Red-throated Loons (Kleinschmidt *et al.* 2019) and other seabird species (McInnes *et al.* 2017), eDNA methods such as described herein could be used to determine the diets of other avian predators, with the requirement that a sufficient reference sequence library for potential prey species exists.

Identifying which potential prey species to include in the reference database was important for this particular eDNA analysis because there are fixed but often limited genetic differences among certain fish species belonging to the same genus (often less than 2% dissimilarity), such as among *Coregonus* spp. Although an eDNA metabarcoding database of 37 fish species was previously created for application in Alaska, we only included 14 fish species in the reference sequence library for our analyses incorporating information only for those species that were previously identified by Laske *et al.* (2016) as inhabiting the lakes sampled. We note that the predominant loon prey species identified through eDNA analyses (Alaska blackfish) matches data identified through fatty-acid diet determination at nearby locations (Haynes *et al.* 2015). Nevertheless, without prior information regarding fish community diversity, future studies that assay lakes in the region for fish species composition, including piscivorous diet analyses, would need to incorporate a more diverse reference library.

Most diet determination methods have biases (Barrett *et al.* 2007) and although we were able to identify the fish prey species that individual Yellow-billed Loons consumed, loons may also eat invertebrates (Haynes *et al.* 2015; Rizzolo 2017; Russell 2020). Freshwater invertebrates can also be identified via metabarcoding techniques (Elbrecht and Leese 2017; Hajibabaei *et al.* 2019), and future diet determination research on loons could incorporate invertebrate genetic information into the potential prey library. Further, the fecal samples collected in our study characterized diet from a single point from a small subset of individuals during the breeding season, reflecting diet specific to when and where the samples were collected, and from which bird. Future work with archived fecal samples from loons in northern Alaska is planned to clarify whether these preliminary diet prey items are representative of the proportion of occurrence within the diet for individuals and for the broader population in the region, whether there are shifts in diets of loons within the breeding season, and if there are differences in the diets of adult sand juvenile loons. Expansion of the reference library to include marine species is also planned for better understanding of the diet of loons that utilize the marine environment during the breeding season (e.g., Red-throated Loons; Rizzolo 2017).

ACKNOWLEDGMENTS

We thank the following individuals for field assistance: H. Beutler, C. Bishop, B. Braden, R. Buchheit, D. Cummings, N. Docken, G. Evans, J. Fair, T. Fondell, S. Garcia, C. Gray, I. Isler, C. Johnson, A. Larned, S. Laske, G. Meese, K. Overduijn, T. Ronningen, T. Shoemaker, T Spivey, H. Uher-Koch, C. VanStratt, and K. Wright. We thank Gordon Brower and Commissioners of the North Slope Borough Planning and Community Services Department and the North Slope Borough Department of Wildlife Management for their assistance and feedback on this research. We thank the members of the NPR-A Subsistence Advisory Panel for their comments on how to best conduct our work in the Arctic to reduce disturbance to wildlife and subsistence activities. We thank Biodiversity Research Institute and the Bureau of Land Management Arctic Office for financial and logistical assistance. The manuscript benefited from thoughtful reviews provided by S. Sonsthagen, A. Ramey, and J. Pearce. This project was funded through the Changing Arctic Ecosystems Initiative of the U.S. Geological Survey Ecosystem Mission Area. Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

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