1 Feeding by *Pseudocalanus* copepods in the Bering Sea: trophic linkages and a potential
2 mechanism of niche partitioning

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6 Running head: *Pseudocalanus* feeding in the Bering Sea

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8 Abstract

9 *Pseudocalanus* copepods are small and abundant zooplankton in the Bering Sea
10 ecosystem which play an important role in transferring primary production to fish and other
11 higher trophic level predators. Four morphologically cryptic species, the primarily arctic *P.*
12 *minutus* and *P. acuspes*, and the more temperate *P. newmani* and *P. mimus*, are found within the
13 Bering Sea. *Pseudocalanus* are generally considered phytoplanktivores. However, their feeding
14 is poorly known, despite their importance to the ecosystem. *In situ* feeding by the three most
15 abundant *Pseudocalanus* congeners, *P. minutus*, *P. newmani*, and *P. acuspes*, was investigated
16 by sequencing partial 18S rDNA (ribosomal Deoxyribonucleic Acid) of gut contents from 225
17 individuals sampled from 8 stations across the Bering Sea in May and June of 2010. The 28,456
18 prey 18S rDNA sequences obtained clustered into 138 distinct prey items with a 97% similarity
19 cut-off, and included diatoms, dinoflagellates, microzooplankton, mesozooplankton, and
20 vascular plants. *Pseudocalanus* diets reflected variations in the environment, with phytoplankton
21 sequences relatively more abundant in copepods from stations with higher water column
22 chlorophyll a concentrations. Feeding differences were observed between species. *P. acuspes*
23 diet contained relatively more heterotrophic dinoflagellate sequences, and was significantly
24 different from that of *P. minutus* and *P. newmani*, which both contained relatively more diatom
25 sequences, and between which no significant difference was observed. Feeding differences
26 between the two primarily arctic species may be a mechanism of niche partitioning between
27 these spatially co-located congeners and may have implications for the effects of climate change
28 on the success of these abundant zooplankters and their many predators in this ecosystem.

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Keywords: *Pseudocalanus*, Bering Sea, trophic interactions, niche partitioning, 18S rDNA

1. Introduction

The competitive exclusion principle in ecological theory suggests that no two sympatric species can occupy precisely the same ecological niche, as one will inevitably eventually outcompete the other (Gause 1934, Hardin 1960). In the Bering Sea, four morphologically cryptic species of *Pseudocalanus* copepods have been identified (Frost 1989, Bailey et al. this issue), which at first glance appear to violate this principle. These four species are similar in both size and overall morphology and traditional morphometric and meristic traits are often unsuccessful at differentiating among them, although they are genetically distinct and can be distinguished by DNA sequencing (Frost 1989, Bailey 2012, Bailey et al. this issue). *Pseudocalanus minutus* and *P. acuspes* are considered primarily arctic, while *P. newmani* and *P. mimus* are considered primarily temperate, but all four species ranges overlap in the Bering Sea (Frost 1989, Coyle et al. 2011). In order for these species to persist in their coexistence in the Bering Sea, they must be different from each other in at least one ecologically meaningful way, such as susceptibility to disease or predators, timing of reproduction, or feeding.

As a location for investigating copepod niche partitioning, the Bering Sea is particularly interesting as it is a region with high levels of interannual variation and alternating 4 to 5 year periods of relatively warm and cool temperatures (Stabeno et al. 2012). Changes in physical forcing may have bottom up ecosystem effects, such as by changing phytoplankton bloom dynamics (Stabeno et al. 2012, Winder & Sommer 2012). Warming has also been associated with a shift from a largely benthic ecosystem to a more pelagic dominated system, giving small

zooplankton such as *Pseudocalanus* spp. an increasingly important role in the transfer of carbon and energy from primary producers to pelagic higher predators (Overland & Staben0 2004).

Although *Pseudocalanus* spp. are small (1-2 mm in length), their abundance makes them important components of food webs and carbon cycling in the Bering Sea ecosystem (Frost 1989, Napp et al. 2002). *Pseudocalanus* are particularly important as prey for walleye pollock, *Theragra chalcogramma*, one of the most commercially important fish stocks in the US, since larval pollock diet consists of up to 60% small copepods (Coyle et al. 2011). Understanding the range of prey consumed by *Pseudocalanus in situ* and the relative importance of different prey types is essential to understanding the role this abundant consumer plays in transferring primary production to higher trophic levels.

Little is known about the specific prey types consumed by *Pseudocalanus in situ*; results published to date have lumped all *Pseudocalanus* species together (Lebour 1922, Marshall 1949, Poulet 1973). Incubation experiments in natural seawater and water from mesocosms have suggested that *Pseudocalanus* spp. copepods are able to feed effectively on particles from 4 to 102 μm diameter, with a potential preference for particles 25 to 57 μm (Poulet 1973, Harris 1982). Microscopic analysis of *Pseudocalanus* gut contents has shown that they consume diatoms, including *Coscinodiscus*, *Paralia*, *Navicula*, and *Thalassiosira* spp., and to a lesser extent, crustaceans, radiolarians, and flagellates (Lebour 1922, Marshall 1949).

The relative paucity of data on *Pseudocalanus* feeding is not surprising, since measuring feeding by small zooplankton is methodologically challenging. Previous work used incubation experiments and particle counters (Poulet 1973), but these may not be representative of feeding *in situ* and give fairly coarse resolution of prey type. Microscopic examination of gut contents

provides *in situ* data, but is strongly biased towards prey with distinctive exoskeletons and typically only limited samples can be analyzed by this labor intensive approach (Lebour 1922, Marshall 1949). Recent advances in DNA analysis and sequencing have allowed for new molecular approaches to understanding zooplankton feeding. DNA barcodes from gut contents can be sequenced, and by comparing these sequences to reference databases of known organisms, the identity of every known eukaryote the predator, in this case *Pseudocalanus*, had consumed in the preceding minutes can be determined (Cleary et al. 2012, Durbin et al. 2012, O’Rourke et al. 2012, Hu et al 2014, Craig et al. 2014). Unlike microscopy-based diet analysis, 18S rDNA sequencing allows for identification of the full range of eukaryotic prey items consumed, including soft bodied and morphologically indistinct prey. Feeding differences have been inferred as mechanisms of niche partitioning in other copepods (e.g. von Vaupel Klein 1997); however, in their study feeding was not directly measured, but rather assumed to be a function of body size and morphology.

This study examined 18S rDNA in *Pseudocalanus* gut contents to elucidate feeding by *P. minutus*, *P. newmani*, and *P. acuspes* in the eastern Bering Sea, to address questions of niche partitioning by these cryptic congeners and improve understanding of food webs and carbon flows through this ecosystem. The gut contents of 225 *Pseudocalanus* individuals from across the shelf and shelf break region were analyzed using Peptide Nucleic Acid (PNA) probes and a high throughput sequencing approach to determine what they had consumed, how environmental factors affected feeding, and whether there were differences in consumption between species which might serve as a mechanism of ecological niche separation.

2. Methods

2.1 Field sampling and species identification

Copepods and environmental data were collected in the eastern Bering Sea between May 19 and June 10, 2010. *Pseudocalanus* spp. copepods were collected in vertically integrated net tows from 60 m (or 1 m above the seafloor where depth <60 m) to the surface at 15-20 m min⁻¹ with a 153 µm mesh 1m ring net at 8 stations (Table 1 and Fig. 1). Mixed plankton samples were immediately preserved in 95% ethanol, to minimize effects of digestion and potential net feeding, and ethanol was changed once after 12 to 24 hours to maintain concentration (Passmore et al. 2006). Temperature, salinity and fluorescence profiles were obtained with a SBE 19+ CTD at the same stations as copepods were collected, with temperature and salinity at the depth of the *in situ* fluorescence maximum used in station comparisons. Total chlorophyll and chlorophyll >5µm were measured fluourometrically from extracted pigments of water collected at this same depth of maximum *in situ* fluorescence (M. Lomas, unpublished data). In the lab, individual *Pseudocalanus* spp. copepods were picked from mixed plankton samples under a dissecting microscope and rinsed thoroughly in clean 95% ethanol (Bailey et al. this issue). DNA was extracted from each whole individual using the DNeasy Blood and Tissue kit (Qiagen) (Bailey et al. this issue). Species identity of each copepod was determined through sequencing of cytochrome oxidase gene fragments by Bailey et al. (this issue).

2.2 Gut contents 18S rDNA amplification and sequencing

Peptide Nucleic Acid Polymerase Chain Reaction (PNA-PCR) was used to amplify the partial 18S rDNA of all eukaryotes in *Pseudocalanus* gut contents from pools of DNA extracts from 5 conspecific individuals from a single net tow (Cleary et al. 2012, O'Rourke et al. 2012). Triplicate pools were analyzed of each species at each of the stations, for 15 copepods in total from each species in each net tow where sufficient individuals were collected (Table 1). One technical replicate, with all stages of analysis after DNA extraction run separately, was run on

one of the pools of 5 copepods from *P. acuspes* at station 99, for an overall total of 46 samples. Each reaction contained 1x GoTaq Green master mix (Promega), 0.5 $\mu\text{mol L}^{-1}$ each 960F and 1200R primers (Gast et al. 2004), 20 $\mu\text{mol L}^{-1}$ PNA (5'-TGCTCAATCTCGTGCGAC-3'), and approximately 0.5 ng μL^{-1} template DNA. An initial denaturation at 95° C for 30s, was followed by 30 cycles of 94° for 30s, 77° for 30s, 58° for 30s, 60° for 45s, and a final extension at 60° for 5 min. To remove any remaining genomic DNA, amplicons were electrophoresed on a 0.8% agarose gel and the entire lane between approximately 2000 and 20 base pairs was excised with a sterile scalpel based on the migration of dye fronts of Crystal 5x DNA Loading Buffer Tri-Color (Bioline).

Amplicons were gel extracted and purified using the Wizard SV kit (Promega) as per manufacturer's directions. 454 sequencing adaptors and 8 base pair sample identification tags for each of the 46 samples were attached to the amplicons in a second PCR, containing 1x GoTaq Green master mix (Promega), 0.5 $\mu\text{mol L}^{-1}$ each 454-tag-1200Rrc Forward and 454-960Frc Reverse primers (modified from Gast et al. 2004), and 30% by volume 18S amplicons from the first round of the PCR. Reverse compliments of the PNA-PCR primers were used here in the addition of 454 adaptors to maximize sequencing resolution of the more variable 3' region of the amplicon. Following an initial denaturation at 95° C for 30s, 9 cycles of 94° for 30s, 65° for 30s, 72° for 45s, were run, with a final extension at 72° for 5 min. No-template controls were carried through all of the above steps along with each set of 6 samples, and visualized at the completion on a 0.8% agarose 1x TAE gel with ethidium bromide – ultra-violet light imaging to confirm the absence of contaminants.

454-tagged amplicons were purified using AmPure (Beckman-Coulter) and sample DNA concentration was quantified using the DNA 1000 kit on a 2100 Bioanalyzer (Agilent).

Equimolar aliquots of amplicons from each sample were combined into one of two template pools (23 samples per pool). Each template pool was sequenced in 1/16th pico-titer plate on a GS FLX+ 454 platform (Roche) at the University of Illinois WM Keck Center following standard protocols.

2.3 Sequence data analysis

Sequence data was denoised to remove sequencing errors (Quince et al. 2009, Edgar 2010), separated by sample tag, and clustered into Operational Taxonomic Units (OTUs) with 97% identity by UClust in MacQiime (Caporaso et al. 2010). A phylogenetic tree was constructed of all prey OTUs using Fasttree in MacQiime (Price et al. 2010) and visualized in FigTree v. 1.4. Subgroups of related prey OTUs from this tree were then combined with related known organisms from GenBank using known phylogeny and BLAST (Altschul et al. 1990) to create 11 sub-trees of OTUs and related organisms. General phylogeny of sub-trees was confirmed with literature comparisons (Baroin-Tourancheau et al. 1992, Collins 2002, Cavalier-Smith & Chao 2003, James et al. 2006).

For each OTU, the nearest taxonomic identity was determined from these sub-trees, and was typically chosen as the taxon into which both the nearest and second nearest neighbor were classified; in some instances where reference sequences were sparse and general tree topology agreed well with known phylogeny, only the nearest neighbor was used. This approach resulted in different degrees of specificity for different prey items. Literature searches were used to identify OTUs whose nearest taxonomic identity was known to be parasitic (Ho & Perkins 1985, Evans et al. 2008, Guo et al. 2012) and these OTUs were excluded from all subsequent dietary analyses. Additionally any OTUs for which the nearest taxonomic identity was *Pseudocalanus*,

suggesting the sequence originated from the copepod itself, or which clustered only with mammalian pseudogenes, suggesting potential trace human DNA contamination, were excluded from prey analysis.

All data were normalized by total sequence abundance per sample prior to analysis and a variety of transformations were tested. Multivariate statistics were run on the abundance of each OTU in each 5-copepod sample in Primer6 (Clark & Gorley 2006). Results are reported for both root-transformed abundance data and presence-absence transformed data. We present both statistical results, because while presence-absence results may be less subject to potential biases arising from variations in 18S rDNA copies per cell, such an analysis will tend to give disproportionately more weight to rare items (Clarke & Warwick 2001). In the case of pyrosequencing, where even very rare trace OTUs are detected, such a presence absence analysis may not provide the most ecologically meaningful comparisons. The relative sequence abundance analysis is similar to previous successful analysis of broadscale diet differences in larger predators (Jarman et al. 2013).

Bray-Curtis distances were calculated as a measure of the similarity of the prey assemblages recovered from each sample. Analysis of Similarity (ANOSIM) was used to determine differences between species and stations, and SIMPER analysis was used to determine which prey items drove observed differences. BioEnv was used to compare diet data with the potential explanatory variables of species, station, on-off shore, total chlorophyll and chlorophyll >5µm (M. Lomas unpublished data), mixed layer temperature, mixed layer salinity, straight line distance to nearest land, water column depth, latitude, longitude, water column stability (the difference between the density at 5m and the density at 100m depths), density at 5 m, and pycnocline depth. Sea ice was not included because none was observed at any of the stations

analyzed. Linear regressions of gut contents and environmental chlorophyll were run in Excel 2007. For all analyses each sample of 5 copepods was considered a separate data point.

3. Results

3.1 Prey spectrum

A total of 28,456 prey sequences, which clustered into 138 OTUs were obtained from the 46 samples (GenBank accession #s KC952737 through KC952871). The PCR and sequencing technical replicates had a Bray-Curtis similarity of 79.75% (*P. acuspes*, station 99). Biological replicates had a lower average similarity of 48.36% between replicate pairs. Overall average similarity across all species and all samples was 29.35%. Each sample contained on average 19.5 different OTUs (± 5.6 stdv, min 10, max 33).

OTUs found in gut contents included a wide phylogenetic range of organisms, including single- and multi-cellular autotrophs and heterotrophs (Fig. 2 and Table 2). Diatoms were among the most abundant and diverse groups of prey items. Centric diatom OTUs included four different *Thalassiosiraceae*, seven different *Chaetoceros*, and one each *Attheya*, *Amphipora*, *Porosira*, *Proboscia*, and *Rhizosolenia*. Pennate diatom OTUs included two different *Achnanthes*, and one OTU each of *Pseudo-nitzschia*, *Navicula*, *Stauroneis*, *Fragilariopsis*, and *Fragilaria*. Ten different diverse dinoflagellate OTUs were found in *Pseudocalanus* gut contents, including groups thought to be predominantly autotrophic (e.g. *Gyrodinium*) and predominantly heterotrophic (e.g. *Polykrikos*, *Amoebophrya*). Green alga in gut contents included *Chlorellaceae*, *Klebsormidiaceae*, and *Prasinococcus*. Sequences from the prymnesiophyte *Phaeocystis* were also present in gut contents. In addition to microalgae,

autotroph sequences in *Pseudocalanus* gut contents included four vascular land plants: *Pinus* (pine) and *Betula* (birch) trees, a *Camellia* (tea/flower) bush and a *Poaceae* (rice/wheat) grass.

Pseudocalanus gut contents also showed evidence of predation by these small copepods, including 18S rDNA from a variety of metazoan prey. Crustacean OTUs found include a *Metridia* (copepod), three different *Euphausacea* (krill), one *Hyperidea* (amphipod), and a *Cirripedia* (barnacle). Other metazoan OTUs included *Sagitta* and *Eukrohnia* (chaetognaths), a *Pteropoda*, an *Ophiuroid* (brittle star), *Bdelloidea* and *Plomida* (rotifers), *Ctenophora*, and *Cnidarians*. Heterotrophic protist OTUs in *Pseudocalanus* gut contents included one *Labyrinthulia*, a *Ciliophora*, a *Centrohelida*, a slime mold, two different *Oomycetes*, two different *Saccharomyces* (yeasts), and 18 other fungi. (A full list of prey items is available in the Web Appendix)

3.2 Parasites within *Pseudocalanus*

Thirteen OTUs were identified as parasitic organisms and excluded from the diet analysis. These parasite OTUs included one *Digenea* trematode, six different gregarines, three *Foettingeridae* (Oligohymenophorea) ciliates, and three *Amoebophrya* dinoflagellates. Overall, parasite OTUs made up 17.7% of the total recovered sequences, with parasites in separate samples of 5 copepods ranging from 0% to 93% of the recovered sequences. *P. minutus* showed the highest percent parasitic sequences, and the highest number of different parasites per sample (mean 27% parasite sequences, 3.1 OTUs), with *P. newmani* showing intermediate parasite abundance and diversity (mean 11% parasite sequences, 2.3 OTUs), and *P. acuspes* with the lowest parasite abundance and diversity (mean 0.7% parasite sequences, 1 OTU) (ANOVA $p < 0.01$ for both % parasite sequences and number of parasite OTUs by *Pseudocalanus* species).

3.3 Environmental effects on diet

Water column chlorophyll a concentration was positively correlated with the percent of gut contents sequences represented by phytoplankton (Fig. 3). This trend was most pronounced in *P. minutus* which was found over the full range of environmental chlorophyll levels (linear regression $r^2=0.93$), but was also present in *P. acuspes* and *P. newmani*. At the stations with the lowest environmental chlorophyll levels, relatively more microzooplankton, mesozooplankton, and gelatinous organism sequences were recovered (Fig. 5). Although the trend of increasing proportion of phytoplankton in *Pseudocalanus* diets with increasing environmental chlorophyll is clear, it is not possible to determine the form of this relationship (linear, exponential, logistic, etc.) without more data from intermediate environmental chlorophyll levels.

Within the diatom OTUs, diet diversity was highest, particularly among the *Chaetoceros* spp., at intermediate chlorophyll levels, while at both high and low chlorophyll concentrations the diatom sequences in gut contents were composed mainly of a single *Thalassiosiraceae* OTU (Fig. 4). It is worth noting, however, that because many species of *Thalassiosiraceae* are identical over the 18S rDNA gene region sequenced, this OTU may contain a complex of related species. *Pseudocalanus* species and water column salinity explained 51% of the variance in BioEnv analysis, with the addition of longitude explaining an additional 1% of the variance.

3.4 Species differences

P. newmani and *P. minutus* diets were not significantly different from each other (root-transformed $p=0.07$, presence-absence $p=0.34$). *P. acuspes* diet was significantly different from *P. newmani* (root-transformed $p=0.001$, presence-absence $p=0.01$), and under the more representative root-transformed data, was also significantly different from *P. minutus* (root-

transformed $p=0.001$, presence-absence $p=0.08$). *P. newmani* and *P. minutus* diet contained relatively more diatoms, while *P. acuspes* diet contained relatively more heterotrophic dinoflagellates (Fig. 5). The OTUs which explained the most of this difference (SIMPER) were a *Thalassiosiraceae*, a *Polykrikos* dinoflagellate, an *Amoebophrya*, and a *Cnidaria*.

4. Discussion

4.1 Prey item diversity

The range of prey items found in *Pseudocalanus* spp. gut contents in this study was very large. 138 different prey item OTUs were identified, and an average of 19 of these prey OTUs were present in each sample of five conspecific copepod individuals. Prey items were taxonomically diverse, and included soft-bodied organisms which could not have been detected with traditional microscopy-based techniques, and unusual prey items which would not have been detected by more targeted molecular approaches.

Diatoms in *Pseudocalanus* spp. gut contents included species known to be common in the Bering Sea, and which have been previously observed in *Pseudocalanus* spp. gut contents. *Thalassiosiraceae* spp. were the most frequent and abundant sequences in gut contents, and are typically one of the major constituents of spring blooms in this region (Aizawa et al. 2005). All of the groups of diatoms that early studies observed microscopically in *Pseudocalanus* gut contents (Lebour 1922, Marshall 1949) were also detected with DNA in this study, with the exception of *Paralia*, a tachypelagic species typically associated with resuspended sediments, and hence more commonly found in shallower waters.

Pine and birch tree DNA in *Pseudocalanus* gut contents likely originated from pollen blowing out to sea and being consumed by these copepods. Birch pollen abundance in southern

Alaska peaks in late May (Municipality of Anchorage, 2013), during the time these *Pseudocalanus* were collected. Terrestrial organic matter represents a large input of carbon to the ocean through both riverine and atmospheric transport, but its fate and potential incorporation into marine food webs is poorly understood (Hedges et al. 1997). Birch tree DNA was found in just under half of the samples and all but one of the stations analyzed, and pine tree DNA was found in nearly a third of the samples and at every station, although overall sequence abundance of both these tree OTUs was much lower than that of the most common diatom prey items. Pine pollen is typically 30 to 50µm in size, while birch pollen is slightly smaller, around 15 to 30µm (Davis & Goodlett 1960). Both of these fall into the 25 to 57µm range previously suggested as optimal for *Pseudocalanus* consumption (Poulet 1973). Further understanding of the quantitative importance of terrigenous material ingested by zooplankton may be useful in modeling carbon fluxes in this ecosystem, particularly the fluxes of carbon from the land to the marine environment, and in predicting Bering Sea ecosystem responses to continuing climate change, with potential changes in the timing and magnitude of inputs, such as pollen, from the terrestrial realm.

4.2 Method assessment

While this data was rigorously quality controlled, it is possible that some of the sequences recovered did not originate from direct ingestion of the sequenced organism by a *Pseudocalanus*. It is possible that trace amounts of prey were adhered to the exoskeleton of the copepods. Copepods used in this analysis were rinsed thoroughly in fresh ethanol prior to DNA extraction and there were no visible organisms attached to the exoskeleton. Although this approach is less rigorous than some which have been suggested (Greenstone et al. 2012, O'Rourke et al. 2013) rinsing with ethanol has been considered to be effective in removing contaminants in

some cases (Greenstone et al. 2012) and given the small size of *Pseudocalanus* and the relative permeability of marine crustacean exoskeletons, this approach reduced surface contaminants without risk of damaging gut contents DNA. It is still possible that some of the “prey” DNA, was in fact adhered to the exterior of the copepod, particularly that of gelatinous organisms, such as ctenophores and chaetognaths (Durbin & Casas 2014, O’Rourke et al. 2013). However, this external contamination is likely to be relatively low in abundance compared with true gut contents. Additionally, external contaminants would be likely to affect all *Pseudocalanus* species equally within a sample; something not supported by our observations of substantial differences between species.

In order to eliminate parasites, we took a conservative approach of excluding from the dietary analysis sequences of any organism known to be parasitic. This may have excluded some parasitic organisms which had been consumed as prey items. Alternately, true parasites not yet reported in the literature as such would have been included as prey. We found that known parasites tended to show relatively low frequency amongst samples but high sequence abundance, since unlike prey, parasites are alive and are not in the process of digestion. On this basis we excluded one additional OTU with very poorly resolved phylogeny which was present in a single sample at high abundance. While starved copepod controls would have been ideal to determine which sequences might originate from symbionts of *Pseudocalanus*, given our conservative approach with the analysis of the available samples, we do not think that any inadvertent inclusion of parasite OTUs as prey significantly affected our diet results.

Although DNA sequences can be used to identify the organisms consumed, they do not provide information on the life stage or body part of the organisms which were consumed. Some of the sequences recovered may thus have originated from feeding on eggs, larvae, fragments of

carcasses, exuviae, or fecal pellets of the sequenced organism either singly, or in aggregates or marine snow. Copepods, including species morphologically similar to *Pseudocalanus* have been observed to feed on marine snow (Green & Dagg 1997), and such feeding may explain some of the unusual prey items observed here. Feeding on fecal pellets might potentially explain euphausiid sequences found in *Pseudocalanus* gut contents. Euphausiids produce thin string-like fecal pellets which are bound by a membrane, and this membrane contains euphausiid DNA. *Pseudocalanus* feeding on euphausiid fecal pellets would consume this membrane, and therefore contain euphausiid 18S rDNA. Prey DNA has been found in feces of copepods (Nejstgaard et al. 2003, Vestheim et al. 2005, Durbin et al. 2012). Although it is possible that the contents of the fecal pellet might also be detected in the *Pseudocalanus* gut contents, this DNA would have already been subject to digestion and degradation in the euphausiid gut, so this doubly digested prey is unlikely to show a strong DNA signature in our analysis. Prey-of-prey has been observed as a potential challenge in studies using species or group specific primers (Sheppard et al. 2005). However, in the universal primer approach applied here, such doubly digested prey-of-prey, derived from fecal pellets or gut contents of consumed prey, is likely to be strongly outcompeted by the more abundant and less degraded prey consumed directly. Thus, though prey-of-prey may be detected, they are likely to occur at very low frequency and abundance and unlikely to be a significant source of error in the diet analysis.

Technical variation from PCR and sequencing was low, with replicates providing very similar gut contents information, lending confidence to the reproducibility of pyrosequencing gut contents. Biological variability between groups of copepods of the same species from the same net tow was higher, with Bray-Curtis similarities on average less than 50%, suggesting difference between individuals in their feeding over the time immediately prior to capture. As

DNA is rapidly digested, this variability is not surprising since individual copepods will vary in their recent feeding history. This variability between biological replicates was still considerably lower than the variability observed between different species and stations sampled, and did not obscure overall patterns in diet.

Direct comparisons of sequence data with ingested biomass suggest that, at least for larger predators, relative sequence abundance offers a broadscale indication of the relative importance of different prey items (Deagle et al. 2010, Murray et al. 2011). In our study the strong correlation between relative abundance of phytoplankton OTU sequences and environmental water column chlorophyll a concentrations suggests these abundances may offer at least a semi-quantitative view of the abundance of each prey item; these OTU abundances may potentially reflect the total prey biomass consumed rather than the number of prey individuals consumed. Additionally, although potential biases due to variations in copy number per cell, digestion, extraction and PCR amplification efficiency are likely to vary between different prey items, they are likely to be consistent across different *Pseudocalanus* individuals. Because these biases are likely to be relatively uniform across all the samples analyzed, relative OTU abundance is used to provide a comparison between species which is less influenced by the rare sequences, and thus may be more representative of actual *in situ* feeding.

4.3 Effects of environmental variation

Pseudocalanus gut contents included a greater proportion of sequences from phytoplankton where environmental chlorophyll a levels were higher. This suggests that when phytoplankton were abundant, *Pseudocalanus* consumed them, whereas when phytoplankton were relatively rare, *Pseudocalanus* fed on alternative prey items such as meso- and

microzooplankton. This ability to feed on alternative prey items may be important to *Pseudocalanus* during periods of low phytoplankton abundance, such as winter and late summer. Switching between herbivory and carnivory has been observed in other copepod species as a function of the relative abundances of different prey types (Landry 1981, Kiørboe et al. 1996). Prey switching may not only buffer the copepods themselves from changing environmental conditions, but also allow for a refuge from predation for low abundance prey items, thus helping to maintain diversity in the plankton (Landry 1981).

Amongst the environmental variables analyzed, salinity was the best predictor of diet. Salinity changes have been previously found to explain temporal variations in copepod assemblages, and it has been hypothesized that this is due to bottom up effects (Pershing et al. 2005, Mountain & Kane 2010, Ji et al. 2012). Salinity variations may drive changes in water column stability with effects on the relative success of diatoms and dinoflagellates; potentially leading to different prey assemblages available for *Pseudocalanus* to feed on (Mountain & Kane 2010, Hinder et al. 2012). However, water column stability, pycnocline depth, and density at 5m were not found to be good predictors of diet here. Thus, in this case, salinity may be acting rather as a tracer for different water masses with varying prey assemblages.

4.4 Niche separation

P. minutus and *P. newmani* showed very similar diets, while *P. acuspes* diet was significantly different from that of *P. minutus* and *P. newmani*. Diet differences were maintained across the range of chlorophyll a concentrations encountered. These diet differences were driven largely by broad differences in the types of prey consumed, with *P. acuspes* consuming relatively more heterotrophic dinoflagellates and to a lesser extent macrozooplankton, while *P. minutus*

and *P. newmani* consumed relatively more diatoms. Of the diatoms consumed, no differences in selection for specific types were observed between any of the three species. Feeding differences between species may result from or be a reflection of small-scale variations in copepod species distribution. Species preferences for locations in the water column are poorly known, and may impact feeding, by affecting the available prey field of different species. Differences in broad prey types consumed suggest feeding differences may also potentially be driven by differences in feeding behavior. Copepods typically catch diatoms and other non-motile prey by maintaining a relatively stationary position and generating a feeding current, whereas motile prey such as dinoflagellates are typically captured by passively sinking copepods detecting hydromechanical signals from the prey items and then capturing them in a form of ambush feeding (Kjørboe et al. 1996). *P. minutus* have been observed to filter water through their mouthparts almost constantly, consistent with our observation of a diet relatively rich in non-motile diatoms (Tiselius & Jonsson 1990). Differences observed between *P. minutus* and *P. newmani* versus *P. acuspes* diets suggest potentially different allocations of effort to these two feeding strategies.

P. minutus and *P. acuspes*, which are both considered primarily arctic (Coyle et al. 2011), and hence occupy largely overlapping geographic ranges, show diet differences, suggesting potential ecological niche partitioning through feeding differences. By contrast *P. minutus* and *P. newmani* which have largely similar diets are more geographically separated, as *P. newmani* is considered to be a primarily temperate species (Coyle et al. 2011), suggesting they may fill similar ecological niches in these different geographic areas. Feeding differences have previously been suggested as a mechanism of niche partitioning amongst co-located copepods (Maly & Maly 1974).

4.5 Potential implications for the future

The Bering Sea is experiencing rapid warming and changes in the patterns of interannual variation in response to anthropogenic climate change; and the effects of these changes have already been observed in both physical and biological components of the Bering Sea (Overland & Stabeno 2004, Stabeno et al. 2012). Overall *Pseudocalanus* abundance has been found to be either negatively correlated with or independent of temperature in the Bering Sea (Coyle et al. 2011, Stabeno et al. 2012). Changes in sea ice and storminess may affect water column stability, with implications for the relative success of diatoms and dinoflagellates (Edwards & Richardson 2004, Edwards et al. 2006, Hinder et al. 2012), and any such changes could have opposite implications for *P. minutus*/*P. newmani* and *P. acuspes*.

Diet has been shown to affect zooplankton fecal pellet sinking speeds, leading to variations in the efficiency of the biological pump and the sequestration of carbon in the deep sea (Atkinson et al. 2012). Diets rich in diatoms are associated with denser faster sinking fecal pellets and more efficient carbon export (Atkinson et al. 2012). *P. minutus* and *P. newmani* feeding may thus be a relatively efficient mechanism for the export of carbon from the mixed layer, while feeding by *P. acuspes* may instead enhance microbial recycling within the upper water column. Interspecific differences in susceptibility to changing environmental variables, and roles in the biological pump suggest that ecosystem monitoring efforts might benefit from differentiating these morphologically cryptic congeners.

Given that *P. minutus* and *P. newmani* have similar diets it is possible that the more temperate *P. newmani* may take over some of the southern part of the range of *P. minutus*, under continued warming. Such a geographic shift in the transition zone between *P. minutus* and *P. newmani* would likely have minimal top down effects since their diet is so similar. However, *P. minutus* stores lipids, while *P. newmani* does not (Coyle et al. 2011), which may change their

nutritional value as prey items to higher trophic levels, with potentially negative implications for commercially important predator species such as walleye pollock, ecologically important groups such as seabirds, and culturally important organisms even higher up the trophic web, such as marine mammals. The high diversity of prey items found in *Pseudocalanus* gut contents suggests these organisms are able to consume a wide range of different prey types, and may potentially be able to feed opportunistically. Opportunistic feeding may allow *Pseudocalanus* populations to help buffer higher trophic levels in this productive ecosystem from bottom-up effects induced by changing climate.

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624 Table 1: Characteristics of sampling locations and species analyzed at each station. Station (Stn)
625 numbers correspond to Bailey (2012), species numbers indicate how many individuals of each
626 species were analyzed, latitude and longitude are in decimal degrees, all dates are in 2010, total
627 bottom depth is in meters and chlorophyll a (Chla) represents the >5 μm size fraction in $\mu\text{g L}^{-1}$ at
628 the depth of maximum chlorophyll a fluorescence.

<i>Stn</i>	<i>P. acuspes</i>	<i>P. minutus</i>	<i>P. newmani</i>	<i>Latitude</i>	<i>Longitude</i>	<i>Date Sampled</i>	<i>Depth</i>	<i>Chla</i>
49	0	30	30	59.8998	-178.8960	May 19	486	20.1
55	0	15	0	58.2043	-174.2357	May 21	381	0.4
87	0	15	15	55.4315	-168.0608	May 29	205	6.2
99	15	0	15	56.8536	-164.5056	May 31	73	3.8
156	0	15	15	62.1890	-175.1521	June 5	79	3.8
163	0	15	0	59.8934	-178.8983	June 7	657	4.1
175	15	0	0	59.9003	-172.2170	June 9	73	0.8
179	15	15	0	58.8301	-168.1589	June 10	46	0.8

Table 2: Most abundant Operational Taxonomic Units (OTUs) from *Pseudocalanus* gut contents, with nearest taxonomic identity, total number of sequences, presence/absence count out of 45 total samples, and cumulative % of total sequences. These top 29 OTUs account for 27,044 sequence reads, which is 95% of the total prey sequence reads found. The complete list of gut contents OTUs can be found in the Web Appendix.

Rank	GenBank Ass. #	Prey Organism	Total	Count	Cumulative %
1	KC952766	<i>Thalassiosiraceae</i> diatom	9451	45	33.2%
2	KC952848	<i>Polykrikos</i> dinoflagellate	4239	29	48.1%
3	KC952832	<i>Cnidaria</i>	2189	30	55.8%
4	KC952803	<i>Ameobophrya</i> dinoflagellate	1974	40	62.7%
5	KC952751	<i>Mertensia</i> cnidarian	1340	31	67.5%
6	KC952857	<i>Fragilaria</i> diatom	1003	19	71.0%
7	KC952818	<i>Fragilariopsis</i> diatom	867	27	74.0%
8	KC952820	<i>Ophiuroid</i> brittle star	857	2	77.0%
9	KC952779	<i>Euphausiid</i> krill	723	37	79.6%
10	KC952790	<i>Polykrikos</i> dinoflagellate	508	15	81.4%
11	KC952860	<i>Sagitta/Krohnitta</i> chaetognath	495	37	83.1%
12	KC952772	<i>Phaeocystis</i> prymnesiophyte	454	21	84.7%
13	KC952833	<i>Chaetoceros</i> sp. diatom	452	18	86.3%
14	KC952738	<i>Chaetoceros</i> sp. diatom	379	12	87.6%
15	KC952837	<i>Chaetoceros</i> sp. diatom	349	23	88.8%
16	KC952807	<i>Chaetoceros</i> sp. diatom	255	15	89.7%
17	KC952745	<i>Pezizomycete</i> fungus	230	26	90.5%
18	KC952802	Grain (rice/wheat/corn)	212	15	91.2%
19	KC952765	<i>Betula</i> sp. Birch tree	205	21	92.0%
20	KC952752	<i>Metridia</i> copepod	124	10	92.4%
21	KC952748	<i>Pinus</i> sp. Pine tree	115	14	92.9%
22	KC952749	<i>Rhizochaete</i> fungus	94	16	93.2%
23	KC952767	<i>Agaromycete</i> fungus	93	17	93.5%
24	KC952867	<i>Navicula</i> diatom	91	10	93.8%
25	KC952824	<i>Porosira</i> diatom	77	4	94.1%
26	KC952756	<i>Semaeostomae</i> cnidarian	76	4	94.4%
27	KC952777	Dinoflagellate	70	8	94.6%
28	KC952791	<i>Cryothecomonas</i> flagellate	63	1	94.8%
29	KC952823	<i>Oligohymenophorea</i> ciliate	59	4	95.0%

634 Figure legends

635 Figure 1: Map of sampling locations in the Bering Sea. The 25, 50, 75, 100, 200 and 1000m
636 isobaths are shown. Stations 49 and 163 were in the same location on different dates.

637 Figure 2: Phylogenetic tree of all prey 18S rDNA sequences recovered from *Pseudocalanus* spp. Circle
638 size indicates in how many of the 45 samples each Operational Taxonomic Unit (OTU) was identified,
639 with no circle indicating less than 10. Branch and circle colors indicate prey identity and correspond to
640 colored text. Gelatinous organisms fall into two distinct clades as this group is defined by morphology,
641 and includes the phylogenetically diverse ctenophores, cnidarian, and chaetognaths.

642 Figure 3: Environmental chlorophyll a concentrations and phytoplankton as a percent of total
643 prey sequences in *Pseudocalanus* gut contents. Circles and solid line show *P. minutus*, triangles
644 and dotted line show *P. acuspes*, squares and dashed line show *P. newmani*. All trend lines show
645 linear regressions.

646 Figure 4: Diatom diversity in gut contents of each sample arranged as a function of chlorophyll a
647 concentration. Colored bars indicate the proportion of the total diatoms in gut contents
648 contributed by each diatom OTU (left-hand axis). White circles show water column chlorophyll
649 a concentration (right-hand axis). Vertical white lines separate each station, while brackets below
650 the x axis indicate the copepod species, with “a” *P. acuspes*, “m” *P. minutus*, and “n” *P.*
651 *newmani*.

652 Figure 5: Differences in diet among species and with chlorophyll a concentration. Pie charts
653 show the relative abundance of sequences belonging to each type of prey in *Pseudocalanus* gut
654 contents.

Fig. 1

656

Fig. 2

657

Fig. 3

Fig. 4

Fig. 5