ASSESSMENT AND APPLICATION OF DNA METABARCODING FOR CHARACTERIZING ARCTIC SHOREBIRD CHICK DIETS

By

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ABSTRACT

Climate change in the Arctic is affecting the emergence timing of arthropods used as food by nesting shorebirds and their young. Characterizing the diets of shorebird young is a prerequisite to evaluate the potential for asynchrony to occur between the timing of arthropod emergence and when shorebird young hatch, an example of trophic mismatch. In this study, DNA metabarcoding was used to identify arthropod remains in feces collected from wild-caught Red Phalarope (*Phalaropus fulicarius*), Pectoral Sandpiper (*Calidris melanotos*), and Dunlin (*Calidris alpina*), young in Utqiaġvik, Alaska between 2014 and 2016. Arthropod specimens were collected at the field site to generate DNA reference sequences from potential prey items. The newly generated sequences in combination with publicly available sequences served as a reference set for species determinations. I assessed the ability of two mitochondrial markers (CO1 and 16s) to detect arthropods in the feces of captive pre-fledged young in controlled feeding experiments. After combining information from both markers, experimental prey taxa were detected in chick feces 82-100% of the time, except for Trichoptera which was never detected. I used the same strategy to characterize the diets of wild-caught shorebird young. The technique detected nearly all prey families documented in historical gut content analyses, as well as 17 novel families. Some of the novel prey diversity may be the result of detecting the prey of prey, known as secondary consumption. We observed that the diets of shorebird young shifted over the course of a summer. Changes in diet generally reflected arthropod composition in the environment estimated from collection of arthropods in pitfall traps. Evidence of diet flexibility by shorebird young suggests that chicks can shift their diets to take advantage of intra-seasonal changes in prey availability. Here, I provide an evaluation and application of DNA metabarcoding to characterize prey resource use by shorebird young for assessing the presence and impacts of trophic mismatch.

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GENERAL INTRODUCTION

High latitude regions in the Northern Hemisphere are experiencing increasing temperatures at higher rates compared to those of lower latitudes, resulting in dramatic changes in the Arctic (Serreze and Barry 2011). Landscape scale shifts on the Arctic Coastal Plain such as loss of snow (Høye et al. 2007), changes in wetlands (Andresen & Lougheed 2015, Liljedahl et al. 2016), and shifts in primary production are underway (Bhatt et al. 2010, Boelman et al. 2015). An ultimate northward shift of the climatic zones is predicted (Walker et al. 2005, 2008).

It is important to study how species seasonally dependent on the Arctic are subject to and respond to these changes. Shorebirds converge from five major flyways including the Pacific, Atlantic, Central, East-Asian Australasian, and Central Pacific (Alaska Shorebird Group 2008) to nest in the Alaskan Arctic where they occur in higher densities than anywhere in North America (Bart et al. 2013). Shorebirds as a group are experiencing worldwide declines. Of the world's 200 shorebird species, 48% are declining (International Wader Study Group 2003). Whether Arctic breeding shorebird declines are linked to forces such as anthropogenic related habitat loss in key stop-over areas (Piersma et al. 2016), hunting pressure in wintering areas (Morrison et al. 2012), or impacts of climate shifts on breeding grounds remains to be determined (Wauchope et al. 2017).

The widespread declines in shorebird populations and expected climate change effects make it urgent to establish any possible connections. There is evidence that shorebird chick growth and survival rates in the Arctic are affected by the availability of prey (McKinnon et al. 2012, Gils et al. 2016, Senner et al. 2017). Rapid climatic change could impact shorebird breeding success in the Arctic if asynchrony between the timing of optimal foraging and chick hatching adversely affects recruitment of offspring. To address this issue requires a thorough characterization of shorebird chick diet. In this thesis, I characterized the diets of wild-caught Red Phalarope, Pectoral Sandpiper and Dunlin young in Utqiagvik, Alaska by identifying remnant prey DNA in feces using a DNA metabarcoding technique. I then used diet information to understand whether pitfall traps are an accurate measure of food available for shorebird young in the environment. The diet information that I produced can be used to assess impacts of climate induced change in the timing and abundance of prey on the growth and survival of shorebird young.

To assess the effectiveness of DNA metabarcoding for chick diet characterization (Valentini et al. 2008, Yoccoz 2012), I carried out an experiential study with captive pre-fledged shorebird young. My aims were to understand if all prey taxa were detected in feces equally following consumption of prey by the chick and if there were biases of technical or biological origin that influenced the detection of prey DNA in feces. The following is a synthesis of relevant background information pertaining to the study species, climate change impacts on shorebirds and the use of environmental DNA for studying bird diets.

Shorebird species under investigation

All three shorebird species in this investigation have experienced recent downward population trends (Andres et al. 2016). The Alaska and Canada subpopulation of Red Phalarope have experienced apparent declines in the last 10 years while Pectoral Sandpipers have experienced significant declines in the last 30 years based on analyses of changes at individual Arctic sites (Andres et al. 2012, B.J. McCaffery, unpubl. data). The *arcticola* subspecies of Dunlin have experienced significant declines in the last 30 years based on changes at individual Arctic study sites. Additionally, they have experienced lower survival (Weiser et al. 2018), and reduced counts in wintering areas (Andres et al. 2012, Andres 2016). It is necessary to evaluate threats to shorebirds on breeding grounds to identify whether climate-related factors are affecting the size of Arctic shorebird populations.

Trophic mismatch

The match/mismatch concept suggests that recruitment for the predator will be high when the most energy costly part of predator breeding is coupled with the peak availability of prey (Durant et al. 2007). This hypothesis has been supported for a handful of bird species including Pied Flycatchers *Ficedula hypoleuca* (Both et al. 2006, Visser et al. 2012). There is no strong evidence yet linking trophic mismatch to large scale population declines for birds (Moller et al. 2008, Dunn and Møller 2014); however, some evidence suggests that this may become increasingly important as the climate continues to warm (Pearce-Higgins et al. 2005, Saalfeld and Lanctot 2017).

During the condensed Arctic summer shorebirds need to establish territories, compete for mates, and carry out subsequent breeding activities. The timing of these events is crucial for

shorebird breeding success, in part, because shorebirds are primarily income breeders (Klaassen et al. 2001), and so rely on the abundance of arthropod prey for egg production (Ruthrauff and McCaffery 2005), and chick growth (McKinnon et al. 2012). Arthropod emergence is temperature-dependent and has been advancing with earlier snow melt (Bolduc et al. 2013, Tulp & Schekkerman 2008, Saalfeld et al. in review). Shorebirds undergo physiological changes, controlled by endogenous and photoperiod cues prior to reaching the Arctic to breed (Piersma et al. 2008). The extent to which shorebirds are physiologically and behaviorally flexible in adjusting to the rapidly advancing snowmelt in the Arctic remains to be determined (Picotin 2007, Grabowski et al. 2013, Liebezeit et al. 2014). The response of Arctic breeding shorebirds to advancing snowmelt may not be consistent among species. Evidence suggests that species with an opportunistic settlement strategy may be advancing nest initiation with snowmelt while those with a conservative settlement pattern are keeping pace less well (Saalfeld and Lanctot 2017).

Environmental DNA

Environmental DNA (eDNA) techniques involve screening environmental samples such as water, soil, feces, etc. to detect evidence of species presence through DNA sequence-based identification. These methods have been growing in application for ecological research since first implemented to study microorganisms in the environment in the 1980s (Ogram et al. 1987). Since then, a number of applications for the technique have been developed such as: food web mapping, biodiversity inventory, tracking changes in species distributions, invasive species monitoring, and investigations of ancient life (Yoccoz 2012, Thomsen and Willerslev 2015, Barnes and Turner 2016). Many eDNA techniques rely on so called DNA barcoding. DNA barcoding targets standardized relatively conserved regions of the genome to delineate taxon based on unique genetic signatures commonly known as 'barcodes'. Although sequencing technology continues to improve and polymerase chain reaction (PCR) free methods are under development (Mason et al. 2011, Liu et al. 2015), current DNA barcoding approaches rely on initial amplification of DNA using PCR. Universal primers PCR amplify multiple species simultaneously by targeting annealing sites that are conserved broadly across lineages. The use of universal primers combined with high-throughput sequencing is known as DNA metabarcoding. In contrast to earlier DNA sequencing technology, high-throughput sequencing

is carried out in a parallel fashion allowing for fast and efficient generation of large and complex datasets. DNA metabarcoding methods are under development to improve recovery of degraded DNA (Claassen et al. 2013, Song et al. 2016, McInnes et al. 2017), develop universal primers to target different groups of organisms (Clarke et al. 2014b, Mong et al. 2015, Cannon et al. 2016, Elbrecht et al. 2017), increase the ease of sequencing (Clarke et al. 2014a, Glenn et al. 2016), and improve accuracy of species identification (Somervuo et al. 2017).

DNA sequence-based analyses offer important advantages over existing diet characterization methods. As an example, for fecal DNA metabarcoding techniques the DNA present in feces is used to identify prey species consumed by the predator. Fecal DNA metabarcoding is non-lethal so allows for re-sampling of individuals, which is ideal for studies of species with a sensitive conservation status. The technique has the potential to reduce the costs of diet studies (Shokralla et al. 2015, Wallinger et al. 2017), and to improve detection efficiency and taxonomic resolution of prey taxa. Diet analyses that rely on identifying partly digested remains of prey from the gastrointestinal tract nearly always include a category of unidentifiable prey (Moreby 1988, Tsipoura and Burger 1999). Diagnostic hard parts are not always recoverable or available from all taxa. This can result in skewed estimates of prey items in the diet (Jenni et al. 1990, Kohn and Wayne 1997, Barrett et al. 2007, Clare et al. 2009). Other techniques for assessing diet such as stable isotopes provide a great deal of information about diet such as assimilation of prey over long time scales and trophic feeding relationships. Stable isotope analysis uses isotopic ratios of the predator that can be compared to broad categories of prey. This approach requires contrasting signatures for potential source foods that are not always available and taxonomic resolution is limited (Hood-Nowotny and Knols 2007). Similarly, diet analyses of fatty acids provide information about broad categories of prey that have unique profiles and are assimilated in the predator's tissues (Traugott et al. 2013). This technique has the benefit of being quantitative however, the method requires predator-specific coefficients for lipid metabolism that do not always exist for the prey of interest and rarely can the technique detect all diet items (Iverson et al. 2004).

Despite the growing body of knowledge on molecular diet characterization techniques, there are aspects of molecular diet characterization that have not yet been evaluated. For example, fecal DNA metabarcoding techniques may be affected by the physiology of the

predator due to differences in the digestion of prey and there may be bias introduced during the various steps in the process of attaining and identifying the prey DNA from the feces of the bird. Therefore, in the first chapter of my thesis I tested the effectiveness of a DNA metabarcoding technique to characterize shorebird chick diets by carrying out a captive study where young were experimentally fed various prey and their feces were analyzed for the presence of those prey. My objective was to feed chicks a variety of prey taxa to test for the presence of those prey in the chick's feces to determine whether prey taxa are equally detected. If prey were not detected equally, I wanted to determine whether detection bias was linked to specific factors associated with the predator, its feces, its prey or the laboratory technique.

In the second chapter of my thesis, I evaluated the diet of the young of three shorebird species using a DNA metabarcoding technique. To date, knowledge of shorebird chick diets in Utqiagvik is based on gut content analyses from the 1960s (Holmes 1966, Holmes and Pitelka 1968, S. F. MacLean unpubl.). While this diet description provides a great deal of valuable information, this representation of diet could be skewed toward prey that are more easily detected and identified. Additionally, the diets of shorebird chick diets may have changed between then and now. Previous studies have assessed how changes in the timing of arthropod emergence are related to chick growth by collecting arthropod in pitfall traps to index shorebird diets (Pearce-Higgins et al. 2005, McKinnon et al. 2013b, Senner et al. 2017). There are a number of reasons why this may not be accurate owing to factors such as prey preferences and diet shifts with age (Poulin and Lefebvre 1997, Roche et al. 2016). To test this assumption, I compared the contents of pitfall traps over a season to chick diets characterized through DNA metabarcoding to understand how well pitfall traps index chick diets. Finally, through this work I present a molecularly derived characterization of diet for the young of three species of shorebirds that nest in the Alaskan Arctic who are potentially under threat of rapid climatic shifts.

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CHAPTER 1. DNA METABARCODING DETECTION OF PREY IN FECES OF CAPTIVE SHOREBIRD YOUNG[1]

ABSTRACT

Diet characterization through DNA sequence-based identification of prey in animal feces offers potential advantages over traditional diet analysis methods. However, there are aspects of the technique that have yet to be evaluated such as the potential for detection bias among different types of prey consumed by the predator. In this study, captive shorebird young were experimentally fed field-collected invertebrate prey followed by systematic collection and analysis of their feces with DNA metabarcoding using two established mitochondrial markers (16s and CO1). Our objectives were to determine whether invertebrate prey items were always detected in feces following consumption of prey, and whether there were biological or technical factors that affected prey detection. Our results support previous findings in the literature showing that using multiple molecular markers improves the overall detection of invertebrate prey. Of the six invertebrate taxa fed to chicks, three were detected every time they were eaten (Chironomidae, Brachycera, and Plecoptera), two were detected between 82-95% of the time they were consumed (Culicidae, Dytiscidae) and one was never detected (Trichoptera). Detection of prey DNA was best predicted by the invertebrate taxon, not by the quantity of prey consumed nor the age of the chick when the fecal samples were collected. Neither fecal sample mass, presence of uric acid, nor DNA yield predicted the abundance of DNA sequences in feces. Our results suggest that DNA metabarcoding usually provides a precise characterization of the prey consumed by shorebird young. However, we detected arthropods that were not part of the experimental diet in captive chick feces. These prey items were likely the result of prey that were consumed by other prey, also known as secondary consumption. Careful consideration of differences in detectability among prey taxa and artificial diversity in the diet due to secondary consumption need to be considered when applying this technique. Future work should investigate differences in detection among prey taxa as well as address mechanisms and solutions for dealing with detection bias.

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Key words: fecal DNA metabarcoding, mitochondrial markers CO1 and 16s, captive feeding experiments, shorebird young

INTRODUCTION

Knowing the ecological requirements of species, such as diet, is essential to understand their biology and manage their populations. Characterizing diets accurately is challenging, particularly for species such as birds that may feed on a diverse assortment of food items that can include poorly known species. Traditional techniques to characterize diets rely on direct observation (Redpath et al. 2001), visual identification of prey that survives digestion (Jenni et al. 1990, Carrière et al. 1999, Tollit et al. 2003, Orłowski et al. 2015), stable isotope and fatty acid composition analyses (Yohannes et al. 2010), or crop flushing (Major 1990). Advances in DNA sequencing technology, gene sequence-based species identification and a growing body of techniques to recover DNA from the environment (water, soil, feces, etc.) offer new options to study bird diets (Symondson 2002). Molecular methods have been used to describe the diets of numerous bird species (Deagle et al. 2007, Trevelline et al. 2016, Jedlicka et al. 2017), uncovering surprising complexity in community food web structure (Wirta et al. 2015), migratory foraging patterns (Novcic et al. 2015), dietary breadth (Gerwing et al. 2016), and impacts of environmental contaminants on bird health (Crisol-martínez et al. 2016).

These studies rely on DNA sequence-based identification of species using a strategy known as DNA barcoding. DNA barcoding uses specified regions in the genome to identify species based on matches to sequences from known sources available in reference databases such as Barcode of Life (BOLD) (Ratnasingham and Hebert 2007), National Center of Biotechnology Information (NCBI), or the SILVA ribosomal RNA gene database (Quast et al. 2013). DNA metabarcoding incorporates the use of universal primers that target multiple species with the use of high-throughput sequencing technology. Fecal DNA metabarcoding is the application of barcoding to DNA isolated from fecal samples. Fecal DNA metabarcoding is ideal for studying species of a sensitive conservation status by allowing for non-lethal re-sampling of individuals over time. Additional advantages are fine scale identification of prey, elimination of observer bias, and no requirement for *a priori* knowledge of diet (Yoccoz 2012).

There remain aspects of molecular diet characterization that are not well understood and need to be addressed to evaluate conclusions drawn from molecular datasets. Researchers have

begun to examine the application of DNA metabarcoding to assess diet in invertebrates (Zaidi et al. 1999, Juen and Traugott 2006, Weber and Lundgren 2009), and vertebrates (Bowles et al. 2011, Oehm et al. 2011, De Barba et al. 2014, Alberdi et al. 2017); however, to our knowledge no published studies have tested the application of universal primers to study bird diets with captive birds. As such, there exist gaps in our understanding of the performance of the technique, which may vary by bird species due to physiology and type of prey.

There are factors of both biological and technical origin that may affect the assessment of diet using DNA metabarcoding techniques at various stages of the processes (Sheppard et al. 2005, Pompanon et al. 2012). For instance, gastrointestinal environments shift with age through changes in enzyme production (Krogdahl and Sell 1989), microbial composition (Grond et al. *in press*), and digestion efficiency (Krijgsveld 2012). This could result in differences in the degradation of prey DNA in the feces through development. Characteristics of the prey itself could affect its detection, including the size, the amount of chitinous tissue (Finke 2007), digestibility of the prey (Custer and Pitelka 1975), and mitochondrial DNA tissue densities (Deagle and Tollit 2007). In addition, the quantity of prey consumed is expected to influence detection of the prey. If more of the prey is consumed, DNA of the prey may have a better chance of surviving digestion. Birds produce and excrete variable amounts of uric acid (Levey and Duke 1992, Jenni et al. 2000). The presence of this substance in bird feces has been proposed to explain lower DNA yields for birds as compared to mammals (Jedlicka et al. 2013). PCR inhibitors are substances that are coextracted and interfere with PCR amplification of DNA (Demeke and Jenkins 2010). A greater quantity of PCR inhibitors may be isolated when starting from larger quantities of feces, thereby influencing the detection of prey DNA. Finally, technical factors such as the existence of reference sequences for a particular gene region, and differences in amplification efficiency among taxa may influence their detection (Clarke et al. 2014b, Deagle et al. 2014, Pinol et al. 2015).

We evaluated the efficacy of a DNA metabarcoding technique to characterize the diets of captive Red Phalarope (*Phalaropus fulicarius*) and Pectoral Sandpiper (*Calidris melanotos*) young using controlled diet experiments. Our objectives were to (1) determine if prey were detected equally, and (2) if prey were not detected equally, whether detection patterns could be connected to the age of the chick, attributes of the sampled fecal matter including mass, visual presence of uric acid, concentration of DNA extracted, factors associated with the prey including

taxon and quantity consumed or technical factors associated with PCR amplification. Additionally, we calculated the average number of sequences per arthropod individual fed to chicks for each taxon to explore potential differences between taxa.

METHODS

Captive Shorebird Experiments

Captive feeding experiments with shorebird young were carried out during the summer of 2015 in Utqiaġvik (formerly Barrow), Alaska. We first located nests of Red Phalaropes (*Phalaropus fulicarius*) and Pectoral Sandpipers (*Calidris melanotos*) by conducting area searches (Saalfeld and Lanctot 2015) near the village of Utqiaġvik. One egg was collected per nest and artificially incubated until hatch, at which time leg bands were attached to chicks using distinct color combinations to uniquely mark individuals. Chicks had access to heat lamps until they were old enough to self-thermoregulate. We provided food *ad libitum* including hydrated poultry and cat feed, *Tenebrio* mealworms, pyralid wax worms, boiled egg whites, pinhead crickets, bloodworms, *Drosophila*, arthropods collected locally and water as a regular diet (AZA Charadriiformes Taxon Advisory Group).

Prey items for feeding experiments were collected from terrestrial and wetland areas near Utqiaġvik using a sweep net and aquatic sampling on a daily or bi-daily basis throughout chick-rearing. Prey included adult and larval non-biting midges (Order Diptera; Family Chironomidae), adult water beetles (Order Coleoptera; Family Dytiscidae), adult flies (Suborder Brachycera; Families Anthomyiidae, Muscidae, Calliphoridae, Syrphidae), larval stone flies (Order Plecoptera), adult mosquitos (Order Diptera; Family Culicidae), larval and adult caddisflies (Order Trichoptera), spiders (Order Araneae), adult and larval crane flies (Order Diptera; Family Tipulidae) and tadpole shrimp (Order Notostraca). All invertebrates were kept either alive or dried until experiments began.

Prior to feeding experiments, chicks had a controlled diet void of experimental prey for 8 hours to avoid carryover of experimental prey in the chick's gastrointestinal tract between experiments. We chose this length of time based on prior work with Carrion Crows (*Corvus corone corone*) that found invertebrate prey were not detectable in feces beyond 4 hours after being consumed (Oehm et al. 2011). Before prey were introduced into the chick's diet, prey items were photographed for later analysis to generate a rough proxy for mass from area (cm^2)

with ImageJ software version 1.49. The quantity of the prey consumed for each taxon during a feeding trial was calculated by summing the area of all prey individuals consumed using the scaled photographs. During feeding experiments, chicks were paired because chicks needed the presence of other chicks to maintain normal feeding behaviors. Likewise, to ensure that chicks maintained normal digestion, chicks were allowed constant access to food *ad libitum* (Wang et al. 2006). Experimental prey items were introduced into the chick's holding area on labeled trays over a 20-minute window of time. Chicks were observed to determine which prey they consumed. Because the transit time from prey ingestion to initial detection in feces as well as the retention time from prey ingestion to final detection in feces are unknown for shorebird young, we collected feces for 90 minutes after introduction of experimental prey into the chick's diet. We estimated this time frame would be sufficient based on a previous report of visual detection of invertebrate prey in feces of Red Knots over a time window spanning from 37 to 75 minutes after prey consumption (Van Gils et al. 2004). We recorded the exact time of when unique prey taxa were eaten and the excretion time for all feces collected during a subset of feeding trials to generate an estimate of when prey items were detectable in shorebird chick feces post consumption. Fecal samples were placed in 1.5 ml cryovials filled with 100% ethanol kept frozen at -20°C in a cryoshipper until transferred to a -80°C degree freezer.

DNA isolation, PCR amplification and high-throughput amplicon sequencing

Prior to DNA extraction, ≤0.25 g of fecal matter was decanted of ethanol preservative, weighed, and scored for visual presence of uric acid using a qualitative estimate. If >30% of the feces appeared white then uric acid was scored as present, while feces <30% white was scored as absent of uric acid. Total genomic DNA was extracted from shorebird chick feces following the MoBio silicon-based PowerSoil DNA Isolation Kit protocol. A bead beating step was included run on a Retsch Mixer-Mill 300 at 20 Hz for 10 minutes. The sample plate was then rotated horizontally and run again for 10 minutes, as recommended by MoBio for samples that require stronger homogenization. The final elution volume in 10 mM Tris was reduced to 25 µL given the expectation of low yields. Extracts were quantified for the presence of DNA using a dsDNA high sensitivity assay kit on a Qubit 2.0 fluorometer and their relative purity assessed using a NanoDrop 1000 spectrophotometer.

To maximize the detection of prey, we combined information from two segments of the mitochondrial genome, cytochrome c oxidase subunit 1 (CO1) and 16s ribosomal RNA subunit (16s) (Elbrecht et al. 2016). The 16s primers selected amplify a wide spectrum of life including vertebrates, fungi and arthropods (Mueller 2006), while the CO1 primers selected target arthropods specifically. Primers were chosen based on their combined coverage of 11 orders of invertebrates and because they target short DNA fragments that occur in feces as the result of degradation (Deagle et al. 2006, Zeale et al. 2011). Neither primer set was reported to amplify significant quantities of bird DNA (Gerwing et al. 2016), which if present could reduce DNA amplification of the arthropod prey (Vestheim and Jarman 2008).

Extracted DNA was used for PCR-based amplification using primer pair (ZBJ-ArtF1c, ZBJ-ArtR2c) targeting segments of the CO1 gene and primer pair (16S1F-degenerate, 16S1R-degenerate) targeting 16s coding regions (Zeale et al. 2011, Deagle et al. 2007, Gerwing et al. 2016). Amplicon products were indexed using custom designed iTru fusion primers (Table 1-A.1 in Appendix 1-A). iTru fusion primers integrate locus specific primers, individual indexes, and regions of compatibility with Illumina primers (Glen et al. 2016; Figure 1-A.1 in Appendix 1-A). PCR conditions for the CO1 marker were: 6 µl of DNA template, 1X of Phusion Green Hotstart II High Fidelity master mix (Thermo Scientific), 3.5 mM Mg^{2+}, 2X of DMSO, 0.5 uM of forward and reverse primers in a 20 µl reaction volume. Temperature cycling conditions were 3 min at 98 °C, followed by 35 cycles of 30s at 98°C, 30s at 45°C, and 15s at 72°C, and a final extension for 10 min at 72°C (modified from Gerwing et al. 2016). PCR conditions for the 16s marker were: 4 µl of DNA template, 1X Phusion Green HF buffer (Thermo Scientific), 0.2 mM dNTP's, 5 mM Mg^{2+}, 1X of BSA, 0.05 U/µl of Phusion Hotstart II DNA Polymerase (Thermo Scientific), and 0.5 uM of forward and reverse iTru fusion primers in a 25 µl reaction volume. Temperature cycling conditions were: 2 min at 94C followed by 35 cycles of 30s at 94C, 30s at 58C, 45s at 68C, and a final extension for 10 min at 68C (modified from Gerwing et al. 2016). PCR products were run on 1.8% agarose gels, normalized with SequalPrep Normalization Plate Kit, pooled, and removed of non-target primer artifacts with HighPrep size-selective SPRI beads (MAGBIO). A second PCR incorporating Illumina adapters and indexes was prepared with non-diluted libraries, and dilutions of 1.5 and 3 followed by a SPRI bead clean up. Amplicons were checked for product size with quantitative PCR and on a bioanalyzer, followed by paired-end

sequencing using 300 cycles on an Illumina MiSeq platform following the manufacturer's protocol.

Bioinformatics and OTU identification

DNA sequences were demultiplexed using Mr_demuxy 1.2.0 (Cock et al. 2009). Paired-end reads were merged with Usearch 9.2.64 fastq_mergepairs, removed of primer regions using Cutadapt version 1.12 (Martin et al. 2011), and then filtered keeping reads with a maximum expected error rate of 1 (Edgar and Flyvbjerg 2015). Reads outside of the expected fragment length were removed from the analysis, followed by dereplication with fastx_uniques, and screening for chimeras with uchime2_denovo. Sequences were clustered into operational taxonomic units (OTUs) with an identity threshold of 97% (Edgar 2013). Taxonomic classification was assigned for each OTU through alignment with Blastn 2.2.26 using a reference database that included all available arthropod sequences from the Barcode of Life (BOLD). The taxonomic assignment of each OTU was based on the following criteria: >85 nucleotides in alignment length (Shokralla et al. 2015), 98% minimum identity to the top hit (Zhou et al. 2009), representation by more than one sequence within a prey family, and a 60 minimum bit score (modified from Gerwing et al. 2016). When species barcode references were unavailable, phylogeny-based inference was used to assign OTUs (<97% identity) to family using Bayesian assignment with the Statistical Assignment Package (SAP) version 1.9.8 (Wilson et al 2011, Munch et al 2008, Leray et al. 2013). For each OTU assignment, we allowed a maximum of 50 homologs to be compiled from the NCBI public genomic database sharing sequence identity of ≥80% to build unrooted phylogenetic trees. A posterior probability of 95% was used for each OTU taxonomic assignment (Munch et al. 2008a). Sequence analyses were carried out on the Arctic Region Supercomputing cluster system based at the University of Alaska Fairbanks. We used only PCR plates with high yields possessing >200,000 mean sequence abundance for analyses. This PCR filtering process was used to distinguish high yield fecal samples that likely captured rarer/difficult to detect taxa from low yield fecal samples that may not have met this criterion.

Sequence Data Analyses

Statistical analyses were carried out using R statistical software version 3.4.1 (R Core Team 2017). To investigate the relationship between attributes of the feces and the abundance of sequences in individual fecal samples, we used generalized linear mixed models (GLMM) with function *glmer* in package *lme4* fit with maximum likelihood estimation using Laplace approximation. A gamma distribution was selected to fit the shape of the response distribution. For computational stability, the response variable, abundance of DNA sequences, was standardized by subtracting the mean and dividing by the standard deviation. Fixed effects included: the concentration of DNA extract as measured by fluorometry, the mass of fecal matter, and visual presence of uric acid. Fecal samples from both shorebird species were used in analyses. We excluded fecal samples with no DNA from the analysis because these feces may have been collected outside of the time interval when prey transited though the shorebird chick gastrointestinal tract. We constructed a GLMM model set that contained all possible combinations of fixed effects including an intercept only model with random effects of the chick individual. We used the Akaike Information Criterion corrected for sample size (AICc) and Akaike weights to select the best approximating models with $\Delta AIC<2$ (Burnham and Anderson 2001, Bates et al. 2015). Confidence intervals were calculated with the function *confint* using the bootstrap method. We determined the mean sequence abundance per individual arthropod eaten to compare the representation among taxa. This was calculated for each taxonomic prey group by averaging the number of pooled sequences in fecal samples collected during each feeding trail by the number of prey individuals fed to chicks during the feeding trial.

Bayesian generalized linear models (BayesGLM) were used to assess the detection (0 or 1) of experimental prey by the CO1 marker in feces pooled for each pair of chicks during feeding trials. Explanatory variables included: chick age ('young' 5-14 days or 'old' 15-20 days), quantity of prey eaten, and prey taxa (Chironomidae, Brachycera, Culicidae, Dytiscidae, Plecoptera, and Trichoptera). Models were constructed with function *bayesglm* in package 'arm' using a binary response with the 'logit' link function and a Cauchy prior. We used BayesGLM models because the method can provide sound estimates when perfect separation of the effects (all 1s or all 0s) occurs by using a weakly informative prior (Gelman et al. 2008). Models with two-way interactions were included in the candidate model set with the corresponding main effect as well as an intercept only model. Best approximating models were selected with the

Akaike Information Criterion corrected for sample size (AIC_c) and Akaike weights. We checked for model overdispersion by dividing the deviance by the residual degrees of freedom. To reduce the number of parameters to be estimated, taxa detected 100% of the time they were consumed were grouped into a parameter 'detected' and taxa that were never detected were grouped into a parameter 'not detected'. Based on the previous GLMM models which included chick individual as a random effect, we found that individual accounted for a marginal amount of variance <10% so pseudo-replication of individual chicks did not likely have a large influence on our results. For BayesGLM models, we report the credible interval, the Bayesian version of the confidence interval. We assessed whether a model was informative based on the width of the parameter estimate's (β) confidence interval. The 85% interval was selected as this is compatible with the information theoretic approach. If a model had an uninformative parameter, it was not considered to have statistical support (Arnold 2010).

To examine whether taxon-specific PCR bias could be linked to the detection of particular taxa in chick feces, we determined the number of primer-template base pair consensuses specific to each taxon and report results of previously published *in silico* PCR values for the ZBJ primer pair (Elbrecht and Leese 2017). The primer-template consensus was determined by downloading all available arthropod sequences from the BOLD database for each experimental prey group, aligning the sequences with Clustal Omega multiple sequence alignment, and then by summing the number of base pair loci where the base matched the primer across all species in the alignment (modified from Pinol et al. 2015). *In silico* PCR values represent the percentage of species from public genomic databases that would be expected to amplify successfully within an arthropod group, determined by the number of primer-template mismatches in an alignment.

RESULTS

High-throughput amplicon sequencing

Red Phalarope (n=13) and Pectoral Sandpiper (n=3) chicks consumed 241 arthropods (Chironomidae n=72, Culicidae n=33, Brachycera n=37, Dytiscidae n=44, Plecoptera n=14, Trichoptera n=41) over the course of 23 captive feeding trials resulting in the collection of 285 fecal samples. The average number of fecal samples collected per feeding trial was 11 (range: 3 – 23). Of all fecal samples analyzed, 85% passed PCR quality filters for further analyses. Of the

samples with DNA, the average quantity of genomic DNA was 95 ng. A sample-based rarefaction was used to evaluate how thoroughly each sample was sequenced. This analysis suggested that most samples were sequenced to saturation (Figure 1.1). The highest proportion of sequences amplified by the 16s marker were arthropod while lesser proportion were of Ascomycota fungi, *Calidris* shorebird, and human DNA origin (Figure 1.2). A higher proportion of OTUs were assigned with the CO1 marker than for the 16s marker (Table 1.1).

Experimental prey detections

The average time between consumption and detection of individual prey in chick feces determined over 8 feeding trials was 48 minutes, with a minimum transit time of 27 minutes, and a retention time of 75 minutes. Five of the six experimental prey taxa were detected by either one or both mitochondrial markers; three prey items were detected 100% of the time by the CO1 marker (Chironomidae, Brachycera and Plecoptera), two were detected less frequently (82-95%) than they were consumed (Dytiscidae, Culicidae), and one taxon was detected with neither marker (Trichoptera) (Table 1.2). Three prey groups fed to chicks (Araneae, Tipulidae, and Notostraca) were dropped from the analysis due to limited sample size. Experimental prey were detected when not observed to have been eaten during 4 experimental feeding trials (2x Plecoptera, 1 Brachycera, and 1 Chironomidae) in addition to 12 non-experimental prey groups including: Trichoceridae (n=40), Chrysomelidae (n=31), Cecidomyiidae (n=20), Carabidae (n=12), Polyphemidae (n=3), Ichneumonidae (n=3), Empididae (n=2), Melyridae (n=2), Staphylinidae (n=2), Curculionidae (n=1), Bibionidae (n=1), and Mycetophilidae (n=1). In parenthesis are the number of fecal samples in which that prey was detected. Prey items from the regular diet were also detected, including mealworms (Tenebrionidae), waxworms (Lepidoptera), and crickets (Orthoptera).

Factors associated with detection of prey DNA in feces

Although present in the top approximating models, uric acid presence, fecal sample mass, and DNA concentration did not show statistical support for describing the abundance of sequences in 84 individual fecal samples (Table 1.3). This conclusion was based on wide confidence intervals of the parameter estimates that suggested the models were non-informative (Arnold 2010). To assess the detection (0 or 1) of experimental prey by the CO1 marker, pooled feces from 19

feeding trials were analyzed during which 154 arthropod individuals were eaten and 226 chick feces were collected. In parentheses is the number of feeding trials in which prey taxa were consumed: Culicidae (n=16), Chironomidae (n=18), Brachycera (n =18), Dytiscidae (n=19), Plecoptera (n=6), and Trichoptera (n=15). The model including taxon fit the data better than the models that included age and quantity of prey consumed, carrying over half (0.57) of the AIC_c weights (Table 1.3). Prey including Chironomidae and Brachycera (β=6.22; 85% CI 3.47-8.79) as well as Dytiscidae (β=3.79; 85% CI 2.3-5.38) had higher odds of detection than Culicidae, while Plecoptera and Trichoptera had lower odds of detection (β=-2.57; 85% CI -4.77- -0.34).

DISCUSSION

Feeding experiments with captive shorebird young demonstrated that the composition of prey in shorebird chick feces generally reflects what was eaten by the shorebird chick within the last 90 minutes. While some prey items were detected every time they were consumed, others were detected less frequently than they were consumed. The use of multiple primer sets improved the overall detection of prey both in terms of taxonomic coverage and the frequency with which prey items were detected. This reinforces previous findings that taxon-specific primer bias can be reduced by targeting multiple gene regions (De Barba et al. 2014, Alberdi et al. 2017). The type of taxon had the strongest influence on the detection of a prey item. We did not find evidence that the age of the chick at the time the fecal sample was collected, nor the quantity of the prey eaten influenced the detection of prey in feces. There was no association between the number of primer/template mismatches and detection among taxa. Characteristics of the fecal sample that we examined did not influence the abundance of DNA in the sample. The presence of non-experimental prey in captive chick feces suggests that secondary consumption can artificially inflate diversity in the diet.

Although we did not detect any difference in detection of prey in feces of younger versus older chicks, effects of age on prey detection may still be present. Feces analyzed for the presence of prey from Shy Albatross (*Thalassarche cauta)* chicks had lower amplification success than feces of adult birds (McInnes et al. 2017). We found that prey items were not detected with greater frequency when consumed in greater quantities despite evidence that arthropod biomass correlates with the abundance of sequences in both laboratory mock communities (Elbrecht and Leese 2015, Saitoh et al. 2016), and to some extent in captive studies

(Bowles et al. 2011). Because the quantity of prey consumed did not influence whether a prey item was detected, this would suggest that prey items are detected regardless of whether the chick ate one or several of the prey item. DNA metabarcoding captured not only the dominant prey in the chick's diet in terms of abundance, but also prey that were rarely eaten.

Although we observed differences in detection among arthropod taxa, it is unclear what contributed to this outcome. Prey that are more degraded by digestion would likely be consistently under-detected regardless of the primer set, however this was not observed. A larger sample size of taxa would be needed to test this hypothesis. Studies using mock communities have shown that detection of taxa in a sample is affected by the amount of biomass from different species (Elbrecht et al. 2017). The ratio of sequence to biomass varies by taxon owing to differences in amplification efficiency (Bowles et al. 2011, Elbrecht and Leese 2015, Thomas et al. 2016). If some prey PCR amplify more efficiently than others, this could result in drastically different representation of prey by sequence abundance in the sample (Elbrecht and Leese 2015). Remnant prey DNA found in avian feces is highly degraded (Deagle et al. 2006). Adding to this, prey species that do not amplify efficiently or are represented by a smaller fraction of biomass in the feces could fall out of detection if there is insufficient sequencing depth (Elbrecht and Leese 2017). Multiple PCR reaction replicates are sometimes necessary to detect all prey in a fecal sample (Alberdi et al. 2017).

Neither *in silico* PCR nor the number of primer-template consensuses showed a consistent pattern in detection of prey taxa in the chick's feces. Previous work has shown that the primer-template consensus explained 73% of the variation in sequence abundance among species (Pinol et al. 2015). The efficiency of a primer pair is highly specific to species (Elbrecht and Leese 2015). There were several species within each experimental prey group fed to chicks. Some species within experimental prey groups may amplify more efficiently than others. Furthermore, the primer-template consensus is only one of multiple factors, such as the composition of nucleotides on the 3' end of the primer, that contribute to the success of a primer pair (Kamel 2003). We did not detect Plecoptera or Trichoptera with the ZBJ primer pair. This result contrasts with studies that have detected these taxa (Zeale et al. 2011). The PCR recipe and conditions of a primer set can influence amplification of taxa (Jusino et al. *in press*). We used different PCR conditions with iTru fusion primers than were used in previous studies (Zeale et al. 2011). This might explain why these taxa were not detected.

Differences in detection of prey in chick feces between mitochondrial markers may be related to the existence of references in genomic databases (Clarke et al. 2014b). The existence of references may have contributed to why a greater proportion of OTUs were assigned taxonomy with the CO1 marker than for the 16s marker. If species sequence references are not available for a prey item, this prey goes undetected. OTUs representing non-prey items amplified by the 16s marker constituted a low proportion of the total reads, meaning that host specific DNA did not likely have a strong influence on the results (Gerwing et al. 2016). We found that parameterization of the bioinformatics pipeline used to process sequences required special attention, particularly fragment length filtering, removal of singletons, and OTU assignment to ensure the removal of false positives. The importance of these specifics have also reached the attention of others (Jusino et al. *in press*, Alberdi et al. 2017). Methods have been proposed to reduce the presence of false positives such as site occupancy-detection modeling (Ficetola et al. 2016).

The detection of prey that were not fed to chicks during feeding trials likely represents prey eaten by the chick's prey, also known as secondary consumption. Feeding relationships of arthropods in the Arctic are highly connected and complex (Sheppard et al. 2005, Wirta et al. 2015). Predacious experimental prey including Dytiscidae, Araneae, Tipulidae, and Notostraca could have consumed prey in the environment prior to being captured. Predaceous prey was observed eating other experimental prey while being housed collectively prior to feeding experiments. Secondary consumption in invertebrates is detectable for a long period of time. In spiders, prey can be molecularly detected up to 60 hours post ingestion (King et al. 2008). Secondary consumption has been documented in other controlled diet experiments including feeding experiments with bats (Jusino et al. *in press*). Future work could explore whether DNA from the ambient environment can transit bird's digestive system through sources such as drinking water. If the research objective is to identify primary consumption, parallel work is required to understand the diet of the bird's prey as was done in an investigation of Atlantic Puffin diets (Bowser et al. 2013).

Our study concludes that there are both unique advantages and limitations of using DNA metabarcoding to characterize shorebird chick diets. We found that prey items were usually detected in chick feces post consumption. The frequency with which prey were detected varied by molecular marker. Employing multiple markers improved both the frequency of detection as

well as the number prey taxa detected. Our results suggest that prey items were detected regardless of the quantity of prey eaten or the age of the chick. Factors associated with the fecal sample had no effect on the amount of DNA in the fecal sample. Non-experimental prey detections in captive chick feces suggested that secondary consumption may artificially inflate diversity of prey in the diet. We recommend carrying out replicate PCR reactions to reduce the potential effects of PCR detection bias. The existence of sequence refences for prey is key to improving the application of DNA metabarcoding for characterizing bird diets. Validation experiments either in the form of captive studies or laboratory simulations can help to calibrate and identify sources of bias in data derived from fecal DNA metabarcoding analyses (Leray and Knowlton 2017). The use of multiple diet characterization methods in parallel can be useful for overcoming drawbacks inherent in any diet technique on its own (Traugott et al. 2013, Nielsen et al. 2018). Through this research, we gained a better understanding of the scope and limitations of DNA metabarcoding to characterize the diets of shorebird young. Finally, we hope that this study will catalyze future investigations to examine the presence, mechanisms and solutions for dealing with prey detection bias.

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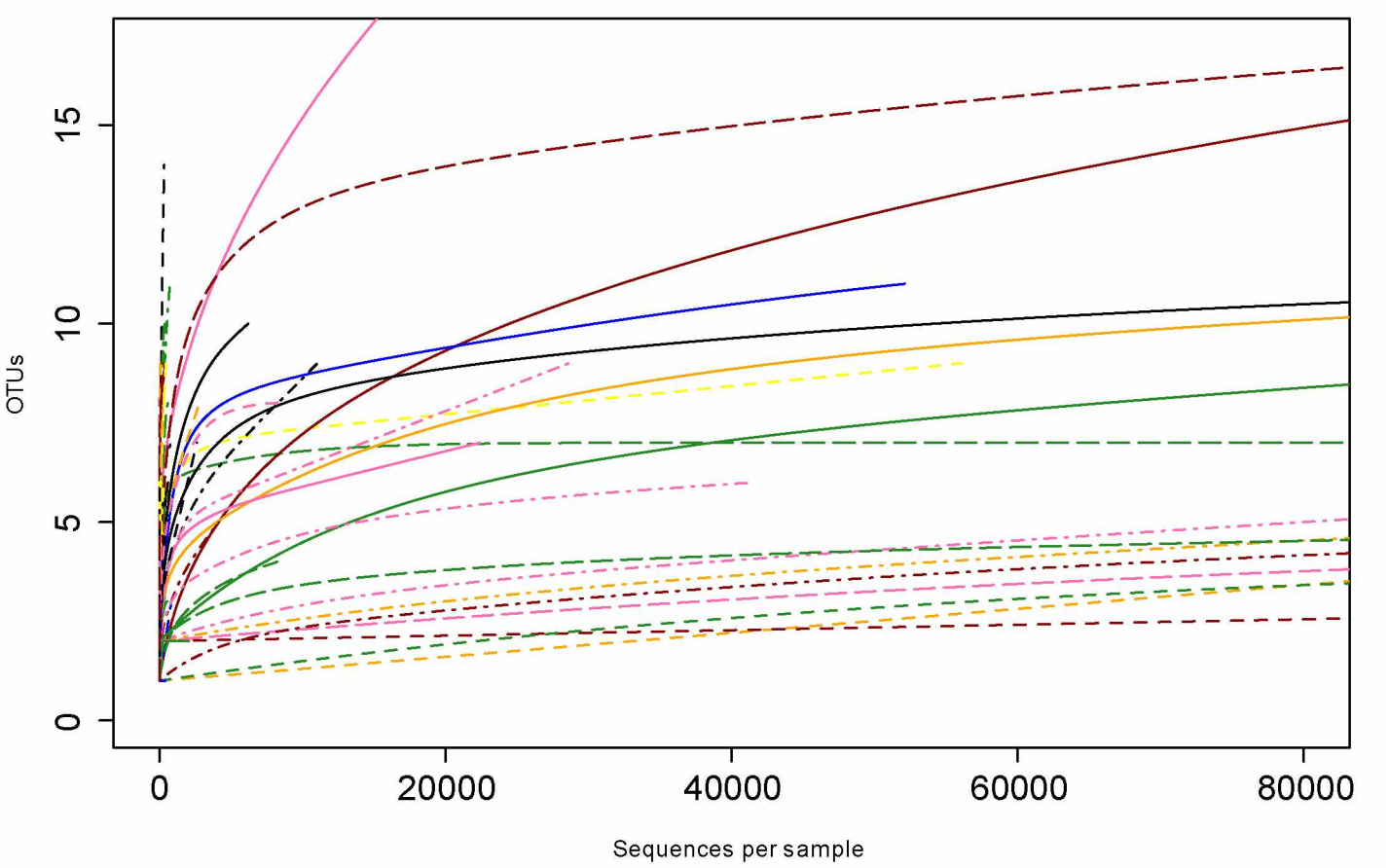


Figure 1.1 Sample-based rarefaction of operational taxonomic units (OTUs) in the feces of captive Red Phalarope and Pectoral Sandpiper young. This includes sequences assigned taxonomy from PCR amplification with two mitochondrial markers (CO1 and 16s).

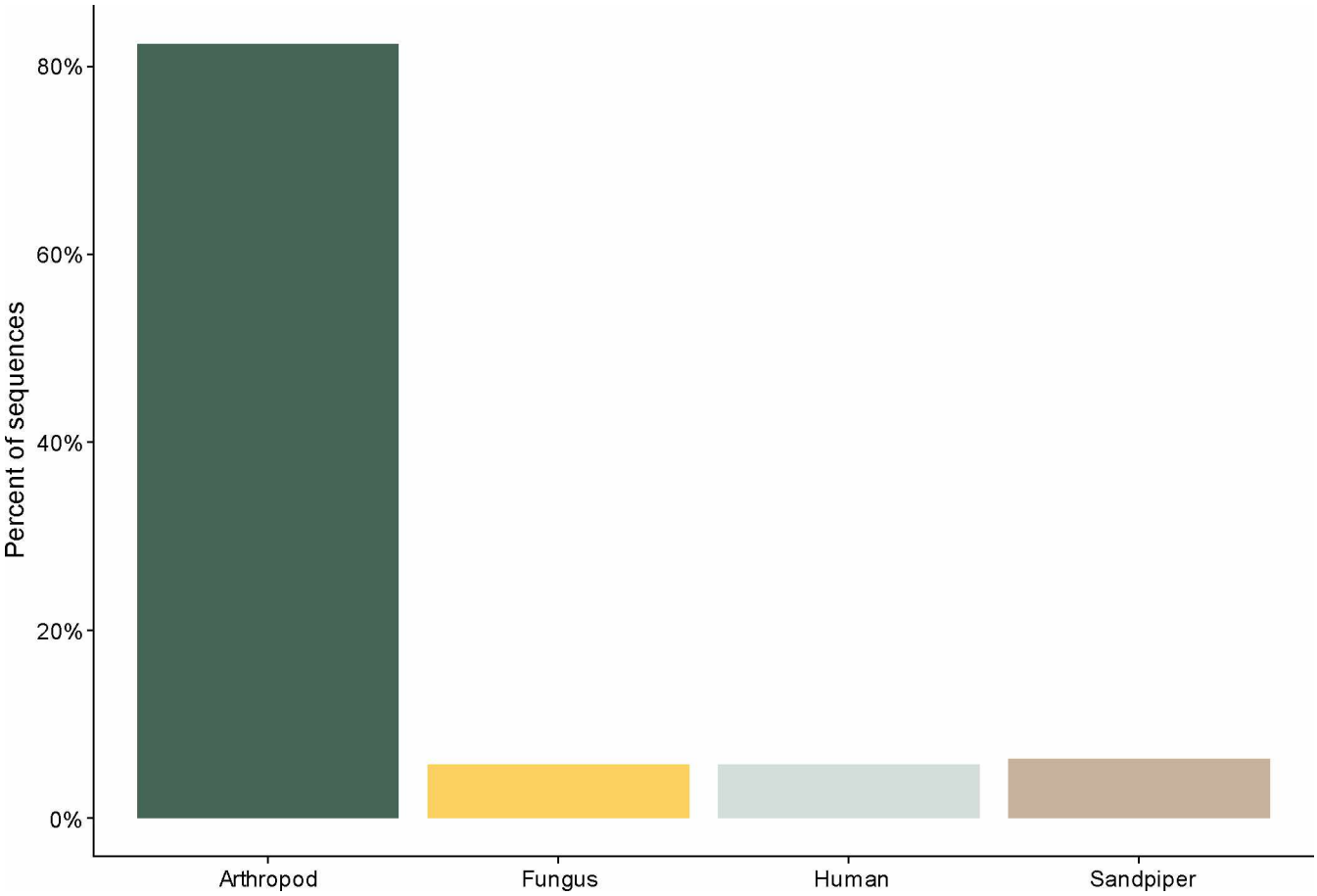


Figure 1.2 Percentage of DNA sequences from arthropod versus non-arthropod origin for the 16s marker.

Table 1.1 Bioinformatics analysis of DNA sequences in feces of captive Red Phalarope and Pectoral Sandpiper young. OTUs were assigned to species with either BLAST or phylogeny-based inference with the statistical assignment package (SAP).

Marker (*n*=num. of fecal samples analyzed)	**Total paired-end reads ($\bar{X}$ ± SD per sample)**	**Merged paired-end reads**	**Trimmed of F & R primer**	**Quality & length filtered**	**Unique sequences**	**Singletons**	**Chimeras**	**Total OTUs (including non-arthropod)**	**Assigned Arthropod OTUs ($\bar{X}$ per sample)**	**Average Arthropod sequences per fecal sample yielding DNA (*n*=num. fecal samples with DNA)**
16s (*n*=101)	1,395,995 (13,399.8 ±12,843.98)	1,310,233 (93.9%)	1,205,825 (92%)	467,544 (38.8 %)	34,555	25,664	96	320	SAP: 55 BLAST: 2 (1.2)	106 (*n*=68)
CO1 (*n*=280)	8,255,753 (35,894 ±100,572)	8,015,432 (97.1%)	8,009,888 (99.9%)	7,842,670 (97.9%)	29,839	17,874	637	331	SAP: 116 BLAST: 78 (2.6)	26,637 (*n*=219)

Table 1.2 The percentage of prey detected with two mitochondrial markers (CO1 and 16s) in shorebird chick feces after observed consumption.

Prey taxa (common name)	**% of prey detected (number of times prey detected/ number of times prey eaten)**		**Sequence abundance per arthropod eaten ($\bar{X}$ ± SD)**		***Forward & reverse primer-template consensus/length of primer**	***In silico* PCR forward/ reverse primer**
Marker	16s	CO1	16s	CO1	CO1	CO1
Diptera; Chironomidae (midge)	82% (9/11)	100% (18/18)	48±131	68113±276696	F14/30: R14/24	35/75
Diptera; Culicidae (mosquito)	82% (9/11)	19% (3/16)	226±165	240±390	F20/30: R22/24	35/75
Diptera; Brachycera (fly)	8% (1/12)	100% (18/18)	7	21362±57167	F19/30: R22/24	35/75
Coleoptera; Dytiscidae (water beetle)	75% (9/11)	95% (18/19)	112±208	20013±73439	F7/30: R11/24	71/60
Plecoptera; Nemouridae (stonefly)	100% (7/7)	0% (0/8)	10±7.6	0	F18/30: R14/24	67/51
Tricoptera (caddisfly)	0% (0/11)	0% (0/9)	0	0	F11/30: R12/24	75/79

*The primer-template consensus was a perfect match for all prey taxa aligned with the 16s marker for both the forward (15 bps) and reverse (22 bps) primers.

Table 1.3 (A) Factors influencing sequence abundance in individual fecal samples amplified by the CO1 marker. (B) Detection of prey (0 or 1) by the CO1 marker in pooled fecal samples of sandpiper chicks experimentally fed prey. The top 4 models ordered by delta AICc (ΔAICc) and the intercept model are reported, as well as model weights (w_i), the number of parameters (K), and the deviance (D). Models with ΔAIC_c <2.0 are considered competitive (Anderson and Burnham 2002).

A. Sequence abundance	**Fixed Effects**	**ΔAIC_c**	**w_i**	**K**	**D**
	Uric acid	0	0.33	4	110.52
	Sample mass + uric acid	1.62	0.25	5	109.84
	Concentration of DNA + uric acid	1.82	0.13	5	110.04
	Intercept	2.62	0.08	3	115.34
B. Detection of prey	Taxon^	0	0.57	4	24.51
	Chick age + taxon	2.23	0.19	5	24.89
	Quantity of prey + taxon	2.24	0.19	5	24.5
	Quantity of prey + taxon + chick age	4.52	0.06	6	24.48
	Intercept	88.35	0	1	119.29

^Model with statistical support based on 85% confidence interval (Arnold 2010).

APPENDICES

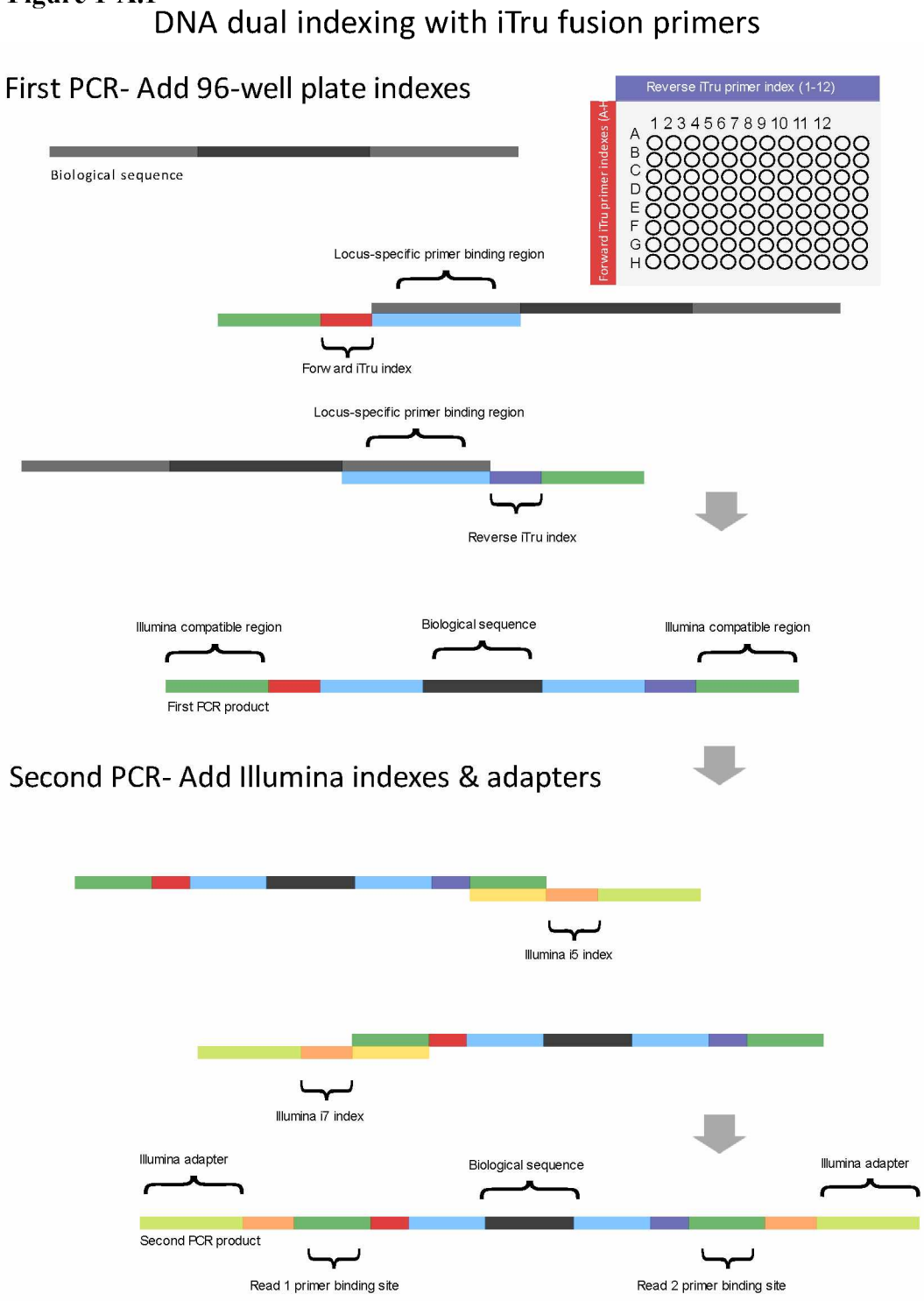
Appendix 1-A

Metabarcoding primers

Table 1-A.1 Project designed iTru fusion primers comprising locus specific primers (capitalized), internal indexes specifying the grid location in the 96 well plate (lowercase), and Illumina compatible region (capital italicized).

Name	Primer sequence
iTru_A_ZBJ-ArtF1c	ACACTCTTTCCCTACACGACGCTCTTCCGATCTggtac*AGATATTGGAACWTTATATTTTATTTTTGG*
iTru_B_ZBJ-ArtF1c	ACACTCTTTCCCTACACGACGCTCTTCCGATCTcaacac*AGATATTGGAACWTTATATTTTATTTTTGG*
iTru_C_ZBJ-ArtF1c	ACACTCTTTCCCTACACGACGCTCTTCCGATCTatcggtt*AGATATTGGAACWTTATATTTTATTTTTGG*
iTru_D_ZBJ-ArtF1c	ACACTCTTTCCCTACACGACGCTCTTCCGATCTtcggtcaa*AGATATTGGAACWTTATATTTTATTTTTGG*
iTru_E_ZBJ-ArtF1c	ACACTCTTTCCCTACACGACGCTCTTCCGATCTaagcg*AGATATTGGAACWTTATATTTTATTTTTGG*
iTru_F_ZBJ-ArtF1c	ACACTCTTTCCCTACACGACGCTCTTCCGATCTgccaca*AGATATTGGAACWTTATATTTTATTTTTGG*
iTru_G_ZBJ-ArtF1c	ACACTCTTTCCCTACACGACGCTCTTCCGATCTctggatg*AGATATTGGAACWTTATATTTTATTTTTGG*
iTru_H_ZBJ-ArtF1c	ACACTCTTTCCCTACACGACGCTCTTCCGATCTtgattgac*AGATATTGGAACWTTATATTTTATTTTTGG*
iTru_1_ZBJ-ArtR2c	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTaggaa*WACTAATCAATTWCCAAATCCTCC*
iTru_2_ZBJ-ArtR2c	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTgagtgg*WACTAATCAATTWCCAAATCCTCC*
iTru_3_ZBJ-ArtR2c	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTccacgtc*WACTAATCAATTWCCAAATCCTCC*
iTru_4_ZBJ-ArtR2c	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTttctcagc*WACTAATCAATTWCCAAATCCTCC*
iTru_5_ZBJ-ArtR2c	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTctagg*WACTAATCAATTWCCAAATCCTCC*
iTru_6_ZBJ-ArtR2c	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTtgctta*WACTAATCAATTWCCAAATCCTCC*
iTru_7_ZBJ-ArtR2c	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTgcgaagt*WACTAATCAATTWCCAAATCCTCC*
iTru_8_ZBJ-ArtR2c	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTaatcctat*WACTAATCAATTWCCAAATCCTCC*
iTru_9_ZBJ-ArtR2c	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTatctg*WACTAATCAATTWCCAAATCCTCC*
iTru_10_ZBJ-ArtR2c	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTgagact*WACTAATCAATTWCCAAATCCTCC*
iTru_11_ZBJ-ArtR2c	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTcgattcc*WACTAATCAATTWCCAAATCCTCC*
iTru_12_ZBJ-ArtR2c	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTtctcaatc*WACTAATCAATTWCCAAATCCTCC*
iTru_A_16S1F-deg	ACACTCTTTCCCTACACGACGCTCTTCCGATCTggtac*GACGAKAAGACCCTA*
iTru_B_16S1F-deg	ACACTCTTTCCCTACACGACGCTCTTCCGATCTcaacac*GACGAKAAGACCCTA*
iTru_C_16S1F-deg	ACACTCTTTCCCTACACGACGCTCTTCCGATCTatcggtt*GACGAKAAGACCCTA*
iTru_D_16S1F-deg	ACACTCTTTCCCTACACGACGCTCTTCCGATCTtcggtcaa*GACGAKAAGACCCTA*
iTru_E_16S1F-deg	ACACTCTTTCCCTACACGACGCTCTTCCGATCTaagcg*GACGAKAAGACCCTA*
iTru_F_16S1F-deg	ACACTCTTTCCCTACACGACGCTCTTCCGATCTgccaca*GACGAKAAGACCCTA*
iTru_G_16S1F-deg	ACACTCTTTCCCTACACGACGCTCTTCCGATCTctggatg*GACGAKAAGACCCTA*
iTru_H_16S1F-deg	ACACTCTTTCCCTACACGACGCTCTTCCGATCTtgatt*GACGACGAKAAGACCCTA*
iTru_1_16S2R-deg	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTaggaa*CGCTGTTATCCCTADRGTAACT*
iTru_2_16S2R-deg	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTgagtgg*CGCTGTTATCCCTADRGTAACT*
iTru_3_16S2R-deg	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTccacgtcc*GCTGTTATCCCTADRGTAACT*
iTru_4_16S2R-deg	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTttctcagc*CGCTGTTATCCCTADRGTAACT*
iTru_5_16S2R-deg	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTctagg*CGCTGTTATCCCTADRGTAACT*
iTru_6_16S2R-deg	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTtgctta*CGCTGTTATCCCTADRGTAACT*
iTru_7_16S2R-deg	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTgcgaagt*CGCTGTTATCCCTADRGTAACT*
iTru_8_16S2R-deg	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTaatcctat*CGCTGTTATCCCTADRGTAACT*
iTru_9_16S2R-deg	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTatctg*CGCTGTTATCCCTADRGTAACT*
iTru_10_16S2R-deg	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTgagact*CGCTGTTATCCCTADRGTAACT*
iTru_11_16S2R-deg	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTcgattcc*CGCTGTTATCCCTADRGTAACT*
iTru_12_16S2R-deg	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTtctcaatc*CGCTGTTATCCCTADRGTAACT*

Figure 1-A.1


DNA dual indexing with iTru fusion primers
First PCR- Add 96-well plate indexes
Biological sequence
Reverse iTru primer index (1-12)
Forward iTru primer indexes (A-H)
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
Locus-specific primer binding region
Forward iTru index
Locus-specific primer binding region
Reverse iTru index
Illumina compatible region
Biological sequence
Illumina compatible region
First PCR product
Second PCR- Add Illumina indexes & adapters
Illumina i5 index
Illumina i7 index
Illumina adapter
Biological sequence
Illumina adapter
Second PCR product
Read 1 primer binding site
Read 2 primer binding site

Appendix 1-B

U.S. Fish and Wildlife Service Region 7
Institutional Animal Care and Use Committee

Assurance of Animal Care Form

IACUC Use Only

IACUC Number: 2015-005 **USDA Classification:** C / D / E (Circle One)

Date Received: 10 MARCH 2015 **Initial Review Date:** 14 APRIL 2015

IACUC Training Complete: ☒ (20 May 2015)

IACUC recommendations: Approved: ☒ **Not Approved:** ☐

Date Revisions Received: 22 April 2015 **Initial Approval Date:** 28 April 2015

Renewal Month: April **First Annual Renewal Date:** _____

Second Annual Renewal Date: _____

IACUC Chair Signature: _____ **Date:** _____

Project Title:

Breeding Ecology of Shorebirds at Barrow, Alaska

Name(s) of Funding Source(s): USFWS, Migratory Bird Management; Arctic LCC

Approximate Starting Date: 25 May 2015 **Completion Date:** long-term **Ongoing** ☒

For continuation of a previously approved Assurance Form, provide the **IACUC number:** 2012006

CHAPTER 2. CHARACTERIZING ARCTIC SHOREBIRD CHICK DIETS: INSIGHTS INTO TROPHIC MISMATCH FROM DNA METABARCODING[2]

ABSTRACT

Climate change in the Arctic is affecting when arthropod prey is available for nesting shorebirds and their young. Characterizing shorebird chick diets is a pre-requisite to evaluating whether temporal changes in food resources impact young through reduced growth and survival. We used fecal DNA metabarcoding to characterize the diets of pre-fledged Red Phalarope (*Phalaropus fulicarius*), Pectoral Sandpiper (*Calidris melanotos*), and Dunlin (*Calidris alpina*) at Utqiaġvik, Alaska during the summers of 2014-2016. Molecular diet characterization of chick feces detected most of the prey families reported in prior gut content analyses. An additional 17 new prey families were identified. We compared the arthropod composition of Red Phalarope and Pectoral Sandpiper diets to collection of prey in pitfall traps during the summer of 2015 to assess how well the contents of traps reflected what chicks were eating. Shifts in diets of shorebird young generally reflected changes in prey composition in pitfall traps. We found that within age groups, half of the variability in prey composition among fecal samples could be explained by the timing of fecal sample collection within the season (p-value=0.07). Chick age and species explained a smaller proportion of the variation, 6% (p-value=0.04) and 5% (p-value=0.05) respectively. Through captive experiments we found that chicks <5 days old may be selecting for prey <9.7 mm possibly due to the exclusion of certain arthropods that may be too large to consume. Our results suggest that shorebird chicks exhibited generalist foraging behavior so were capable of adjusting to intra-annual variation in prey availability; however, climate-mediated changes in the timing of food resources in the Arctic could still result in insufficient quantities of food for chicks to achieve adequate growth rates.

Keywords: shorebird young, trophic mismatch, fecal DNA metabarcoding, Red Phalarope, Pectoral Sandpiper, Dunlin, molecular diet analysis

[2] Gerik, D. E., Lanctot, R. B., Gurney, K. E. B., Spangler, M. A., Saalfeld, S. T., López, J. A., Characterizing arctic shorebird chick diets: insights into trophic mismatch from DNA metabarcoding. Prepared for submission to The Auk.

INTRODUCTION

Changing climate conditions are affecting the timing of key ecological events (Both and Visser 2001, Visser and Both 2005, Visser et al. 2012). This is particularly pronounced in the high Arctic where the phenology of spring is advancing (Høye et al. 2007, Stendel et al. 2008, Saalfeld and Lanctot 2017). Because shorebirds undertake long distance migrations to breeding areas from distant wintering sites, altering the timing of their arrival and nest initiation may be challenging (Moller et al. 2008, Both et al. 2010). Their self-feeding young have a strong reliance on arthropods (Holmes 1966, Holmes and Pitelka 1968, Schekkerman et al. 2003), whose emergence is occurring earlier and is dictated by local conditions (MacLean and Pitelka 1971, Tulp and Schekkerman 2008, Bolduc et al. 2013). Temporal decoupling of prey emergence and the timing of chick growth has been linked to reduced growth and survival of shorebird young (McKinnon et al. 2012, Gils et al. 2016, Senner et al. 2017, Saalfeld et al. *in review*). Understanding the susceptibility of a species to changes in food availability is relatively straightforward for species that rely strongly on a narrow set of prey items (Cresswell and McCleery 2003, Both et al. 2006); however, shorebirds often feed on a diversity of prey types (Tulp and Schekkerman 2008), creating additional layers of complexity. The degree of flexibility in chick diets may be an indicator of how well individual species are able to adjust to changes in prey resources occurring in the Arctic (Tulp and Schekkerman 2008, Bolduc et al. 2013).

An essential component in assessing trophic mismatch is the need to estimate how much food is available to chicks in the environment over time. In the Arctic, prey availability has been typically measured by sampling arthropod biomass with pitfall traps that readily capture surface active arthropods, the main food of shorebird chicks (Holmes and Pitelka 1968, Meltofte et al. 2007b). However, arthropod data derived from traps may not be reflective of food availability for a variety of reasons (Poulin and Lefebvre 1997). In addition, chicks may have preferences for particular prey, there may be intra- and interspecific diet preferences, or shifts in diet through development. Thus the composition of prey items eaten by chicks may differ from that of the arthropods collected in traps at any given time (Roche et al. 2016). However, no formal analyses have examined whether the contents of the traps used to assess food availability directly reflect the composition or proportions of prey in shorebird chick diets. To date, chick diet information has been based on gut content analyses of collected chicks (Holmes 1966, Holmes and Pitelka 1968, S. F. MacLean unpubl.) or adults of the same species (McKinnon et al. 2012, 2013a;

Reneerkens et al. 2016). Visual examination of prey remains from the upper GI tract of shorebirds can have limited taxonomic resolution and are biased toward identification of prey that survive digestion and have diagnostic hard parts (Gales 1988, Tollit et al. 2003). Thus, there are limitations to using stomach content analysis to determine the diets of shorebird young. A genetic-based approach for identifying chick diet has the potential to enhance our understanding of what chicks eat. Fecal DNA metabarcoding, a method that identifies prey in feces based on their unique genetic signatures, has been used to characterize the diets of a growing list of bird species (Novcic et al. 2015, Wirta et al. 2015, Crisol-martínez et al. 2016, Trevelline et al. 2016). Molecular diet characterization provides a minimally invasive means to re-sample individuals over time as well as fine scale taxonomic resolution of prey.

We collected and analyzed feces from young of three shorebird species (Red Phalarope, *Phalaropus fulicarius*, Pectoral Sandpiper, *Calidris melanotos*, and Dunlin, *Calidris alpina*) using DNA metabarcoding. We then compared molecular diet characterization to records of diet composition through gut content analyses from the literature. Based on prior studies, we predicted that fecal DNA metabarcoding would resolve some of the unidentified small adult flying insects previously documented in the diet for these species (Holmes 1966). We examined biological and temporal factors that could influence chick diet composition including the shorebird species, the date of capture within the season, and the age of the chick. To address whether chicks may be limited by the sizes of prey they can eat with age, we carried out a captive feeding study where shorebird young were fed prey of different sizes to evaluate prey size selection through development. Finally, we tested the use of pitfall traps as a proxy for chick diet by relating chick diet composition derived from DNA metabarcoding to the temporal biomass of their prey present in the environment based on field trapping. Assuming shorebird young are opportunistic and follow optimum foraging theory (Griffiths 1975), we predicted that young would eat prey according to their temporal availability.

METHODS

Study Area and Collection of Shorebird Feces. Fecal samples were collected from shorebird young near Utqiaġvik (formerly Barrow), Alaska (71° 17′ N 156° 47′ W) during the summers of 2014-2016. The tundra around Utqiaġvik is characterized by a mosaic of landforms, including predominantly high- and low- center polygons, shallow oriented lakes and

nonpatterned tundra underlain by acidic soils (Brown et al. 1980, Walker et al. 2005). To determine the age of chicks at the time of feces collection, we tracked chicks from their nest sites. Shorebird nests were located by conducting area searches and rope drags (Saalfeld and Lanctot 2015). Nest hatch dates were estimated by adding the number of days a given species typically incubated a clutch to nests found during laying or by floating eggs (Liebezeit et al. 2007, Saalfeld and Lanctot 2015). Nests were visited near hatch and newly hatched chicks were banded with unique U.S. Geological Survey metal bands, allowing for identification of chicks whose feces were collected when incidentally re-found during routine field work. A subset of chicks, one per brood, were equipped with VHF radios shortly after hatch (Saalfeld et al. *in review*) so chicks, and frequently their brood mates, could be tracked and recaptured every three days. We also collected feces from unbanded chicks whose ages were estimated using species-specific growth curves (Saalfeld et al. *in review*). Once chicks were captured, feces we collected by placing each chick in their own sterilized container lined with wax paper for up to 15 minutes. Fecal samples were placed in 1.5 ml cryovials filled with 100% ethanol kept frozen at -20°C in a cryoshipper until transferred to a -80°C degree freezer.

Molecular Identification of Prey in Shorebird Feces. Arthropod specimens were collected at the field site to generate reference DNA sequences from potential prey items in chick feces. The newly determined sequences in combination with publicly available sequences served as a reference set for species determinations. Detailed methods generation of DNA sequence references, including DNA extraction, PCR amplification, and sanger sequencing are described in Appendix 2-A. Arthropod sequence references generated through this project are listed in Table 2-A.1 in Appendix 2-A.

DNA was extracted from shorebird feces, PCR amplified with two established mitochondrial markers cytochrome c oxidase subunit 1 (CO1) and 16s ribosomal RNA subunit (16s) (Deagle et al. 2007, Gerwing et al. 2016), and sequenced with high-throughput amplicon sequencing on an Illumina MiSeq. The resulting paired-end sequences were quality filtered and clustered into operational taxonomic units (OTUs) for taxonomic assignment. If species references were unavailable, OTUs were assigned to a family using Bayesian phylogeny-based inference. We categorized fecal samples in PCR plates into high and low yield (>200,000 or <200,000 mean sequence abundance respectively). This PCR filtering process was used to distinguish fecal samples that likely captured rarer/difficult to detect taxa from low yield fecal

samples that may not have met this criterion. Low yield fecal samples were used exclusively for inventory of prey in shorebird chick diets. Detailed methods of fecal DNA extraction, PCR amplification, high-throughput amplicon sequencing and identification of diet items are described in Appendix 2-A.

Arthropod Collection in the Environment. We generated an index of surface-active arthropod availability by sampling arthropods in mesic and xeric habitats using modified Malaise pitfall traps from early June until late July in 2015 (Brown 2014). Two traps per habitat type were placed at four locations over the approximately 25 km^2 study area. Traps were composed of ~38 cm x 5 cm x 7 cm plastic containers placed at ground level with a 36 cm x 36 cm mesh screen placed perpendicular above the container to capture aerial arthropods that hit the screen and fell into the trap (Brown 2015). Arthropods were collected from traps every three days and stored in 100% ethanol. Samples were transferred to Aquatic Biology Associates, Inc. for identification to family or order. Arthropod biomass was estimated using standard linear regressions from arthropod lengths and widths to the nearest 0.25 mm for individuals <2 mm and to the nearest 0.5 mm for individuals >2 mm. Arthropod biomass was correlated amongst traps in different part of the study area (Saalfeld et al. *in review*). We calculated daily arthropod availability by averaging biomass across sampling traps and habitat for each prey group within each 3-day sampling period. Prey biomass was estimated by interpolation for days when arthropods were not sampled using the *na.approx* function in the package 'zoo' in R statistical software version 3.4.1 (R core team 2017). Best fit lines of arthropod biomass were generated for each arthropod taxa using a 6^{th} degree polynomial with the stat_smooth option in the 'ggplot2' package (R core team 2017).

Statistical Analyses. We examined diversity in the diet as a function of fecal sample size for each shorebird species and for two age groups ("young" 0-3 or "old" 4-16 days) using rarefaction curves with the function *specaccum*. Age groups were selected based on sample size. A sample-based rarefaction was constructed to examine whether read depth was sufficient to capture prey diversity in fecal samples using the function *rarecurve* in R package 'vegan'. We used permutational multivariate analysis of variance (perMANOVA) to assess the influence of shorebird species (Phalarope and Pectoral Sandpiper), collection date, and chick age group ("young" 0-3 or "old" 4-16 days) on the prey composition of shorebird chick feces collected during the summer of 2015 with the function *adonis* using 999 permutations in the 'vegan'

package. Prior to running the analysis, we randomly removed re-sampling events from the same individual to account for autocorrelation. We found through captive feeding experiments that sequence abundance can vary drastically among prey taxa. Therefore, we did not rarify by sequence abundance across samples because this could result in the removal of prey from the data set that yield less DNA sequences. A Bray-Curtis distance matrix was constructed with the function *vegdist* on a presence-absence dataset of prey families. Because perMANOVA assumes homogeneity of variances (Anderson and Walsh 2013), we tested for differences in dispersion within each of the effects of interest using the function *betadisper* in the 'vegan' R package (Clarke 1993). The order of parameters in the perMANOVA model were permuted to test whether the order of the parameters affected the outcome.

To determine whether the size of prey eaten by captive-reared chicks was related to their age, we calculated an electivity index for prey lengths to compare the proportion of prey selected to the proportion of prey offered within age classes (2-4, 5-7, 8-10, and 11-16 days) (see details in Appendix 2-C). To examine whether the availability of prey in the environment reflects prey in the diets of shorebird chicks, we divided the summer of 2015 into time intervals 'early' 23 June-2 July, 'mid' 3 July-15 July, and 'late' 16 July-27 based on major shifts in prey biomass in the environment. We then compared the proportion of occurrence of prey in chick diets during these time periods to the proportion of prey biomass collected in pitfall traps on days that fecal samples were collected. When multiple fecal samples were collected on the same day, the biomass over each fecal sample collected was summed. Prey taxa detected in chick diets with DNA metabarcoding were binned into the taxonomic level of the invertebrates identified in pitfall traps for compatibility between data sets. Statistical analyses were carried out using the R statistical software version 3.4.1 (R core team 2017).

RESULTS

Prey Detected in Shorebird Chick Feces. We collected and analyzed 140 fecal samples from 122 shorebird chicks for the presence of prey DNA with DNA metabarcoding (Table 2.1). Of these, prey was identified in 92 fecal samples collected from 84 individual chicks (Table 2-A.2 in Appendix 2-A). A rarefaction analysis suggested that read depth, a measure of how thoroughly the sample was sequenced, was sufficient to sequence most samples to saturation (Figure 2-A.1 in Appendix 2-A). Prey identified in chick feces collectively included 62

genetically distinct species representing 3 classes, 7 orders, 27 families, and 39 genera within the phylum Arthropoda (Table 2-B.1 in Appendix 2-B). Pectoral Sandpiper chicks ate a total of 22 prey families, Red Phalarope chicks 17 prey families and Dunlin chicks 14 prey families. Differences in prey diversity between shorebird species may, in part, reflect sample size (Figure 2.1-A). DNA metabarcoding detected 10 of the 12 prey families found in the diets of Red Phalarope, Pectoral Sandpiper, and Dunlin through gut content methods carried out in Utqiaġvik in the 1960s, as well as an additional 17 previously-undocumented families. Most of the novel prey groups (11 of 17) occurred in the environment in the 1940s-1960s (Weber 1950, MacLean and Pitelka 1971), but were not found in the stomachs of collected birds (Table 2.2). There was variable coverage of prey between mitochondrial markers. More unique prey taxa were identified by the CO1 marker than by the 16s marker (16 and 4 prey families respectively). A total of 6 prey families were detected independently with both markers (Table 2.2).

Factors Influencing Prey Composition in Feces. The perMANOVA analysis included 14 Red Phalarope and 22 Pectoral Sandpiper fecal samples. Based on tests for multivariate dispersion, neither shorebird species ($p=0.44$) nor chick age group ($p=0.58$) had significant within group variance while capture date showed some within group variance ($p=0.05$). Because the order of the variables in the perMANOVA model affected the results for chick age group and species, we ran two separate models, one in which chick age group was held constant as a blocking factor and the other in which species was held constant as a blocking factor. Within age groups, capture date explained over half of variability in the composition of fecal samples. Age group explained 6% and shorebird species 5% of the variability (Table 2.3).

We found that by the time chicks reached 3 days of age, 89% of the total prey families detected in Red Phalarope, Pectoral Sandpiper and Dunlin young collectively were present in chick diets. There were more prey families unique to the diet of chicks 0-3 days old versus 4-16 days old, including Dipteran flies (Anthomyiidae, Empididae, Scathophagidae), parasitoid wasps (Braconidae), soft-winged flower beetles (Melyridae), and sawflies (Tenthredinidae). Prey families unique to chicks 4-16 days old included mites (Crotoniidae), gall midges (Bolitophilidae), and springtails (Sminthurididae). A rarefaction analysis suggested that the lower diversity in chicks aged 4-16 days was likely related to sample size (Figure 2.1-B).

Prey Proportion of Occurrence. Red Phalarope and Pectoral Sandpiper prey during the summer of 2015 were mainly composed of flies (Diptera; 79% of feces), followed by beetles

(Coleoptera; 78%), spiders (Araneae; 30%), stoneflies (Plecoptera; 20%), wasps (Hymenoptera; 7%), mites (Sarcoptiformes; 2%) and springtails (Symphypleona; 2%). These were represented by families including non-biting midges (Chironomidae; 83%), flies (Muscidae; 49%), ground beetles (Carabidae; 37%), gall midges (Cecidomyiidae; 29%), rove beetles (Staphilinidae; 24%), winter crane flies (Trichoceridae; 24%), mosquitos (Culicidae; 24%), dwarf-weaver spiders (Linyphiidae; 24%), stone flies (Nemouridae; 20%), water beetles (Dytisctidae; 17%), leaf beetle (Chrysomelidae; 15%), dagger flies (Empididae; 15%), crane flies (Tipulidae; 10%), hover flies (Syrphidae; 7%), crab spiders (Philodromidae; 7%), parasitoid wasps (Ichneumonidae; 5%), fungus gnats (Mycetolphilidae; 5%), dark-winged fungus gnats (Sciaridae; 5%) and long-legged flies (Dolichopodidae; 5%), while the remaining families were detected in less than 5% of feces.

Diet Compared to Temporal Biomass of Prey in the Environment. All the prey groups detected in Red Phalarope and Pectoral Sandpiper diets during the summer of 2015 could be classified into arthropod groups collected in invertebrate pitfall traps with the exception of Bolitophilidae, Dytiscidae, Crotoniidae, and Melyridae. Taxa collected in invertebrate pitfall traps that were absent in chick diets according to DNA metabarcoding included Curculionidae, Psychodidae, Ceratopogonidae, Trichoptera, Saldidae, Lepidoptera. Except for Trichoptera, all taxa absent from chick diets that were collected in invertebrate pitfall traps were also absent in gut content analyses of chick diets (Table 2.2). There was some variation in the proportional biomass as well as the composition of prey between mesic and xeric habitats sampled by invertebrate pitfall traps (Figure 2-D.1 in Appendix 2-D). We observed that some prey taxa were consistently available over the summer while other prey groups were sometimes available. We limited our investigation to those prey groups present in both chick diets and invertebrate pitfall traps. Results suggest that chicks switch between prey types since the proportion of occurrence of various prey changed in the diet during the time intervals 'early', 'mid' and 'late'. The degree to which this was reflected by changes in composition and proportional biomass of those prey collected in invertebrate pitfall traps varied by prey group (Figure 2.2). In an analysis of prey size limitations with captive young, we found that chicks 2-4 days old did not select for prey sizes greater than 9.7 mm. Therefore, we excluded Tipulidae and Brachycera individuals >9.7 mm from estimates of prey biomass available for chicks 4 days and younger (Table 2-C.1 in Appendix 2-C). For more details on chick prey size selection see Appendix 2-C.

DISCUSSION

Molecular diet characterization revealed a wide breadth of prey taxa at a fine scale taxonomic resolution in the diets of pre-fledged Red Phalarope, Pectoral Sandpiper and Dunlin young in Utqiaġvik, Alaska. Over half the families were previously undescribed as prey for shorebird young at the site based on gut content analysis. The temporal availability of prey in the environment collected through pitfall traps generally matched the major prey groups eaten by Red Phalarope and Pectoral Sandpiper chicks during the summer of 2015, although there were some exceptions. The date of collection explained a great deal of the variability in Red Phalarope and Pectoral Sandpiper diets while chick species and age accounted for a small amount of the variation. Chicks acquired most of the prey diversity present in their diets soon after hatch. Therefore, they appear to opportunistically forage immediately upon leaving the nest. We observed differences in prey diversity between shorebird species. We determined through captive experiments that chicks may be constrained in the size of prey they can eat up to 4 days after hatch. Young chicks may not have access to some of the larger prey despite their presence in the environment. This suggests that prey biomass collected in pitfall traps needs to be adjusted to account for prey size restrictions to avoid overestimating prey availability.

Molecular diet characterization may have detected prey in chick diets that were previously undetected in gut content analysis for a variety of reasons. First, there may have been a shift in the composition and abundance of particular arthropods present in Utqiaġvik since the 1960s, when gut content analyses were conducted. Changing arthropod abundances linked to a changing climate has been documented in other parts of the Alaskan Arctic (McDermott 2017). Major shifts in arthropod composition seems unlikely, however, because most of the arthropod prey in chick diets documented through molecular techniques were present in gut content analyses from the 1960s (Table 2.2). Second, additional prey types may reflect secondary or tertiary consumption from the prey in the stomach of prey consumed by shorebirds. For example, detections of *Phygadeuon trichops* and *Microplitis sp.*, both parasitoid wasps, may have been due to these wasps parasitizing the prey eaten by the shorebird chicks. We did not attempt to separate primary from secondary consumption because chicks fed on prey from a variety of trophic levels and food webs in the Arctic are highly connected and complex (Wirta et al. 2015). Third, DNA metabarcoding likely improves detection of soft bodied arthropods that are difficult to identify with gut content methods due to degradation and difficultly recovering diagnostic

hard parts for identification. The ability to detect soft-bodied prey is vital for characterizing shorebird chick diets given that Diptera is the most prevalent prey group in Utqiaġvik based on collection of arthropods in pitfall traps. Diptera represents the most important prey group by proportion of occurrence in shorebird chick diets both in this study and others (Holmes 1972, Pearce-Higgins and Yalden 2004, Buchanan et al. 2006). Despite the increase in diversity revealed though molecular methods, previous gut content analyses documented families not found using DNA metabarcoding including a family of mites (Erythraeidae) and caddisflies (Trichopetera). A different family of mite was detected with molecular techniques (Crotoniidae). The absence of Trichoptera in chick diets may be attributed to the inability of the molecular techniques to detect the prey (Gerik unpublished).

Among factors influencing chick diet composition, the date of collection within the season explained the most variation in prey composition for Red Phalarope and Pectoral Sandpipers. In the model in which permutations occurred within each species separately, collection date had less support (p-value= 0.15), possibly because of a difference in the phenology of the species; Red Phalarope terminated nesting earlier than Pectoral Sandpipers in 2015 and therefore Red Phalarope fecal samples were restricted to the 'early' time period (Figure 2.2). Because of this, Red Phalarope may not have had the opportunity to eat as many types of prey taxa as Pectoral Sandpipers. Differences in foraging strategies of the chicks could also contribute to variation in diet between species. Red Phalarope chicks sometimes spin feed whereas Pectoral Sandpiper chicks do not. In the model where age groups were held constant, collection date, with a p-value of 0.07, was short of the conventional 0.05 level of significance. Being close to significant, the result indicates that collection date is important for explaining diet composition. Within group heterogeneity of collection date may have contributed to some of the variance explained in the models. The composition of prey in chick feces likely reflects the daily availability of prey because prey DNA was detectable in feces as soon as 27 minutes after they were eaten in controlled feeding experiments. It appears to be adaptive for shorebird chicks to shift their diet to take advantage of the prey currently available given the high variability in prey abundance between years, both within and among prey groups (MacLean and Pitelka 1971).

Shifts in chick diets over the season corresponded with shifts of certain prey groups in invertebrate pitfall traps better than others (Figure 2.2). There are a number of factors that could contribute to lack of correspondence between chick diets and the collection of arthropods in

pitfall traps. Temperature and wind, as well as differences in the ease of catching different types of arthropods, influence the collection of arthropods in pitfall traps. This method serves as more of measure of arthropod activity than true abundance in the environment (MacLean and Pitelka 1971, Poulin and Lefebvre 1997, Høye and Forchhammer 2008). We observed differences in the proportion of prey in mesic and xeric habitats (Figure 2-D.1 in Appendix 2-D). Chicks may be moving between habitats or prey patches. DNA metabarcoding does not always detect prey when eaten by shorebird chicks so some prey items are likely going undetected.

Because nearly all of the diversity of prey in the chick diets was present 3 days after hatch, chicks are likely eating almost any prey they encountered prior to becoming self-thermoregulating. This is surprising given that shorebird chicks are limited in their movements early on due to thermal dependence (Krijgsveld et al. 2003, Schekkerman et al. 2003), and likely go through a period where they must learn to catch prey (Pearce-Higgins and Yalden 2004). The point in maturation when shorebird chicks are most susceptible to food shortage has not been firmly established and likely varies by species. This information may be important when considering the impacts of food availability on chick growth and survival predicted under trophic mismatch scenarios. Although previous studies have found that younger chicks (< 5 days) have overall lower survival (Hill MS thesis 2012), other studies have found that reduced food impacted older (>5 days), rather than younger shorebird chicks, through slower growth (McKinnon et al. 2013a) or reduced survival (Senner et al. 2017). Chicks in our study were ate nearly all prey taxa, although not all prey sizes, in the environment within 3 days of leaving the nest. This suggests that susceptibility of young chicks to starvation is not likely due to an inability of chicks to consume prey but rather an ability to access sufficient prey resources.

Our results suggest that measures of prey availably in the environment through pitfall trapping track the temporal usage of at least the major prey groups in Red Phalarope and Pectoral Sandpiper diets. We found that differing phenology between shorebird species impacts the diversity of available prey. This could be important in terms of health or for growth of shorebird chicks because not all prey have equal caloric and nutritional value (Razeng and Watson 2015, Twining et al. 2016). Shorebird species tend to nest in a fairly consistent order from year to year (Saalfeld and Lanctot 2015). This behavior could be adaptive for species that have evolved to time their breeding activities with the emergence of certain types of prey. The relationship between chick diets and the collection of arthropods in pitfall traps shows some support for

studies that have relied on invertebrate trapping to evaluate how the timing of food resources affect growth and survival of shorebird young (Saalfeld et al. *in review*, Schekkerman et al. 2003, McKinnon et al. 2012, Mckinnon et al. 2013, Reneerkens et al. 2016). To improve the index of prey availability through collection of arthropods in pitfall traps, we suggest adjusting for prey size limitations through chick development. Our results suggest that shorebird chicks have converged on a flexible foraging strategy. This may be advantageous because arthropod prey is often fleetingly abundant in the Arctic. Despite the ability of shorebird chicks to shift their diet, prey resources may not be available during the narrow window of time to meet the demands of rapid growth if a trophic mismatch occurs (Schekkerman et al. 2003). Future work should focus on characterizing the diets of adult shorebirds using DNA metabarcoding techniques. This could improve understanding of how the timing of food availability for adults impacts their ability to lay eggs, subsequent nest success, as well as the survival of their chicks.

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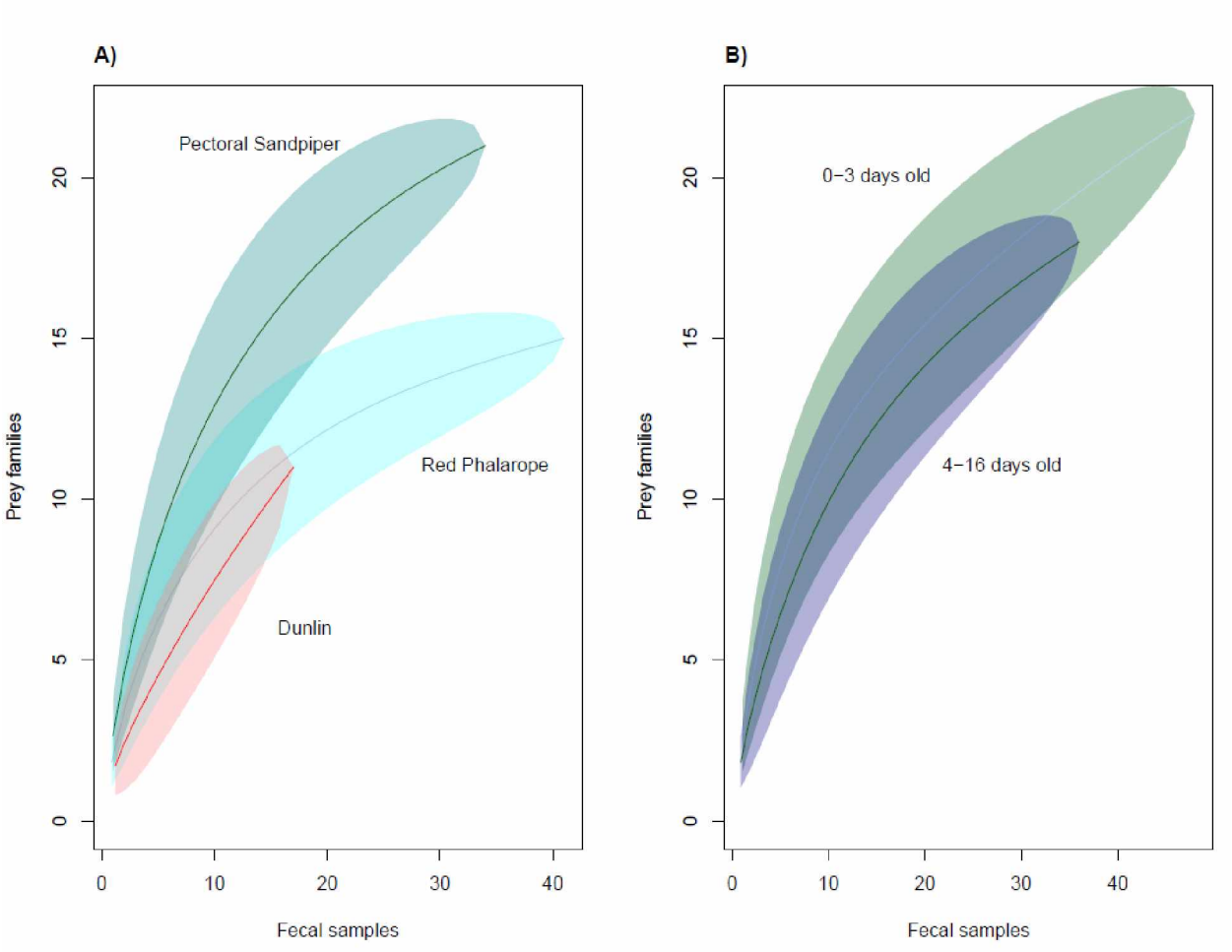

Figure 2.1 (A) Rarefaction analysis of prey families present in fecal samples for three species of shorebird young and (**B**) chick age groups for all shorebird species combined. Envelopes represent 95% confidence intervals.

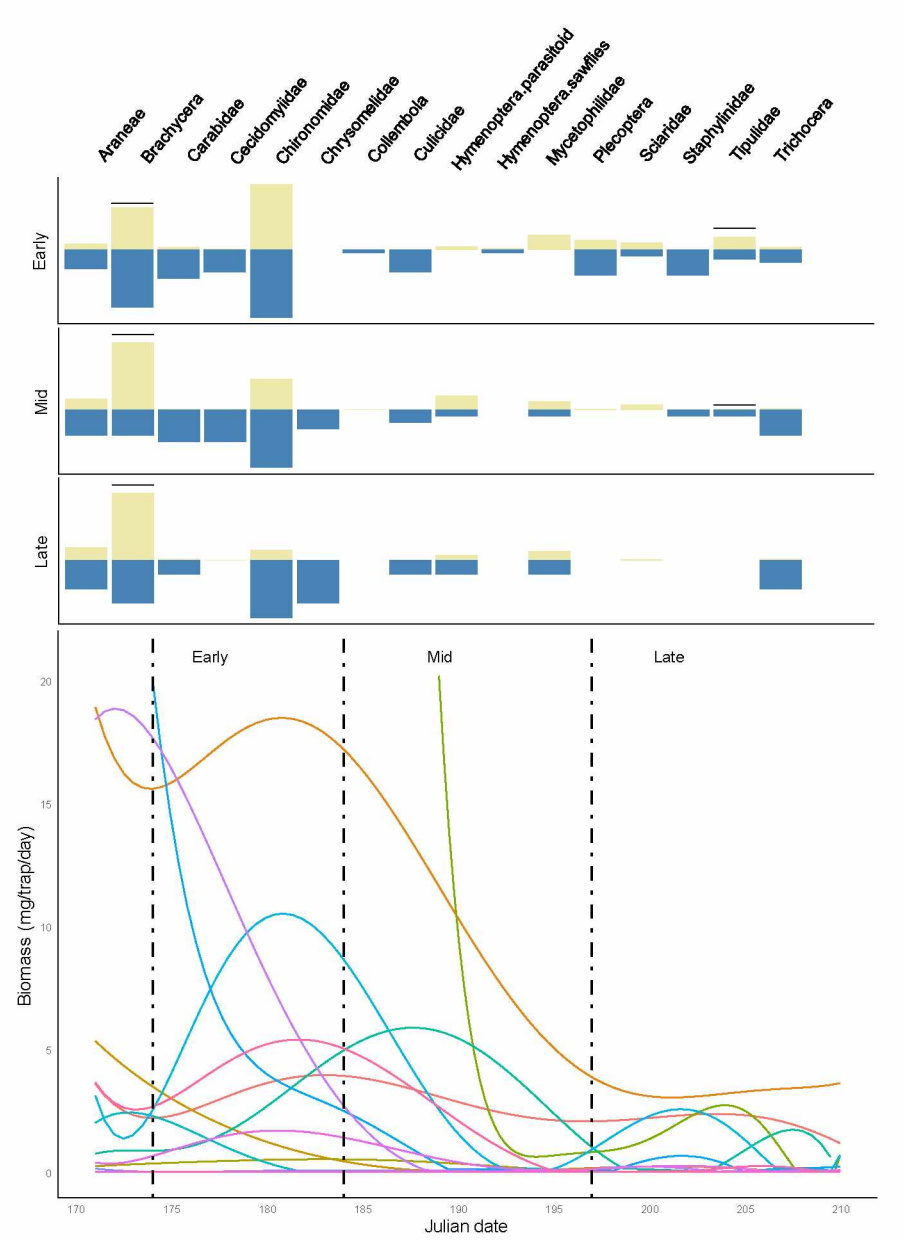

Figure 2.2 (Above) Proportion of arthropod biomass sampled in pitfall traps (in tan) during early-, mid- and late summer of 2015 compared to the proportion of occurrence of those prey detected in feces of Red Phalarope and Pectoral Sandpiper young (in blue). Black bars indicate the arthropod biomass had prey size not been accounted for chicks <5 days old (prey restricted to <9.7mm). For prey biomass >0, a constant was added for visibility in the figure. **(Below)** Best fit lines of arthropod biomass over the time period that feces were collected during the summer of 2015. Prey groups depicted are limited to those found in the chick feces and pitfall traps. The Julian date 170=June 19th.

Table 2.1 Fecal samples analyzed with DNA metabarcoding by shorebird species and year.

Species	No. fecal samples collected by year			No. individuals
	2014	2015	2016	All years
Red Phalarope	9	26	18	48
Pectoral Sandpiper	0	33	12	38
Dunlin	26	0	16	36

Table 2.2 Prey families in the diet of Red Phalarope, Pectoral Sandpiper, and Dunlin young in Utqiaġvik determined through DNA metabarcoding analysis of feces (DNA) and gut content analysis (Gut) (Holmes & Pitelka 1968, Holmes 1966a, S. F. MacLean unpub). The final column depicts arthropods present in the environment in the 1940-1960s (Hist) (Weber 1950, MacLean and Pitelka 1971), and currently (Now) present on the Arctic Coastal Plain of Alaska (Lackman pers comm, BOLD reference database).

Class	Order	Family	Red Phalarope		Pectoral Sandpiper		Dunlin		Molecular marker		Environment	
			DNA	Gut	DNA	Gut	DNA	Gut	CO1	16s	Hist	Now
Insecta	Diptera	Chironomidae	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
		Sciaridae	✓				✓	✓		✓	✓	✓
		Muscidae	✓		✓		✓	✓	✓		✓	✓
		Anthomyiidae			✓		✓	✓		✓	✓	✓
		Mycetophilidae			✓		✓	✓	✓	✓	✓	✓
		Tipulidae	✓	✓	✓	✓		✓	✓		✓	✓
		Trichoceridae	✓		✓		✓		✓	✓	✓	✓
		Cecidomyiidae	✓		✓				✓		✓	✓
		Culicidae	✓		✓		✓		✓	✓	✓	✓
		Empididae	✓		✓				✓		✓	✓
		Syrphidae			✓				✓			✓
		Bolitophilidae			✓				✓			✓
		Dolichopodidae	✓						✓		✓	✓
		Scathophagidae					✓		✓			✓
	Coleoptera	Dytiscidae	✓		✓		✓		✓	✓	✓	✓
		Staphylinidae	✓		✓	✓	✓	✓	✓	✓	✓	✓
		Chrysomelidae	✓		✓		✓	✓		✓	✓	✓
		Carabidae	✓		✓	✓	✓	✓	✓		✓	✓
		Melyridae			✓				✓			
	Hymenoptera	Ichneumonidae			✓				✓		✓	✓
		Tenthredinidae	✓						✓		✓	✓

Table 2.2 continued…

		Braconidae			✓			✓		✓	✓
	Trichoptera	Limnephilidae		✓			✓			✓	✓
	Plecoptera	Nemouridae	✓		✓	✓			✓	✓	✓
Arachnida	Araneae	Linyphiidae	✓		✓	✓	✓	✓		✓	✓
		Philodromidae			✓			✓			✓
		Erythraeidae				✓				✓	✓
	Sarcoptiformes	^Crotoniidae			✓			✓			
Collembola	Symphypleona	Sminthurididae	✓					✓			✓

^Taxon found in northern Canada (BOLD reference database).

Table 2.3 Permutational multivariate analysis (perMANOVA) testing for differences in prey composition in feces collected during the summer of 2015 between shorebird species (Red Phalarope and Pectoral Sandpiper), collection date, and chick age.

Blocking factor	Fixed effect	R^2	P
Shorebird species	Sample collection date	0.50	0.15
	Age group	0.06	0.04
Age group	Sample collection date	0.53	0.07
	Shorebird species	0.05	0.05

APPENDICES

Appendix 2-A

Genetic methodologies

Generation of Arthropod DNA References

Arthropods were opportunistically collected by field crews in Utqiaġvik during the summers of 2013 and 2014. Specimens were stored in 70-100% ethanol. DNA was extracted from arthropod specimens collected in Utqiaġvik using either HotSHOT or PureGene DNA extraction kits, followed by PCR amplification primers targeting the cytochrome c oxidase subunit 1 region (CO1). Primers included LCO1490, HC02198, Chelicerate_R2, C1-J-1718_USNM, and TL2-N-3014 (Simon et al. 1994, Barrett and Hebert 2005, Zhou et al. 2009). Primers were selected based on taxon specificity and for overlap with the metabarcoding region for primers (ZBJ-ArtF1c, ZBJ-ArtR2c) (Zeale et al. 2011). PCR products were visualized on a 1.5 agarose gel for presence of the correct length DNA fragment followed by sanger sequencing with Eurofins Genomics. Paired-end forward and reverse sequences were merged into contigs, removed of locus specific primer, and screened for a minimum Phred score of Q20 over the entire length of the sequence in CodonCode aligner. Sequences were aligned with Clustal Omega multiple sequence alignment to check for gaps (Sievers et al. 2011), and a minimum of 100 base pairs overlap with the ZBJ region. Sequences were then assigned to species based on a 98% match to the top hit in the Barcode of Life Database (Zhou et al. 2009). References with <98% match were assigned taxonomy using phylogeny-based inference using a Bayesian assignment with the Statistical Assignment Package (SAP) version 1.9.8 (Munch et al. 2008b, Wilson et al. 2011, Leray et al. 2013). Homologs were compiled from sequences available in NCBI sharing sequence identity of ≥80% to build unrooted phylogenetic trees. A posterior probability of 95% was used for OTU taxonomic assignments (Munch et al. 2008a).

DNA isolation, PCR amplification and high-throughput amplicon sequencing

Prior to extraction ≤0.25 g of shorebird chick fecal matter was decanted of ethanol preservative. Total genomic DNA was extracted from feces following the MoBio silicon-based PowerSoil DNA Isolation Kit following the manufacturer's protocol. A bead beating step was included run

on a Retsch Mixer-Mill 300 at 20 Hz for 10 minutes. The sample plate was then rotated horizontally and run again for 10 minutes, as recommended by MoBio for samples that require stronger homogenization. The final elution volume in 10 uM Tris was reduced to 25 µL given the expectation of low yield. A subset of extracts were quantified for the presence of DNA using dsDNA high sensitivity assay kit on a Qubit 2.0 fluorometer and measured for quality with NanoDrop 1000 spectrophotometry.

Extracted DNA was used for PCR-based amplification using primer pair (ZBJ-ArtF1c, ZBJ-ArtR2c) targeting segments of the CO1 gene and primer pair (16S1F-degenerate, 16S1R-degenerate) targeting 16s coding regions (Zeale et al. 2011, Deagle et al. 2007, Gerwing et al. 2016). Primers were selected for combined coverage of 11 orders of invertebrates and for targeting short fragments for working with degraded DNA (Deagle et al. 2006, Zeale et al. 2011, Elbrecht et al. 2016, Gerwing et al. 2016). Neither primer sets were reported to amplify bird DNA which, if present, can reduce target DNA amplification (Vestheim and Jarman 2008). To address variation in detection of prey among primers (Clarke et al. 2014b), we combined information gained from the 16s maker which amplifies a wider spectrum of life (Mueller 2006), with the CO1 maker which has a more extensive reference database (Folmer et al. 1994, Deagle et al. 2014).

Amplicon products were indexed using custom designed iTru fusion primers. PCR conditions for the CO1 marker were: 6 µl of DNA template, 1X of Phusion Green Hotstart II High Fidelity master mix (Thermo Scientific), 3.5 mM Mg^{2+}, 2X of DMSO, 0.5 uM of forward and reverse primers in a 20 µl reaction volume. Temperature cycling conditions were 3 min at 98 °C, followed by 35 cycles of 30s at 98°C, 30s at 45°C, and 15s at 72°C, and a final extension for 10 min at 72°C (modified from Gerwing et al. 2016). PCR conditions for the 16s marker were: 4 µl of DNA template, 1X Phusion Green HF buffer (Thermo Scientific), 0.2 mM dNTP's, 5 mM Mg^{2+}, 1X of BSA, 0.05 U/µl of Phusion Hotstart II DNA Polymerase (Thermo Scientific), and 0.5 uM of forward and reverse iTru fusion primers in a 25 µl reaction volume. Temperature cycling conditions were: 2 min at 94C followed by 35 cycles of 30s at 94C, 30s at 58C, 45s at 68C, and a final extension for 10 min at 68C (modified from Gerwing et al. 2016). PCR products were run on 1.8 agarose gel, normalized with SequalPrep Normalization Plate Kit, pooled, and removed of non-target primer artifacts with HighPrep size-selective SPRI beads (MAGBIO). A second PCR incorporating Illumina adapters and indexes was carried out in triplicate with non-

diluted libraries, and dilutions of 1.5x and 3x followed by a SPRI bead clean up. Amplicons were checked for product size with quantitative PCR and on a bioanalyzer, followed by paired-end sequencing using 300 cycles on an Illumina MiSeq platform following the manufacturer's protocol.

Bioinformatics and OTU identification

DNA sequences were demultiplexed using Mr_demuxy 1.2.0 (Cock et al. 2009). Paired-end reads were merged with Usearch 9.2.64 fastq_mergepairs, primer regions removed using Cutadapt version 1.12 (Martin et al. 2011), and then filtered keeping reads with a maximum expected error rate of 1 (Edgar and Flyvbjerg 2015). Reads not passing filters of expected fragment length were removed from the analysis, followed by dereplication with fastx_uniques, and screening for chimeras with uchime2_denovo. Sequences were clustered into operational taxonomic units (OTUs) with an identity threshold of 97% (Edgar 2013). OTUs were aligned with Blastn 2.2.26 to all available arthropod sequences from the Barcode of Life (BOLD). Taxonomy was assigned using the following criteria: >85 nucleotides in alignment length (Shokralla et al. 2015), 98% minimum identity to the top hit (Zhou et al. 2009), representation by more than one sequence within a prey family, and a 60 minimum bit score (modified from Gerwing et al. 2016). Phylogeny-based inference was used to assign OTUs (<97% identity) to family using Bayesian assignment with the Statistical Assignment Package (SAP) version 1.9.8 when species barcode refences were unavailable (Wilson et al 2011, Munch et al 2008, Leray et al. 2013). For each OTU assignment, homologs were compiled from the NCBI public genomic database by SAP sharing sequence identity ≥80% to build unrooted phylogenetic trees. A posterior probability of 95% was used for OTU taxonomic assignments (Munch et al. 2008a). Sequence analyses were carried out on the Arctic Region Supercomputing cluster system.

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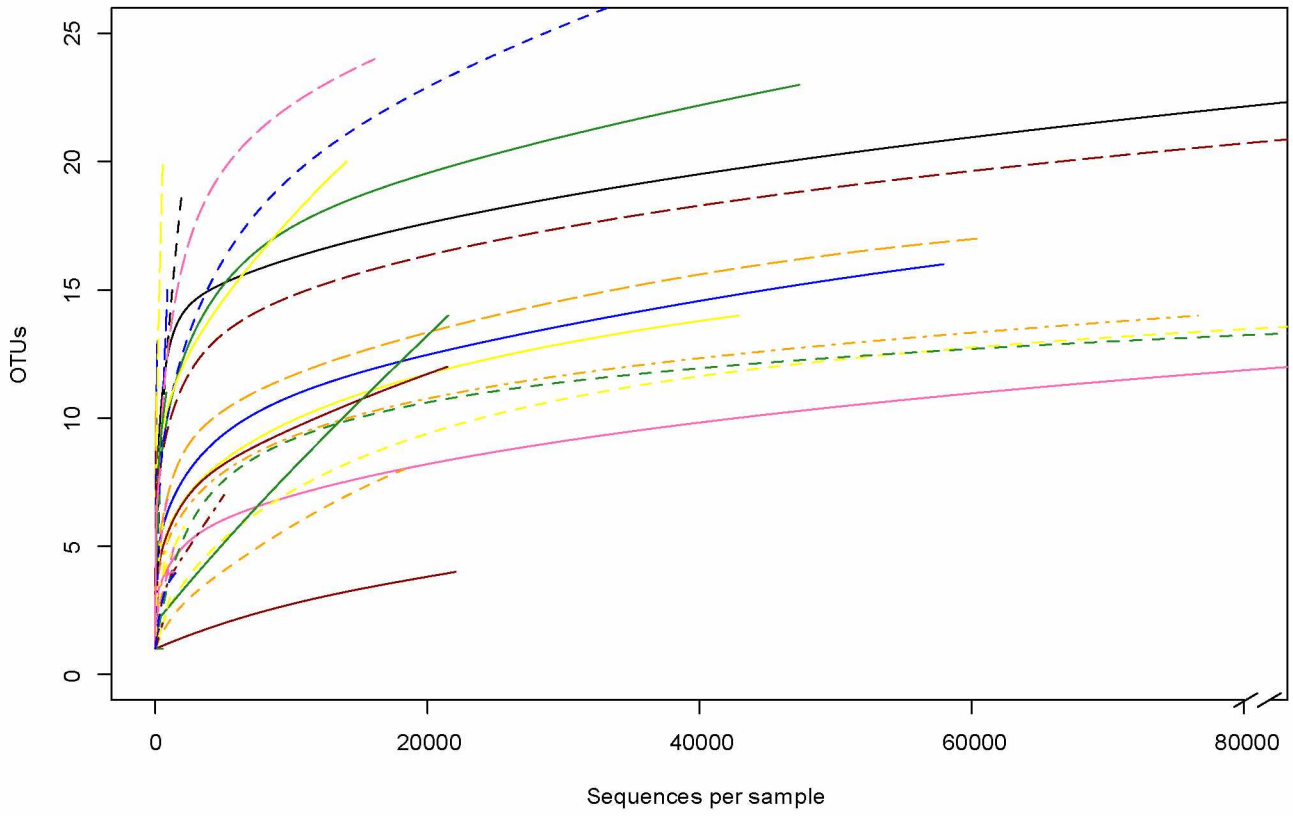

Figure 2-A.1 Sample-based rarefaction of operational taxonomic units (OTUs) in the feces of Red Phalarope, Pectoral Sandpiper and Dunlin young.

Table 2-A.1 DNA references generated from arthropods collected in Utqiaġvik, Alaska for the CO1 marker.

Class	Order	Family	Genus species	Common name	Percent similarity
Arachnida	Araneae	Linyphiidae	*Hilaira proletaria*	dwarf-weaver spider	99.83
			Hilaira vexatrix	dwarf-weaver spider	99.83
			Hilaira vexatrix	dwarf-weaver spider	99.65
			Silometopoides pampia	dwarf-weaver spider	100
			Silometopoides pampia	dwarf-weaver spider	99.82
			Silometopoides pampia	dwarf-weaver spider	99.84
			Diplocephalus barbiger	bearded sheet-web weaver	99
			Erigone psychrophila	dwarf-weaver spider	100
			Gibothorax tchernovi	dwarf-weaver spider	100
			Hybauchenidium aquilonare	dwarf-weaver spider	99.83
			Hybauchenidium aquilonare	dwarf-weaver spider	99.68
			Hybauchenidium aquilonare	dwarf-weaver spider	100
			Masikia indistincta	dwarf-weaver spider	99.32
			Unassigned sp.[1]		94
Insecta	Diptera	Mycentophilidae	*Exechia frigida*	fungus gnat	100
			Exechia sp.	fungus gnat	100
			Exchia sp.	fungus gnat	96.74
			Exechia sp. GS01	fungus gnat	99.46
			Phronia egregia	fungus gnat	99.12
		Empididae	*Clinocera nivalis*	dagger fly	100
			Clinocera nivalis	dagger fly	98.73
		Tipulidae			93.37
		Calliphoridae	*Protophormia atriceps*	blow fly	100
			Protophormia atriceps	blow fly	99.73
		Dolichopodidae	*Hydrophorus alpinus*	long-legged fly	100
			Hydrophorus alpinus		99.15
			Hydrophorus alpinus		99.74

Table 2-A.1 Continued…

Alphaproteobacteria	Rickettsiales	Rickettsiacae	*Wolbachia*[2]	proteobacteria	100

[1] DNA references without representative species matches in public genomic databases NCBI or BOLD were assigned to the finest taxonomic level possible using phylogeny-based inference with the Statistical Assignment Package.

[2]Proteobacteria *Wolbachia,* known to infect arthropods (Hilgenboecker et al. 2008), was PCR amplified from an arthropod specimen.

Table 2-A.2 Bioinformatics analysis of DNA sequences PCR amplified with mitochondrial markers (CO1 and 16s) from feces of Red Phalarope, Pectoral Sandpiper and Dunlin young.

Locus	Total paired-end reads ($\bar{X}$ ± SD per sample)	Merged paired-end reads	Trimmed of F & R primer	Quality & length filtered	Unique sequences	Singletons	Chimeras	Total OTUs	Assigned OTUs ($\bar{X}$ per fecal sample yielding DNA)	Average sequences per fecal sample yielding DNA
16s	2,708,905 (26,548.65 ±15,602.3)	2,596,002 (95.83 %)	2,275,097 (87.64%)	918,822 (40.39%)	88,123	65,392	611	970	*SAP: 343 BLAST: 1 (3.7)	1,529
CO1	3,342,201 (39,246.52 ±120,546.1)	3,222,893 (96.43%)	3,216,944 (99.82%)	3,092,574 (96.13%)	14,118	6,912	269	176	SAP: 37 BLAST: 80 (7.1)	45,146

*Non-arthropod OTUs identified included only sandpiper (Scolopacidae).

Appendix 2-B

Prey detected in feces of shorebird young

Table 2-B.1 Arthropod taxa detected in feces with DNA metabarcoding for three species of shorebird young: Red Phalarope (REPH), Pectoral Sandpiper (PESA) and Dunlin (DUNL). For each shorebird species *n* represents the number of fecal samples that yielded arthropod DNA.

Class	Order	Family	Genus Species	Common Name	REPH (*n*=41)	PESA (*n*=34)	DUNL (*n*=17)
Insecta	Diptera	Chironomidae	*Cricotopus tibialis*	non-biting midge	✓	✓	
			Cricotopus sp. 4ES	non-biting midge	✓		
			Cricotopus sp. 8ES	non-biting midge	✓		
			Cricotopus tricinctus	non-biting midge	✓		
			Cricotopus	non-biting midge	✓	✓	
			Psectrocladius oxyura	non-biting midge	✓	✓	
			Psectrocladius fennicus	non-biting midge		✓	
			Procladius dentus	non-biting midge	✓	✓	✓
			Paratanytarsus	non-biting midge	✓		
			Chironomus sp. 1TE	non-biting midge	✓	✓	✓
			Chironomus sp. TE11	non-biting midge	✓	✓	
			Chironomus pilicornis	non-biting midge		✓	
			Orthocladius sp. TE13	non-biting midge	✓	✓	✓
			Orthocladius subletteorum	non-biting midge		✓	
			Orthocladius	non-biting midge	✓	✓	
			Orthocladiinae	non-biting midge	✓	✓	
			Tanytarsus telmaticus	non-biting midge		✓	
			Tanytarsus	non-biting midge	✓	✓	
			Tanytarsus inaequalis	non-biting midge		✓	

Table 2-B.1 Continued…

		Metriocnemus sp. 1ES	non-biting midge		✓	✓
		Sergentia sp. 2TE	non-biting midge	✓		
		Allocladius sp. 1ES	non-biting midge		✓	
		Limnophyes asquamatus	non-biting midge		✓	
		Smittia	non-biting midge			✓
		Corynoneura	non-biting midge	✓		
		(subfamily: Chironominae)	non-biting midge		✓	
		Unassigned	non-biting midge	✓	✓	✓
		Unassigned	winter crane flies	✓	✓	
	Muscidae	*Spilogona atrisquamula*	house flies	✓	✓	✓
		Spilogona suspecta	house flies	✓	✓	
		Spilogona trigonata	house flies	✓		
		Spilogona tundrae	house flies	✓		
		Spilogona	house flies	✓		✓
	Cecidomyiidae	*Unassigned*	gall midge	✓	✓	
	Bolitophilidae	*Unassigned*	gall midge		✓	
	Empididae	*Clinocera nivalis*	dagger flies	✓	✓	
	Mycetophilidae	*Boletina*	fungus gnat		✓	✓
	Culicidae	*Aedes*	mosquito	✓	✓	✓
		Aedes impiger	mosquito	✓	✓	✓
	Syrphidae	*Platycheirus scambus*	hover fly		✓	
		Platycheirus scamboides	hover fly		✓	
	Sciaridae	*Unassigned*	fungus gnat	✓		✓
	Dolichopodidae	*Hydrophorus altivagus*	long-legged fly	✓		
	Anthomyiidae	*Unassigned*	root-maggot fly		✓	✓
	Scathophagidae	*Gonarcticus arcticus*	dung flies			✓
	Tipulidae	*Prionocera ominosa*	crane fly	✓	✓	
		Tipula limbata	crane fly	✓		
		Angarotipula tumidicornis	crane fly		✓	
Coleoptera	Dytiscidae	*Colymbetes dolabratus*	water beetle		✓	

Table 2-B.1 Continued…

			Colymbetes densus	water beetle		✓	
			Agabus congener	water beetle	✓	✓	✓
		Staphylinidae	*Boreostiba sibirica*	rove beetle		✓	
			Unassigned	rove beetle	✓	✓	✓
		Carabidae	*Pterostichus pinguedineus*	ground beetle	✓	✓	✓
			Pterostichus	ground beetle		✓	
		Chrysomelidae	*Unassigned*	leaf beetle	✓	✓	✓
		Melyridae		soft-winged flower beetle		✓	
	Hymenoptera	Ichneumonidae	*Phygadeuon trichops*	parasitoid wasp		✓	
		Tenthredinidae	*Pachynematus vagus*	sawflies	✓		
		Braconidae	*Microplitis sp.*	parasitoid wasp		✓	
		Unassigned	*Unassigned*	membrane-winged insect			✓
	Plecoptera	Nemouridae	*Unassigned*	stonefly	✓	✓	✓
Arachnida	Araneae	Linyphiidae	*Gibothorax tchernovi*	dwarf-weaver spider	✓	✓	✓
			*Hilaira proletaria**	dwarf-weaver spider	✓	✓	✓
			*Silometopoides pampia**	dwarf-weaver spider		✓	
			*Unknown**	dwarf-weaver spider		✓	
			Erigone psychrophila	dwarf-weaver spider			✓
			Masikia indistincta	dwarf-weaver spider	✓	✓	
		Philodromidae	*Tibellus maritimus*	slender carb spider		✓	
			Thanatus striatus	crab spider		✓	
		Unassigned	*Unassigned*	spider sp.		✓	
	Sarcoptiformes	Crotoniidae	*Neonothrus sp. nov.*	mite		✓	
Collembola	Symphypleona	Sminthurididae	*Sminthurides aquaticus L2*	springtail	✓		

*Project generated Arthropod DNA references

Appendix 2-C

Captive shorebird prey size selection

We determined the size of prey selected by chicks as they aged through feeding experiments of captive-reared Red Phalarope and Pectoral Sandpiper young. Sixteen eggs (13, Red Phalarope and 3, Pectoral Sandpiper) were collected in proximity to Utqiaġvik, one egg per nest, and artificially incubated eggs until hatch. Leg bands were attached to chicks after hatch using distinct color combinations to uniquely mark individuals. Chicks were reared in indoor facilities during the summer of 2015 in Utqiaġvik, Alaska and provided with heat lamps until they were old enough to self-thermoregulate, at which time they were housed at ambient outdoor temperatures. Food (hydrated poultry and cat feed, *Tenebrio* mini mealworms, Pyralid wax worms, boiled egg whites, pinhead crickets, *Drosophila*, and arthropods from the environment), and water was provided *ad libitum* as a regular diet.

Feeding experiments were carried out in indoor facilities and consisted of observing shorebird chicks offered experimental arthropod prey. Experimental prey items were introduced into the chick's holding area on trays and chicks were then observed for 20 minutes to determine which prey were consumed. Multiple chicks were included in each feeding trial because chicks required the presence of other chicks to maintain normal feeding behaviors. Data were grouped by chick age classes (2-4, 5-7, 8-10, and 11-16 days), where prey that were not selected were included in all age classes for which chicks belonging to the age class were present. The experimental taxa included: Chironomidae (adult and larvae), Coleoptera; Dysicidae (adult), Brachycera (adult), Plecoptera (larvae), Tipulidae (adult and larvae), Araneae, and Trichoptera (larvae). All prey items were collected opportunistically in Utqiaġvik through sweep net and aquatic sampling. Prior to feeding experiments, experimental prey items were photographed. Photo images were later uploaded later into ImageJ software (version 1.49) to measure body lengths of prey.

A total of 39 experimental feeding trials were performed, during which 307 arthropod individuals ranging from 1.6 to 22.8 mm in length were offered to chicks 2-4,5-7, 8-10, 11-16 days old. An electivity index was calculated for each prey length, rounded to the nearest whole number, among each age group using the equation: $E = (r-p)/(r+ p)$, where r = the fraction of the prey size class selected, and p = fraction of that same prey size class offered (Ieno et al. 2004).

Based on this analysis, chicks aged 2-4 days selected for prey sizes up to 10 mm (rounded from 9.7), chicks 5-7 selected for prey sizes up to 18 mm (rounded 17.7), chicks 8-10 days old selected for prey sizes up to 18 mm (rounded from 17.8), while chicks 11-16 days selected for prey sizes up to 21 mm (rounded from 21.4) (Figure 2-C.1 in Appendix 2-C). By species, the maximum prey size selected for chicks 2-4 days old was 7.3 mm for Red Phalarope and 9.7 for Pectoral Sandpiper. By age 5 both species selected for prey greater than 10 mm. These results were not due to the way we provided prey to chicks as there were no differences in the lengths of prey offered to the different age groups (one-way ANOVA, $F= 0.33$, $P=0.81$) which could confound size selection analyses using an electivity index if biased (Chesson 1983).

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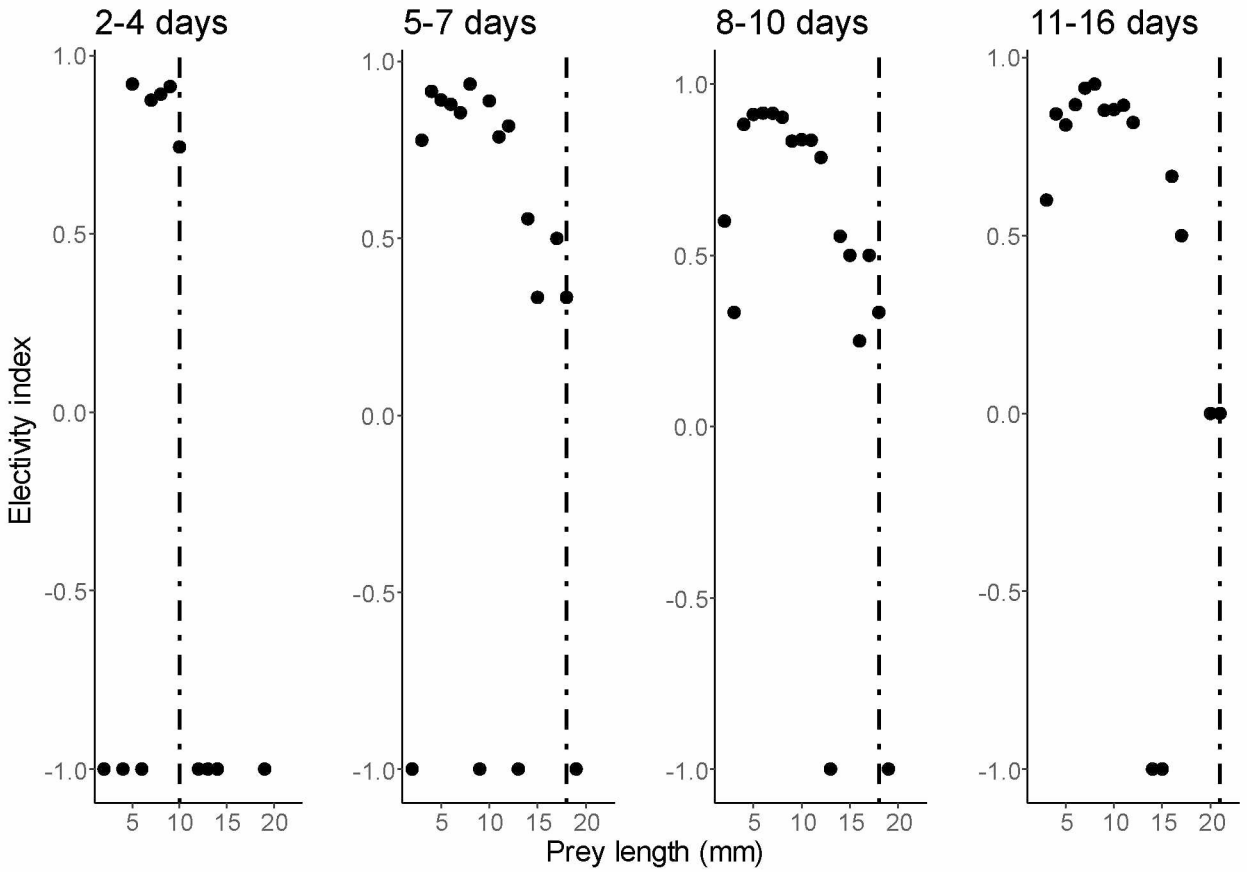

Figure 2-C.1 Electivity index describing prey size selection by captive Red Phalarope and Pectoral Sandpiper chicks that were 2-4, 5-7, 8-10, and 11-16 days old. A positive electivity index indicates a size preference, 0 indicates no preference, and a negative value indicates prey size rejection. Dashed lines indicate the maximum prey size selected in each age class rounded to the nearest whole number.

Table 2-C.1 Length range of arthropods collected in mesic and xeric invertebrate pitfall traps in Utqiaġvik during the summer of 2015.

Arthropod taxa	Length range (mm)
Araneae	1-7
Brachycera	2-13
Chironomidae	1-10
Collembola	1-3.5
Cecidomyiidae	1-3.5
Hymenoptera parasitoid	1-9
Sciaridae	2.5-4
Ceratopogonidae	2
Psychodidae	1-3
Mycetophilidae	3-5
Trichocera	3-6
Culicidae	5-7
Carabidae	4-8
Staphylinidae	3-8
Saldidae	3-6
Lepidoptera	15
Hymenoptera sawflies	3-14
Plecoptera	4-8
Trichoptera	4-8
Tipulidae	6-17
Chrysomelidae	9-16
Curculionidae	1.5-2

Appendix 2-D

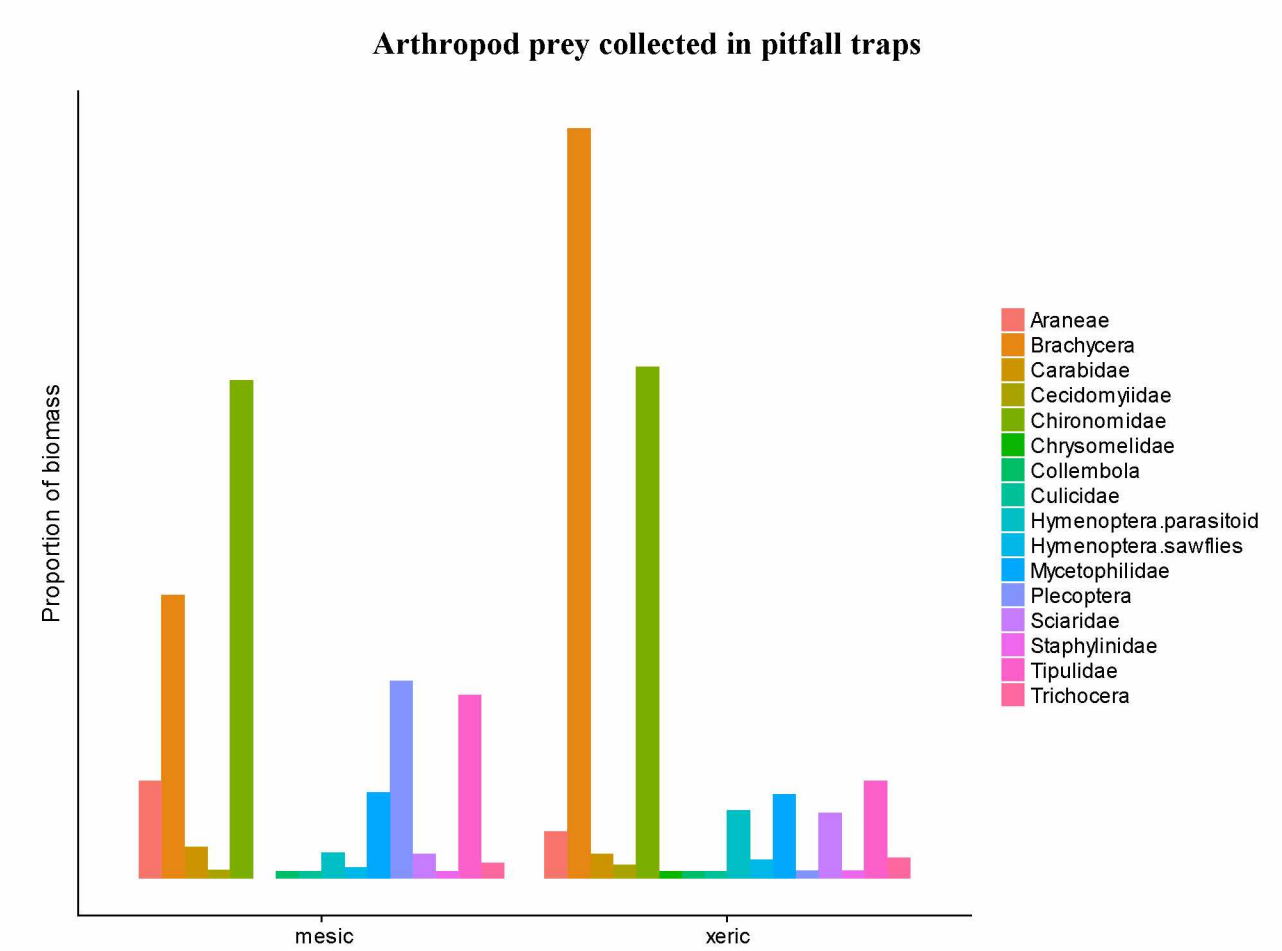

Figure 2-D.1. Proportion of prey biomass by habitat collected in modified malaise pitfall traps during the summer of 2015. For prey biomass >0, a constant was added for visibility in the figure. Prey were limited to those groups found in chick diets.

GENERAL CONCLUSIONS

My research provides a characterization of diet for the young of three shorebird species including Red Phalarope, Pectoral Sandpiper and Dunlin using fecal DNA metabarcoding. Potential biases of the molecular technique were examined through captive feeding experiments with shorebird young. Through combined information from two molecular markers (CO1 and 16s), these experiments showed that prey items were detected in chick feces nearly every time they were consumed. Technical bias associated with the chick's age, its feces or the quantity of prey eaten was not detected; however, differences were observed in the detection of prey among taxa and between molecular markers. When DNA metabarcoding was applied to characterize the diets of wild shorebird young, novel prey families were detected, previously undocumented in gut content analyses. Secondary consumption proved to be a limitation of the molecular technique because the method cannot differentiate detection of DNA from prey consumed by the prey of the target organism. I tested whether fecal DNA metabarcoding traced chick diets through time by comparing the composition of prey in chick feces during a season to the composition of arthropods in the environment collected by pitfall traps. I found that arthropod prey of shorebird young was generally a reflection of prey collected in pitfall traps for the major prey groups in chick diets. This suggests that arthropod pitfall trapping can be used as method to assess the presence and impacts of trophic mismatch on shorebird young. Below is a discussion of how these results fit into ongoing research on the fecal DNA metabarcoding techniques and shorebird chick diets pertaining to trophic mismatch.

DNA metabarcoding provided a minimally invasive means to inventory and track changes in shorebird chick diet over the course of a summer season. With the aid of a well-developed library of DNA sequences for Arctic arthropods, the molecular technique identified 62 genetically distinct species in shorebird feces, a leap in diversity from previous gut content analyses. Captive feeding experiments with shorebird young provided confidence in the technique's effectiveness, as well as an idea of the limitations of the molecularly derived dataset. Some level of PCR bias is present in all DNA metabarcoding studies, but can be mitigated through careful experimental design (Elbrecht et al. 2016).

Molecular methods continue to move away from PCR as sequencing technologies become sensitive enough to eliminate this step. Shotgun sequencing, the use of probes to target conserved regions of DNA and sequence capture methods (Mason et al. 2011, Zhou et al. 2013,

Liu et al. 2015, Dowle et al. 2016) could ultimately eliminate the effects of PCR-based bias. In the meantime, applications of DNA metabarcoding for monitoring and inventory of biodiversity is being rapidly adopted (Bohmann et al. 2014, Tang et al. 2015, Aylagas et al. 2016). Work is underway to develop quantitative applications for DNA metabarcoding (Deagle and Tollit 2007, Bowles et al. 2011, Saitoh et al. 2016, Thomas et al. 2016). Here, we contribute new data to a growing body of information to understand and establish molecular diet characterization methods.

DNA metabarcoding analysis suggested that the wild shorebird young examined were able to eat a wide array of prey types and could adjust to changing prey resources during the summer by shifting their diet. Whether dietary plasticity is sufficient to buffer climate induced changes in prey resources will depend on whether prey is present in sufficient quantities to allow for adequate growth of shorebird young. The effects of climate change on shorebird prey may vary spatially (Tulp and Schekkerman 2008, Bolduc et al. 2013, Reneerkens et al. 2016), being particularly pronounced in the high Arctic (Meltofte et al. 2007a) where the timing of emergence may be condensed to the point of limiting chick growth if mistimed (Saalfeld et al. *in press*).

Food availability may be an important driver in multiple aspects of shorebird breeding success such as chronology, nest site selection, distributions, and movements (Holmes 1966, Meltofte et al. 2007a). It is unclear whether climate shifts will improve or exacerbate the availability of prey resources for shorebirds. The response of Arctic arthropods to climate change is complex and will likely vary by taxa (Ellwood et al. 2012, Høye and Sikes 2013, Bowden et al. 2015, Loboda et al. 2017). This could be important in terms of shorebird chick food because not all arthropods have equal nutritional and caloric value (Razeng and Watson 2015, Twining et al. 2016). Interannual variation in the abundance of arthropod prey may be a larger driver of chick growth than the timing of prey availability (Reneerkens et al. 2016). Longer and warmer summers mediated by changes in climate may reduce thermoregulatory costs for chicks (McKinnon et al. 2013b), and could increase the length of time when food is sufficiently abundant for chick growth. The amount of time chicks have to forage could increase if the chicks required less brooding by adults (Krijgsveld et al. 2003). Shorebirds are highly abundant, feeding at the same trophic level across the Arctic and therefore serve as an indicator species of change in Arctic ecosystems. Understanding threats to shorebirds is basic for broad enquiries on climate impacts in the Arctic (Piersma and Lindström 2004).

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