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Utilizing DNA metabarcoding to characterize the diet of marine-phase Arctic lamprey (*Lethenteron camtschaticum*) in the eastern Bering Sea.

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Abstract

To understand the marine feeding ecology of Arctic lamprey (*Lethenteron camtschaticum*) in the eastern Bering Sea, visual observations and DNA metabarcoding of gut contents (*N* = 250) were used to characterize Arctic lamprey diet composition in 2014 and 2015. Differences among individual diets were evaluated by collection year, capture site, and fish size. Hard structures and tissues were observed during visual examinations of gut contents, and 10 ray-finned fish taxa were identified by DNA metabarcoding. The most frequent taxa included capelin (*Mallotus villosus*), Pacific herring (*Clupea pallasii*), Pacific sand lance (*Ammodytes hexapterus*), and gadids. Six taxa were reported for the first time as prey for Arctic lamprey. Individual diets differed between collection years, among capture sites, and among size classes; however, both collection year and size explained only a small portion of diet variability (*R^2* = 0.01 and 0.04, respectively) relative to station site (*R^2* = 0.49). These study results indicate that Arctic lamprey are flesh-feeding species, and highlighted the value of DNA metabarcoding to characterize the diet of a poorly understood lamprey species.

Introduction

Characterizing the diets of marine-phase lamprey poses a special challenge to researchers. Diet analysis of fishes has largely relied upon morphological identification of prey remains and/or undigested hard structures within digestive tracts (Madenjian et al. 1998; Creque and Czesny 2012; Whitney et al. 2017). However, digested blood and/or tissue masses consumed by lamprey generally yield limited details on prey composition. Observations of lamprey wounds on teleost fishes and occurrences of hard, undigested structures within lamprey intestinal contents have been routinely used to identify prey (Beamish 1980; Maitland et al. 1984; Novomodnyy and Belyaev...
While observations of lamprey wounds provide insights into lamprey feeding interactions, identified prey may be biased toward highly valued and frequently encountered commercial fishes (Hardisty and Potter 1971). In addition, undigested hard structures, while taxonomically informative, often have variable recovery and digestion rates and may not be regularly ingested during predation, which can lead to biased or misleading dietary inferences (Tollit et al. 1997; Bowen 2000; Cottrell and Trites 2002). As a result, trophic interactions of marine-phase lamprey remain poorly understood (Mesa and Copeland 2009).

The food habits of closely related species of lamprey can vary from blood to the flesh of their prey (Potter and Hilliard 1987; Renaud et al. 2009). Flesh-feeding species are generally characterized with having smaller buccal glands, a smaller oral disc with fewer teeth, and having an enlarged median cusp on the U-shaped transverse lingual lamina atop the ‘tongue-like piston’ (Potter and Hilliard 1987; Renaud et al. 2009). Lampreys that exhibit flesh-feeding food habits target smaller-bodied fishes and inflict serious damage that often results in the death of the prey (Roos et al. 1973; Beamish 1980; Maitland et al. 1984; Renaud et al. 2009). Flesh-feeding species are known to ingest large pieces of flesh (including, on some occasions, whole fish) and have been shown to penetrate the prey’s body cavity to consume internal organs (Beamish and Williams 1976; Beamish 1980; Maitland et al. 1984). In contrast, blood-feeding lampreys primarily target larger fish species that are less susceptible to damage because wounds from blood feeders are characterized by a single hole or slide where blood can continuously be extracted (Potter and Hilliard 1987; Renaud et al. 2009; Patrick et al. 2009). Previous research has largely inferred the food habits of different lamprey species based on morphological characteristics of the oral disc and dentition (Potter and Hilliard 1987; Renaud et al. 2009). Despite food habits and feeding
mechanisms ranging from blood-feeding (i.e., parasitic) to flesh-feeding (i.e., predatory), the term ‘parasitic’ is regularly used to describe the feeding ecology of all lampreys.

The Arctic lamprey (*Lethenteron camtschaticum*) is hypothesized to be a flesh-feeding lamprey species (Potter and Hilliard 1987; Renaud et al. 2009). This conclusion was based on morphological similarities in dentition to known flesh-feeding species (e.g., the European river lamprey [*Lampetra fluviatilis*] and Western river lamprey [*Lampetra ayresii*]) and not explicit evaluations of the diet (Potter and Hilliard 1987; Renaud et al. 2009). Visual examination of the intestinal contents of known flesh-feeding species revealed the presence of tissue masses, fins, scales, eggs, and internal organs (Beamish and Williams 1976; Beamish 1980; Maitland et al. 1984; Renaud et al. 2009). Although visual observations provided insights into the food habits and feeding mode of these lamprey species, unidentifiable remains were reported in up to 56% of the lampreys examined (Beamish 1980; Maitland et al. 1984; Beamish and Neville 1995). To date, no studies have examined the intestinal contents of marine-phase Arctic lamprey, and the potential occurrence and frequency of these structures in the diet is currently unknown.

Much of what is known about Arctic lamprey diets originated from visual observations of lampreys attached to fish and incidences of lamprey wounds on fishes (Nikol’skii 1956; Gritzenko 1968; Heard 1966; McPhail and Lindsey 1970; Nursall and Buchwald 1972; Novomodnyy and Belyaev 2002; Shevlyakov and Parensky 2010). Arctic lamprey have been found attached to Chinook salmon (*Oncorhynchus tshawytscha*), sheefish (*Stenodus nelma*), starry flounder (*Platichthys stellatus*), and smelt (Osmeridae) within watersheds in southwestern Alaska and in the Beaufort Sea (McPhail and Lindsey 1970). Within Russian estuaries, Arctic lamprey wounds have been observed on forage fishes (e.g., Clupeidae, Osmeridae) and juvenile Pacific salmon,
suggesting the importance of these taxa as prey (Nikol’skii 1956; Gritzenko 1968; Novomodnyy and Belyaev 2002; Shevlyakov and Paresky 2010).

The application of molecular techniques to characterize prey species in predator diets improves detection and taxonomic resolution of prey relative to traditional morphological methods (Braley et al. 2010; Carreon-Martinez et al. 2011; Moran et al. 2016). Continued development and refinement of ‘DNA metabarcoding’ approaches for accurate species identification has made it possible to characterize diet components that lack taxonomic characteristics with little a priori information on predator diets (Valentini et al. 2009; Pompanon et al. 2012; Taberlet et al. 2012). Prey DNA can be isolated from fecal or gastrointestinal tract samples and used for targeted sequencing of taxonomically informative genome regions (reviewed in Pompanon et al. 2012). However, DNA metabarcoding has only been used in a limited number of studies involving predatory fish diet evaluations (Leray et al. 2013, Berry et al. 2015; Leray et al. 2015; Harms-Tuohy et al. 2016).

The Bering Sea is an important and complex food web that supports both commercial and subsistence fisheries, yet the role of lampreys as predators in this region is poorly understood. The aim of this study was to characterize the diet of marine-phase Arctic lamprey in the eastern Bering Sea through a combination of visual inspection and DNA metabarcoding of intestinal contents. The specific objectives of this study were to: (i) assess if diets of Arctic lamprey change as a function of capture year, capture site, and/or size class; and (ii) evaluate the relative performance of diet composition inferred from previous reports of visual observations of lamprey scars to that revealed by DNA metabarcoding. These results will help to provide a more objective evaluation of Arctic lamprey predation and further our understanding of predator/prey interactions in the eastern Bering
Methods

Lamprey Collections and Processing

Marine-phase Arctic lamprey \((N = 250)\) were collected during the U.S. Bering-Aleutian Salmon International Survey (BASIS) on the eastern Bering Sea shelf in 2014 \((n = 122)\) and 2015 \((n = 128)\) (Fig. 1) and stored at -20°C until further processing. In the laboratory, whole Arctic lamprey were thawed and measured for total length \((L_T; \text{nearest 1 mm})\) and total weight \((W_T; \text{nearest 0.01 g})\) prior to dissection. Whole intestinal tracts were removed from each specimen. Intestinal contents were examined using a Leica M125C stereoscope (Leica Microsystems, Wetzlar, Germany) for the presence of undigested hard structures. To act as a validation measure for DNA metabarcoding sequences, a subset \((n = 61)\) of tissues (e.g., sizable tissue masses, internal organs) were removed and preserved in 96% molecular-grade ethanol to be used in targeted Sanger sequencing. Upon completion of visual observations, anterior and posterior intestinal contents were placed in separate 15 mL vials and frozen at -20°C.

DNA Extractions

Genomic DNA \((gDNA)\) was isolated from recovered tissues using the Gentra Puregene Tissue Kit (Qiagen, California, USA). Because predator gut contents contain semi-digested, highly degraded prey DNA, contents from the anterior intestine were used in an effort to maximize the quality of isolated prey DNA. Anterior intestinal contents were thawed and mechanically homogenized to reduce intra-sample variability and facilitate DNA isolation. Total gDNA was extracted from four 200 mg sub-samples of homogenized anterior content using the DNeasy® mericon food kit (Qiagen, California, USA) following the manufacturer’s short fragment recovery
When a total of 800 mg could not be recovered from the anterior content, a combination of anterior and posterior content was used. Extraction negative controls were systematically incorporated during extractions.

**PCR – Sanger Sequencing**

Polymerase chain reactions (PCRs) for Sanger sequencing of sub-sampled tissues were conducted using universal DNA barcode primers. Primers FishF1 – 5’ TCAACCAACCACAAAGACATTGGCAC 3’ and Fish R1 – TAGACTTCTGGGTGGCAGAATCG 3’ were used to target a 655 base pair (bp) region of the cytochrome c oxidase subunit I (COI) in fishes (Ward et al. 2005). The COI PCR was conducted in 25 μL reaction volumes with 1 μL template DNA and the following reagent concentrations: 1X GoTaq polymerase buffer, 0.4 μM of each primer, 0.8 mM dNTP’s, 2.0 mM Mg²⁺, and 0.025 U/μL of GoTaq polymerase. Optimized temperature cycling conditions for PCRs were an initial denaturation at 94°C for two minutes followed by 32 cycles of 94°C for 45 seconds, 51°C for 30 seconds, and 72°C for 45 seconds, followed by a final extension at 72°C for seven minutes and 30 seconds then held at 4°C. A PCR negative control was included in all amplifications. Sanger sequencing was conducted on an ABI 3730XL at Eurofins MWG Operon (Louisville, KY, USA). Sequences were visually inspected and analyzed with the CodonCode Aligner software (Dedham, MA, USA) and compared to publically available DNA sequences in GenBank using the basic local alignment search tool (BLAST; Altschul et al. 1990).

**PCR – DNA Metabarcoding**

A vertebrate-specific primer set targeting a 106 base pair (bp) segment of the mitochondrial genome coding the 12s ribosomal RNA gene was used for DNA metabarcoding (Riaz et al. 2011).
Eight forward and 12 reverse primers with internal sequence tags (Table S1) were generated following the approach described in Glenn et al. (2016) to preserve the ability to assign sequence reads back to individual lamprey specimens. The PCR reactions were conducted in 25 μL reaction volumes with 5 μL template DNA and the following reagent concentrations: 1X Go Taq polymerase buffer, 0.4 μM of each primer, 0.8 mM dNTP’s, 2 mM Mg²⁺, 10 ug/mL of bovine serum albumin (BSA), and 0.025 U/μL of Go Taq polymerase. We adjusted original PCR conditions described in Kelly et al. (2014) to optimize amplifications. The optimized PCR conditions were an initial denaturation at 95°C for five minutes followed by 28 cycles of 95°C for 30 seconds, 57°C for 30 seconds, and 72°C for 30 seconds. A PCR negative control was included in all amplifications.

Indexed PCR products were combined into four pools that contained samples with a unique combination of indexes, one randomly selected DNA extraction negative control, and one PCR negative control. Pooled libraries were multiplexed and 150PE sequenced on an Illumina MiSeq. The run included 10% PhiX DNA spike-in control to improve the data quality of low-diversity samples.

**Bioinformatics**

Initial performance of the MiSeq run was evaluated with FastQC v.0.11.5 (Andrews 2010). Individual sequencing reads were demultiplexed using BBDuk within BBTools package (J. Bushnell, Joint Genome Institute, unpublished data) and a modified PERL script by Eric Collins (University of Alaska Fairbanks, https://github.com/rec3141/demult/blob/master/run-bbduk.sh) allowing no mismatches per barcode. Primers were trimmed from demultiplexed reads using CUTADAPT v.1.12 (Martin 2011). Sequencing reads that contained no primer, contained greater than 10% error rates (> 1 primer mismatch), or fell outside of the target read length (96 – 116 bp)
were discarded. Paired-end reads were merged with a minimum overlap of 30 bp using PEAR v.0.9.6 (Zhang et al. 2014). Trimmed and merged reads were then run through a VESEARCH v.2.4.0 (Rognes et al. 2016) pipeline that included (1) de novo chimera checking, (2) dereplicating 100% identical sequences, and (3) clustering sequences at a ≥ 96% similarity threshold into operational taxonomic units (OTUs). To exclude sequencing noise and/or artifacts, dereplicated sequences assigned to individual samples were removed from downstream analyses using VSEARCH. Dereplicated sequences were classified as noise and/or artifacts when a sequence occurred a fewer number of times in an individual sample when compared to the frequency with which it occurred in DNA and PCR negative controls.

A custom BLAST database of complete mitochondrial fish genomes was generated for this study using the downloaded fish genome files that were compiled in the Mitochondrial Genome Database of Fish (MitoFish; Iwasaki et al. 2013). The database was created using the ‘makeblastdb’ option within BLAST+ v.2.6.0 (Camacho et al. 2009) and contained 2,148 unique fish mitochondrial genome sequences.

Final OTU sequences were queried against the custom BLAST database using the command-line tool ‘blastn’ within BLAST+. Search parameters specified an e-value threshold of 10^-5, 90% or greater sequence identity, and a maximum retention of 10 sequence alignments. BLAST files were imported into MEGAN (Huson et al. 2016) to visualize taxonomic assignments using customized least common ancestor (LCA) parameters (min score = 100, top percent = 8, min support = 1) and the LCA algorithm weighted at 80%. A sequence similarity of ≥ 98% was considered to be a species level match. Otherwise, OTUs were assigned to the highest taxonomic classification that encompassed all significant matches. The final taxonomic incidence table (e.g.,
presence/absence) contained all individual samples and was exported from MEGAN for subsequent analyses.

**Statistical Analysis**

Rarefaction analysis and the Chao2 species richness estimator was calculated to assess the effect of sample size on the number of detected host species and estimate the number of additional samples needed to fully describe the diet components of Arctic lamprey. Sample-based estimates and 95% confidence intervals were calculated in EstimateS v.9.1.0 (Colwell 2013) using 1,000 sample-order randomizations.

Multivariate statistical analyses were conducted using the VEGAN package (Oksanen et al. 2013) in R (R Core Team 2013). The incidence table was used to generate distance matrices among samples using the Jaccard distance measure. Permutational Multivariate Analysis of Variance using distance matrices (PERMANOVA) was run with 999 permutations using the R-VEGAN function ADONIS to examine the statistical significance and percentage of dietary variation that could be explained by collection year, station sites where Arctic lamprey were captured, and total length. For total length analyses, lampreys were grouped into 12 size-class intervals of 25 mm, which corresponded to 1-inch length measurements. Nonmetric multidimensional scaling (NMDS) plots were used to visually investigate patterns in the diets of individuals for each of the above factors. Finally, binomial general linear mixed models (GLMMs) were used to test whether the presence/absence of a prey species in the gut contents of lamprey was significantly related to the biomass of prey species in trawl catches at station sites surveyed in 2014 and 2015. Separate GLMMs were conducted for the 6 most common prey items using the lme4 package in R. In each model, the variable ‘station’ was included as a random effect to account for multiple resamples.
Results

Intestinal contents were recovered from all Arctic lamprey \((N = 250)\). The \(L_T\) of examined specimens ranged from 187 – 465 mm, while \(W_T\) ranged from 8.0 – 192.1 g. Recovered diagnostic structures included eggs, fins and/or fin rays, internal organs, otoliths, scales, vertebrae, and uncategorized bone fragments. Diagnostic structures were recovered from 103 (84\%) and 112 (88\%) intestinal tracts in 2014 and 2015, respectively. Fins and/or fin rays were the most abundant structure in both years, while otoliths were the most infrequent structures (Fig. 2).

Sanger Sequencing

Genomic DNA from 28 of the 61 (46\%) tissue samples was successfully amplified by PCR. Of those successful amplifications, 27 sequences were taxonomically identified to species based on the criteria of \(\geq 98\%\) sequence similarity to publicly available sequences in the NCBI database. Eight species were detected in 2014 and 2015, respectively (Table 1). Two species, Chinook salmon and yellowfin sole \((Limanda aspera)\), were detected only from samples collected in 2014. One species, pink salmon \((O. gorbuscha)\), was detected only in 2015 samples. The remaining five species \([walleye pollock \((Gadus chalcogrammus)\), capelin \((Mallotus villosus)\), Pacific herring \((Clupea pallasii)\), Pacific sand lance \((Ammodytes hexapterus)\), and saffron cod \((Eleginus gracilis)\)] were detected in samples from both study years.

DNA Metabarcoding

The high throughput sequencing run produced 21,590,316 raw reads of which 18,862,344 were assigned back to unique index tags. A small proportion of the reads (0.1\%) were assigned to one of the eight negative control samples; however, no sequencing reads remained in the negative control samples after the filtering process. A total of 7,557,159 high quality reads (Phred score \(\geq\)
Q38) were demultiplexed among 219 (88%) samples and used in downstream analyses. The OTU clustering approach implemented in VSEARCH delineated 261 OTUs in the intestinal contents. All OTUs were identified to the level of taxonomic family, genus, or species. The sample-based rarefaction curve appeared to reach a plateau, which indicated that the number of sampled individuals provided an adequate representation of species in the diet of Arctic lamprey in the eastern Bering Sea. However, the Chao2 estimator suggested that the dietary extent of Arctic lamprey had not been fully described (Fig S1).

A total of 10 ray-finned fish taxa were detected. These taxa were comprised of eight orders, with four taxa taxonomically identified to family, one identified to genus, and five identified to species (Table 2). Capelin, Pacific herring, Pacific sand lance, and Gadidae occurred most frequently in the diet of Arctic lamprey (Table 2). Capelin and Pacific herring were the dominant taxa for 2014 and 2015, respectively (Fig. 3). The number of taxa detected within individual Arctic lamprey intestinal contents ranged from one (66%) to four (0.5%), but two and three taxa were also observed within individual gut contents (27% and 6%, respectively).

Only 27 of 61 (44%) tissue samples yielded high quality sequences. All taxonomic groups that were identified by sequencing a 655 bp region of COI from sub-sampled tissues were represented in the high-throughput final sequence library. However, three taxonomic groups [daubed shanny (Leptoclinus maculatus), sculpins (Cottidae), and sticklebacks (Gasterosteidae)] were only detected in the high-throughput dataset. Identical taxa were detected by both methods in 20 of 27 (74%) samples. The DNA metabarcoding approach detected more than one taxonomic group in 10 of 27 (37%) individual gut contents.

Statistical analysis
The diets of Arctic lamprey were significantly different between collection years (ADONIS: $R^2 = 0.011$, $P = 0.009$) and among the 12 size classes (ADONIS, $R^2 = 0.037$; $P = 0.020$), but each factor accounted for only a small proportion of diet variability. Diets of individual Arctic lamprey were also significantly different among station sites and accounted for a moderate proportion of diet variability (ADONIS: $R^2 = 0.487$, $P = 0.001$). Although NMD scaling produced clustering in a two-dimensional plot and provided a good representation of the data (Kruskal’s stress value = 0.04), visual inspections of each plot did not reveal obvious patterns or clusters solely represented by individuals belonging to different spatial groups (Fig. 4). Of the 6 GLMMs, the presence/absence of Pacific sand lance in the gut contents of Arctic lamprey was significantly associated with the biomass of Pacific sand lance in trawl catches at station sites ($z = 2.37$; $P = 0.01$; Table S2). However, the presence/absence of a species in Arctic lamprey diets was not related to biomass for the remaining 5 species examined.

**Discussion**

This study is the first to implement gene-based identification of lamprey diet composition. Results from DNA metabarcoding detected frequent occurrences of capelin, Pacific herring, Pacific sand lance, and gadids in Arctic lamprey diets. Of the taxa detected in this study, capelin and Pacific herring had the highest frequency of occurrence among samples. This supports previous reports of lamprey predation of clupeids and osmerids (Nikol’skii 1956; Maitland et al. 1984; Beamish and Williams 1976; Beamish 1980; Beamish and Neville 1995; Siwicke 2014). While our results provided further support that forage fishes are a common prey item for Arctic lamprey, these results also indicated notable differences in Arctic lamprey diet when compared to previous studies. The relative importance of Pacific salmon as prey for Arctic lamprey was not reflected in the
results of this study. Observations of lamprey wounds on Pacific salmon have been widely reported (Beamish and Youson 1987; Heard 1966; Beamish and Neville 1995; Novomodnyy and Belyaev 2002; Shevlyakov and Parensky 2010; Siwicke 2014). As a result, many researchers have concluded that flesh-feeding lampreys are predators of Pacific salmon and that has led to speculation about the impact of lamprey predation on salmon stocks (Beamish and Youson 1987; Beamish and Neville 1995; Novomodnyy and Belyaev 2002; Shevlyakov and Parensky 2010). Surprisingly, the overall contribution of Pacific salmon in the diet of Arctic lamprey in the eastern Bering Sea was much lower than previous studies have suggested with DNA detected in only 3.5% of samples. The results of previous studies may reflect biased reports of lamprey wounds on a frequently encountered taxon of economic importance. However, limited spatial and temporal sampling efforts in this study may not fully capture annual and/or geographic variability in the diet of Arctic lamprey because sampling efforts were limited to a three-week period in the fall for both years. Further, the location of trawl sites may not have been in close enough proximity to major Alaskan estuaries where lamprey predation on juvenile Pacific salmon has primarily been reported. Additional sampling efforts are necessary to evaluate if the trends reported in this study are consistent among seasons and throughout the range of Arctic lamprey.

While this study found minor contribution of Pacific salmon, five species (capelin, Pacific sand lance, walleye pollock, daubed shanny, and yellowfin sole) and one family (Cottidae) are reported for the first time in the diet of Arctic lamprey. Interestingly, capelin, Pacific sand lance, and gadids had a relatively high occurrence indicating the importance of these species as prey for Arctic lamprey. Although daubed shanny, yellowfin sole, and Cottidae were reported in Arctic lamprey diets for the first time, DNA was detected in less than 1% of samples. This suggests the
remaining taxa are an infrequent prey for Arctic lamprey in the eastern Bering Sea. Overall, these results highlighted the utility of DNA metabarcoding to support previous observations, reveal unreported dietary components, and detect rare predation events.

A number of species previously reported as prey for Arctic lamprey were not detected in this study. This may be largely influenced by different assemblages of host communities in freshwater lakes and rivers where these predation events were documented. Within the Great Slave Lake, Northwest Territories and the Naknek River system, Alaska, populations of freshwater-resident, parasitic Arctic lamprey have been documented feeding on whitefishes and ciscoes (*Coregonus* spp.), lake trout (*Salvelinus namaycush*), rainbow trout (*O. mykiss*), pygmy whitefish (*Prosopium coulterii*), and threespine stickleback (*Gasterosteus aculeatus*; Heard 1966; Nursall and Buchwald 1972). Although the focus of this study was on anadromous Arctic lamprey predation in marine waters, these observations highlight the diet variability within a single lamprey species.

The presence of hard structures and tissue masses within Arctic lamprey intestinal tracts are indicative of a flesh-feeding approach. The feeding mode of parasitic lampreys has been hypothesized to be determined by morphological structure of the dentition and size of the oral disc (Potter and Hilliard 1987; Renaud et al. 2009). Previous studies that examined morphological differences among lamprey oral discs classified Arctic lamprey as a flesh-feeding species. However, this conclusion was inferred by morphological similarities to known flesh-feeding species and not visual examinations of intestinal contents (Potter and Hilliard 1987; Renaud et al. 2009). Although the occurrence of hard structures and internal organs in intestinal tracts have been widely documented for other flesh-feeding lamprey species (reviewed in Hardisty and Potter 1971; Maitland et al. 1984; Beamish and Williams 1976; Beamish 1980; Renaud et al. 2009), these results
are the first to visually confirm tissue masses, internal organs, and undigested hard structures in the intestinal tracts of Arctic lamprey. The frequency and type of undigested hard structures observed within the gut contents offer limited insight into the specific feeding behavior(s) of this species because skeletal structures may be ingested during predation or scavenging events. If Arctic lamprey predominantly exhibits predatory behavior, the frequency with which vital skeletal structures and organs were observed suggests that prey attacked in the eastern Bering Sea sustain high mortality rates. Ultimately, the study results supported the hypothesis that Arctic lamprey is a flesh-feeding species; however, without direct observations, the feeding modality of marine-phase Arctic lamprey remains unknown.

The observation of entire gastrointestinal tracts within the gut contents of Arctic lamprey increased the probability of detecting signals of secondary predation (i.e., prey of prey) in this study. Secondary predation has been documented in other dietary studies, and is recognized as a limitation of DNA metabarcoding (Deagle et al. 2009; O’Rorke et al. 2012; Bowser et al. 2013; De Barba et al. 2014; Pinol et al. 2014). Many of the prey species identified in this study have multifaceted roles as both predator and prey in the Bering Sea (Robards and Piatt 1999; Sturdevant et al. 2000; Hjermann et al. 2004; Godiksen et al. 2006; Davis et al. 2009). For example, both pink salmon (O. gorbuscha) and Chinook salmon consume juvenile walleye pollock, Pacific herring, and Pacific sand lance (Davis et al. 2009). Therefore, identification of multiple taxa within intestinal tracts could be instances of secondary predation and may be heavily influencing the results of this study. The four taxa with the highest frequency of occurrence were detected both individually and with other prey items, further confounding inferences of secondary predation. Ultimately, the ambiguity of Arctic lamprey feeding behavior and food-web interconnectivities in the eastern
Bering Sea highlights a limitation of DNA metabarcoding. Without direct visual observations of feeding behaviors under natural conditions, key aspects of the feeding ecology of Arctic lamprey remain speculative. The use of DNA metabarcoding in combination with visual observations and stable-isotope techniques may circumvent the limitations of any one technique and provide additional insight into the trophic position of Arctic lampreys relative to other fish species in the Bering Sea ecosystem.

Predator sample sizes were large enough to examine potential biological, temporal, and seasonal variability in Arctic lamprey diet composition. Although both biological and temporal variables were significant, they explained only a small portion of the differences between diets (1 and 4%, respectively). Of the factors examined, station site (i.e., spatial) explained the greatest proportion of diet variability (49%). Similarly, diet inferred from stable isotopes identified regional differences among invasive sea lamprey (*Petromyzon marinus*) in Lake Superior (Harvey et al. 2008). In the eastern Bering Sea, overlapping patterns of abundance inferred from catch-per-unit effort (CPUE) data have shown co-occurrence between Arctic lamprey and Pacific herring, capelin, and juvenile Pacific salmon (Siwicke 2014). Siwicke (2014) concluded these species were important prey items for Arctic lamprey, and hypothesized that occurrences of these species influenced the distribution of Arctic lamprey in this region. While this study confirmed the importance of Pacific herring and capelin in the diet of Arctic lamprey, the underlying mechanisms driving spatial diet variability among Arctic lamprey remain speculative. Except for Pacific sand lance, the presence/absence of a prey species in the diet of Arctic lamprey was not correlated with the biomass of that prey species in trawl catches. This suggests that consumption of frequent prey items identified in this study is not solely opportunistic. Arctic lamprey may be exhibiting selective
feeding and targeted these taxa regardless of localized abundance. Although studies in regions of
the Laurentian Great Lakes concluded sea lamprey wounds were most frequently observed on fish
species with the highest seasonal abundance, recent studies have documented selective feeding on
specific prey over more abundant species for both invasive sea lamprey and river lamprey (Harvey
et al. 2008; Inger et al. 2010). Thus, selective feeding and shifting distributions of preferred prey
may be underlying mechanisms that explain the diet variability among Arctic lamprey at different
station sites.

Both gene-based identification techniques produced similar dietary results, however, there
were discrepancies and biases associated with each approach. Species identification using DNA
metabarcoding and the COI gene fragment detected identical taxonomic groups for 74% of
individuals. Additional taxa were detected with DNA metabarcoding in 37% of individuals,
suggesting that the tissue samples that could be analyzed with the COI gene fragment did not fully
capture the dietary variability within individual lampreys. This also proved to be a limitation of
DNA metabarcoding. Samples where different taxonomic groups were identified with different
methods indicated that all taxa within individual intestinal tracts were not always detected. This
may be an unintended result of isolating DNA from only the anterior portion of intestinal contents.
When sampling predator feces for prey DNA, different meals were reflected depending on which
portion of the feces were sampled (Deagle et al. 2005). Thus, additional species within individual
Arctic lamprey may not have been detected by excluding the posterior content during DNA
homogenization and isolation.

The taxonomic resolution of the DNA metabarcoding primer set was unable to discriminate
among sequences from fishes in the families Cottidae, Gadidae, Gasterosteidae, and Pleuronectidae
and the genus *Oncorhynchus*. However, the fine-scale taxonomic resolution of the COI gene fragment provided additional trophic insight. Sequenced tissue samples identified two species within the families Gadidae (walleye pollock and saffron cod) two species within the genus *Oncorhynchus* (pink and Chinook salmon). While it cannot be confirmed that the remaining Gadidae and Pacific salmon sequences were those specific species, it provided additional taxonomic resolution to the trophic dataset. Ultimately, the large sample size and little taxonomic variability observed in this study characterized the dietary variability of Arctic lampreys in the eastern Bering Sea.

We improved our understanding of the food habits of Arctic lamprey in the eastern Bering Sea. Indeed, visual observations of intestinal contents confirmed the flesh-feeding approach of this species (Potter and Hilliard 1987; Renaud et al. 2009), while the DNA metabarcoding approach identified the importance of pelagic schooling fishes in the diet. However, additional study is needed to investigate if diet varies by season as well as throughout their geographic distribution. While this approach cannot explicitly ascertain the feeding behavior modality or ‘secondary predation’, it provided insights into the food-web dynamics in the eastern Bering Sea and the need for additional observations of lamprey feeding behavior under both environmental and laboratory conditions. Finally, the term ‘parasitic’ has been used to describe fishes that consume tissue and/or internal fluids of a host species without killing their host (Elliott et al. 2002). While this description may apply to some lamprey species, the frequent occurrence of hard structures and diagnostic tissues observed in this study suggest Arctic lamprey exhibit a predatory feeding behavior, and the term ‘parasitic’ should not be used as a generalization to describe the feeding ecology of all lampreys.
Acknowledgements

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TABLE 1. Comparison of taxonomic assignments from select tissue samples (Sanger sequencing) and whole gut contents (DNA metabarcoding). One tissue type was recovered per intestinal tract. Bold taxa correspond to agreement in the taxonomic assignments.

<table>
<thead>
<tr>
<th>SANGER SEQUENCING</th>
<th>WHOLE GUT DNA METABARCODING</th>
</tr>
</thead>
<tbody>
<tr>
<td>Year</td>
<td>Tissue type</td>
</tr>
<tr>
<td>2014</td>
<td>Flesh</td>
</tr>
<tr>
<td>2014</td>
<td>Flesh</td>
</tr>
<tr>
<td>2014</td>
<td>Organ</td>
</tr>
<tr>
<td>2014</td>
<td>Flesh</td>
</tr>
<tr>
<td>2014</td>
<td>Flesh</td>
</tr>
<tr>
<td>2014</td>
<td>Flesh</td>
</tr>
<tr>
<td>2014</td>
<td>GI tract</td>
</tr>
<tr>
<td>2014</td>
<td>Pyloric caeca</td>
</tr>
<tr>
<td>2014</td>
<td>Flesh</td>
</tr>
<tr>
<td>2015</td>
<td>GI tract</td>
</tr>
<tr>
<td>2015</td>
<td>Pyloric caeca</td>
</tr>
<tr>
<td>2015</td>
<td>Organ</td>
</tr>
<tr>
<td>2015</td>
<td>Pyloric caeca</td>
</tr>
<tr>
<td>2015</td>
<td>Organ</td>
</tr>
<tr>
<td>2015</td>
<td>Flesh</td>
</tr>
<tr>
<td>2015</td>
<td>Organ</td>
</tr>
<tr>
<td>2015</td>
<td>Flesh</td>
</tr>
<tr>
<td>2015</td>
<td>Flesh</td>
</tr>
<tr>
<td>2015</td>
<td>Flesh</td>
</tr>
<tr>
<td>2015</td>
<td>Organ</td>
</tr>
<tr>
<td>2015</td>
<td>Organ</td>
</tr>
<tr>
<td>2015</td>
<td>Flesh</td>
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<tr>
<td>2015</td>
<td>Flesh</td>
</tr>
<tr>
<td>2015</td>
<td>Flesh</td>
</tr>
<tr>
<td>Year</td>
<td>Location</td>
</tr>
<tr>
<td>------</td>
<td>----------</td>
</tr>
<tr>
<td>2015</td>
<td>Flesh</td>
</tr>
<tr>
<td>2015</td>
<td>Flesh</td>
</tr>
<tr>
<td>2015</td>
<td>GI tract</td>
</tr>
</tbody>
</table>
TABLE 2. Taxonomic assignment of prey items found in Arctic lamprey intestinal contents. 
Species-level assignments were based on criteria of > 98% sequence similarity to sequences in the reference database. Taxa in bold occurred in ≥15% of the gut contents.

<table>
<thead>
<tr>
<th>Order</th>
<th>Family</th>
<th>Genus</th>
<th>Species</th>
<th>Frequency of occurrence (%)</th>
<th>Total number of samples (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clupeiformes</td>
<td>Clupeidae</td>
<td>Clupea</td>
<td><em>pallasii</em></td>
<td>25.0</td>
<td>78</td>
</tr>
<tr>
<td>Gadiformes</td>
<td>Gadidae</td>
<td></td>
<td></td>
<td>16.0</td>
<td>50</td>
</tr>
<tr>
<td>Gasterosteiformes</td>
<td>Gasterosteidae</td>
<td></td>
<td></td>
<td>0.3</td>
<td>1</td>
</tr>
<tr>
<td>Osmeriformes</td>
<td>Osmeridae</td>
<td>Osmerus</td>
<td><em>dentex</em></td>
<td>1.6</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Mallotus</em></td>
<td><em>villosus</em></td>
<td>30.8</td>
</tr>
<tr>
<td>Perciformes</td>
<td>Ammodytidae</td>
<td>Ammodytes</td>
<td><em>hexapterus</em></td>
<td>21.8</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>Stichaeidae</td>
<td>Leptoclinus</td>
<td><em>maculatus</em></td>
<td>0.3</td>
<td>1</td>
</tr>
<tr>
<td>Pleuronectiformes</td>
<td>Pleuronectidae</td>
<td></td>
<td></td>
<td>0.3</td>
<td>1</td>
</tr>
<tr>
<td>Salmoniformes</td>
<td>Salmonidae</td>
<td>Oncorhynchus</td>
<td>spp.</td>
<td>3.5</td>
<td>11</td>
</tr>
<tr>
<td>Scorpaeniformes</td>
<td>Cottidae</td>
<td></td>
<td></td>
<td>0.3</td>
<td>1</td>
</tr>
</tbody>
</table>
Figure 1. Map of sample sites in the eastern Bering Sea in 2014 (black) and 2015 (gray). Each circle denotes a station where surface trawls were conducted. The diameter of each circle represents the number of lampreys captured at each locality.

257x203mm (300 x 300 DPI)
Figure 2. Number of individuals with diagnostic hard structures and tissues within intestinal tracts of Arctic lamprey. The percentages above each column are the frequency of occurrence (e.g., presence/absence) relative to the number of individual lamprey for both collection years combined.

168x146mm (300 x 300 DPI)
Figure 3. The cumulative frequency with which prey taxa were detected within Arctic lamprey diets by sampling year and overall.

388x439mm (300 x 300 DPI)
Figure 4. Nonmetric Multidimensional Scaling (NMDS) ordination constructed from a Jaccard matrix of diet dissimilarities. Station sites (N = 34) are represented by grey scale. Loading vectors of significant prey types \( (p \leq 0.05) \) are displayed in the ordination.