Feeding by Antarctic krill *Euphausia superba* in the West Antarctic Peninsula: differences between fjords and open waters

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ABSTRACT: Antarctic krill *Euphausia superba* are key components of Antarctic ecosystems, serving as the major prey item for most of the megafauna in the region. Coastal fjords along the West Antarctic Peninsula have been identified as biological hotspots, areas in which high biomasses of both *E. superba* and their megafauna predators are consistently observed. We investigated feeding by *E. superba* in fjords and adjacent open waters of the West Antarctic Peninsula. Next generation sequencing of stomach contents from 174 krill indicated a diverse diet, with broad patterns consistent with previous understanding of *E. superba* feeding. Diatom sequence reads were frequent and abundant, indicating a largely diatom-based diet, while the occasional presence of high abundances of copepod sequence reads suggests carnivory supplemented the diet. Striking differences were observed between the stomach contents of krill collected in fjords and those of krill collected in adjacent open waters. *Chaetoceros* spp. diatoms made up 71% of the stomach contents sequences of krill collected in fjords, but less than 10% of the stomach contents sequences of krill collected in open waters. These differences could not be explained by differences in the surface water phytoplankton communities, as in both open waters and fjords *Chaetoceros* spp. made up less than 10% of the surface water sequence read assemblages. These feeding differences highlight the importance of taking into account regional differences in krill feeding when considering *E. superba*'s roles in Southern Ocean ecosystems, and suggest krill in fjords may make use of vertical structure in phytoplankton assemblages.

KEY WORDS: *Euphausia superba* · Feeding · Stomach contents · 18S rDNA · West Antarctic Peninsula · Fjords · *Chaetoceros* spp.

INTRODUCTION

Antarctic krill *Euphausia superba* is a keystone species in the wasp–waisted Antarctic ecosystems (Quetin & Ross 1991, Croxall et al. 1999, Atkinson et al. 2012, Stowasser et al. 2012). *E. superba* are preyed upon by most of the charismatic megafauna in the region, making up over 90% of the diet for baleen whales, squid, crabeater seals and brush-tailed penguins, and contributing to the diet of other vertebrates (Quetin & Ross 1991, Knox 2006, Trathan & Hill 2016).

E. superba are distributed around Antarctica, and occupy a broad swath of latitudes from the ice edge at 75° S up to the sub-Antarctic islands of South Georgia and Bouvetøya at 53° S (Schmidt & Atkinson 2016). Across this immense range, they encounter diverse environmental conditions and prey fields and, unsurprisingly, their diet differs regionally. These regional differences in diet have implications for the role of *E. superba* in carbon cycling, as well as for their role as prey for megafauna, with krill in the West Antarctic Peninsula (WAP) and South Georgia region growing to much larger sizes than those in the

Lazarev Sea and other open ocean areas (Schmidt et al. 2014). Geographic differences in krill feeding also have implications for their value to the growing fishery, as krill which have been feeding heavily on phytoplankton are considered to be of lower quality due to their grassy taste (Nicol et al. 2000). Understanding these spatial differences in feeding thus has implications for krill's role in the carbon cycle and for spatial fisheries management, as well as having the potential to help elucidate underlying environmental drivers of differences in krill feeding.

Within the WAP region are a series of over a dozen deep fjords extending from Charlotte Bay at 64.5° S near the tip of the peninsula to Neny Fjord at 68.25° S. Most research programs on krill, including the time series programs of Antarctic Marine Living Resources (AMLR) and Palmer Long Term Ecological Research (Palmer LTER), have their shoreward-most stations outside of the fjords, and relatively little work has been done on krill within these coastal fjords. These coastal WAP fjords create a unique habitat with their complex bathymetry, ice and freshwater inputs, and retentive circulation (Grange & Smith 2013). Compared to more open shelf areas, biomass of many groups is higher within these fjords, with benthic megafaunal abundances 15-fold greater within fjords than over the open shelf, and the highest reported densities of humpback whales found within fjords (Nowacek et al. 2011, Grange & Smith 2013). *E. superba* are similarly highly abundant within these coastal fjords, with observations of large super-swarms of krill filling nearly the entirety of certain fjords in winter (Nowacek et al. 2011, Cleary et al. 2016) and observations of frequent dense schools in summer (E. G. Durbin unpubl. data). It has been suggested by some that these unique environmental conditions and high organism densities may lead fjords to have unusual food webs (Espinasse et al. 2012, Grange & Smith 2013), while others have suggested these very inshore areas may be amongst the areas which best exemplify the stereotypical Antarctic food web of diatom–krill–megafauna (Garibotti et al. 2003).

E. superba consume a diverse range of prey types including phytoplankton, microzooplankton, mesozooplankton, ice algae and sediments. The relative importance of these different prey types varies spatially and seasonally. Diatoms are the most widely recognized prey of *E. superba*, and krill have been observed to consume a diverse range of genera including *Chaetoceros*, *Eucampia*, *Fragilariopsis*, *Porosira*, *Rhizosolenia* and *Thalassiosira* spp. (Hopkins 1985, Martin et al. 2006, Schmidt et al. 2006). Diatoms are most often observed as the dominant prey

close to the continental coast (Garibotti et al. 2003). Diatoms have been observed to be important dietary components in both the Lazarev and Scotia Seas, but to be relatively less important further north near South Georgia (Schmidt et al. 2014). In the Lazarev Sea, gut contents microscopy found a diatom-rich diet close to the continent, and a more ciliate-rich diet in krill to the north (Perissinotto et al. 1997). Microzooplankton, including tintinnids, heterotrophic dinoflagellates, aloricate ciliates and armoured flagellates have been observed to make up significant fractions of krill diet, and even dominate the stomach contents in some samples, particularly away from the coast (Perissinotto et al. 1997, Schmidt et al. 2006).

Mesozooplankton can also be important prey for *E. superba.* Because mesozooplankton are often common within phytoplankton blooms, carnivory by *E. superba* has been thought to supplement their diet even in the phytoplankton-rich spring and early summer period (Hernández-León et al. 2001, Polito et al. 2013, Schmidt & Atkinson 2016). Copepods have frequently been observed as prey for krill, including *Metridia* and *Oithona* spp. (Schmidt et al. 2006, 2014, Töbe et al. 2010). Mesozooplankton have been particularly noted as important prey near South Georgia and under sea ice (Nishino & Kawamura 1994, Atkinson & Snÿder 1997, Ju & Harvey 2004, Töbe et al. 2010, Schmidt et al. 2014).

At the edges of the pelagic habitat, krill feed on surfaces. Krill have been observed feeding on ice algae in the Weddell Sea and near the WAP, among other locations (Marschall 1988, Stretch et al. 1988). Sea ice is typically more prevalent further south, and closer to shore (Holland & Kwok 2012). Sediment food resources are more accessible to krill in shallower waters, but krill have been observed feeding on sediment both at relatively shallow depths of 200 to 300 m (Schmidt et al. 2011, 2014, Cleary et al. 2016), and at abyssal depths of 3500 m (Clarke & Tyler 2008).

To date, research into feeding by *E. superba*, though extensive, has been hampered by methodological challenges. Adult krill are fast-swimming organisms, classified more accurately as micronekton than macrozooplankton, and are able to exploit a diverse range of habitats including both the water column and the sediment and sea ice interfaces. Replicating such an environment, let alone scaling it appropriately, is near impossible in a laboratory setting. Thus, while incubation experiments have provided valuable insight into the feeding capabilities of krill, it is difficult to assess their relevance for *in situ* krill feeding. Various *in situ* measures of krill feeding have been

applied, including stomach contents microscopy, stomach fluorescence, stable isotopes, fatty acids and immunochemical assays (Hopkins 1985, Haberman et al. 2002, Schmidt et al. 2003, Reiss et al. 2015). These methods have provided new and complimentary insights into krill feeding, but all are limited in the resolution of prey types they can detect, and have various biases for or against different prey groups.

In the past decade, DNA has shown promise as a dietary biomarker in krill. DNA sequences recovered from krill stomachs can be compared to a database of reference sequences to determine what organisms the krill consumed immediately prior to capture. DNA has the advantage of being present in all living prey. Unlike some biomarkers such as stable isotopes, which integrate over months and thus obscure signals from seasonally varying diets (Schmidt et al. 2003), DNA is rapidly digested and provides a 'snapshot' of the prey ingested in the minutes to hours prior to capture (Durbin et al. 2012). Early studies investigating DNA as a marker of krill gut contents applied a variety of approaches. Group-specific primers for phytoplankton identified 13 distinct prey items including *Fragilariopsis*, *Thalassiosira* and *Chaetoceros* spp. diatoms in krill stomach contents (Passmore et al. 2006). Species-specific primers investigating carnivory in krill indicated that *Oithona* spp. were the most important copepod prey for larval krill in the Lazarev Sea (Töbe et al. 2010). Denaturing gradient gel electrophoresis with universal primers identified 26 distinct prey from larval krill stomachs, including various groups of phytoplankton, micro-zooplankton and mesozooplankton (Martin et al. 2006). Most promisingly, a krill-blocking primer overlapping universal primers was applied to 13 krill individuals and resulted in 96 putative prey sequences including 4 types of phytoplankton and a ciliate (Vestheim & Jarman 2008).

DNA sequencing technology has been advancing at an incredible rate, such that the amount of data available for a given effort and budget from modern Next Generation Sequencing (NGS) platforms is now 4 orders of magnitude greater than with the conventional sequencing which was available when the above-mentioned studies were conducted. We combined this new sequencing power with universal primers and a peptide nucleic acid (PNA) krill-specific blocking probe. PNA probes offer advantages over blocking primers because they can align to the predator's DNA anywhere along the amplicon of interest, allowing for greater flexibility in experimental design, and the use of more conserved (and thus more likely to be truly 'universal') primer sites.

The use of universal primers combined with a predator-specific blocking probe minimizes *a priori* assumptions about diet, as the only types of feeding which are not observable are cannibalism and feeding on prokaryotic aggregates. As neither cannibalism nor bactivory are considered likely to be important components of krill diet (Schmidt & Atkinson 2016), this approach should provide indications of the full range of prey consumed by *E. superba in situ*. A universal-primer based approach also minimizes the potential confounding effect of detecting secondary predation. Unlike with targeted approaches, which detect even trace amounts of the DNA of interest, with universal primers, prey-of-prey will be largely outcompeted by the vastly more abundant DNA from direct predation.

Sequence read abundance offers an indication of differences across predator individuals in the relative importance of different prey. Sequence read abundance is not a quantitative measure of food consumed, due to differences between prey in ease of digestion, amplification efficiency and copies of the target 18S gene per cell (Zhu et al. 2005, Martin et al. 2006). Despite these potential biases, ground-truthing experiments with larger predators have shown sequence read abundance offers at least a semi-quantitative indication of the importance of different prey items (Deagle et al. 2010, Murray et al. 2011). Because the potential biases between different prey types are consistent, regardless of which individual of a predator species consumes the prey, differences between predators in the fraction of their diets made up by different prey item sequences likely reflect true differences in diet. For example, if 2 krill individuals were to eat 2 different prey types, with each prey type having distinct 18S copy numbers, digestion rates and amplification efficiencies, it would be impossible to know with certainty what fraction of each krill's diet was made up of each prey type. However, because the 18S copy numbers, digestion rates and amplification efficiencies of each prey type will be the same, regardless of which krill individual consumed it, we can make comparisons of the relative importance of the different prey types to our 2 different krill individuals.

In this work, we investigated *E. superba* feeding in fjords and adjacent shelf areas in the central WAP using NGS with universal primers and a predator-blocking probe. The goals of this research were to investigate differences in feeding between krill in fjords and those in open waters, and to establish an NGS-based methodology for the analysis of krill diet. To these ends, we sequenced the stomach contents of

174 *E. superba* individuals, as well as 11 environmental water samples with which to contextualize krill feeding, from 6 stations. Over 4 million sequences were generated, offering new insights into krill feeding in this region.

MATERIALS AND METHODS

Field collections

Euphausia superba and their environment were sampled on cruise NBP1410 of the US RV Ice Breaker 'Nathaniel B. Palmer' between 10 and 21 December 2014 (Table 1, Fig. 1). Because of the over 4 orders of

magnitude differences in size across the range of organisms sampled (2 μm phytoplankton cells through 6 cm krill), a variety of sampling gear was employed to efficiently capture each size fraction.

Krill were collected from the water column with oblique tows of a 1 m^2 multiple opening closing net and environmental sensing system (MOCNESS) with 333 μm mesh nets (Wiebe et al. 1985). In order to minimize krill net avoidance, the MOCNESS was outfitted with black nets and with 3 LED strobe lights, which flashed twice s^{-1} with a nominal light output of 3 W throughout the tows (Brightwater Instruments) (Sameoto et al. 1993, Lawson et al. 2004, Wiebe et al. 2004). All tows were short (less than 30 min) and krill were processed immediately

Table 1. Krill and water sample collections. MOC: MOCNESS; ICE: samples from sea ice; CTD: Niskin bottle on a CTD-rosette; Date: day in December 2014; time is local. Letters after locations correspond to Fig. 3

| Type	Date	Time (h)	Location	Region	Depth (m)	Tow No.	Lat (°S)	Long (°W)	No. of krill
MOC	10	09:38	Andvord Bay (F)	Fjord	0–150	8	64.8308	62.6388	5
MOC	17	11:04	Palmer Deep (A)	Open water	84–99	15	64.9297	64.3410	5
MOC	17	20:42	Bismarck Strait-East (B)	Open water	80–110	16	64.9127	63.6710	40
MOC	18	13:20	Anvers Island (C)	Open water	0–115	17	64.8612	63.8557	36
MOC	18	22:36	Flandres Bay (E)	Fjord	0–40	18	65.0528	63.2149	42
MOC	21	12:36	Andvord Bay (G)	Fjord	40–70	24	64.8406	62.5927	33
ICE	13	14:00	Renaud Island (D)	Open water	0	1	65.6132	66.4412	8
CTD	10	08:58	Andvord Bay (F)	Fjord	10	1	64.8245	62.6412	–
CTD	17	09:40	Palmer Deep (A)	Open water	10	2	64.9364	64.3579	–
CTD	18	09:00	Bismarck Strait-West (I)	Open water	10	3	64.8954	63.7190	–
CTD	19	00:25	Flandres Bay (E)	Fjord	10	4	65.0567	63.2035	–
CTD	21	14:20	Andvord Bay (G)	Fjord	10	5	64.8149	62.6721	–
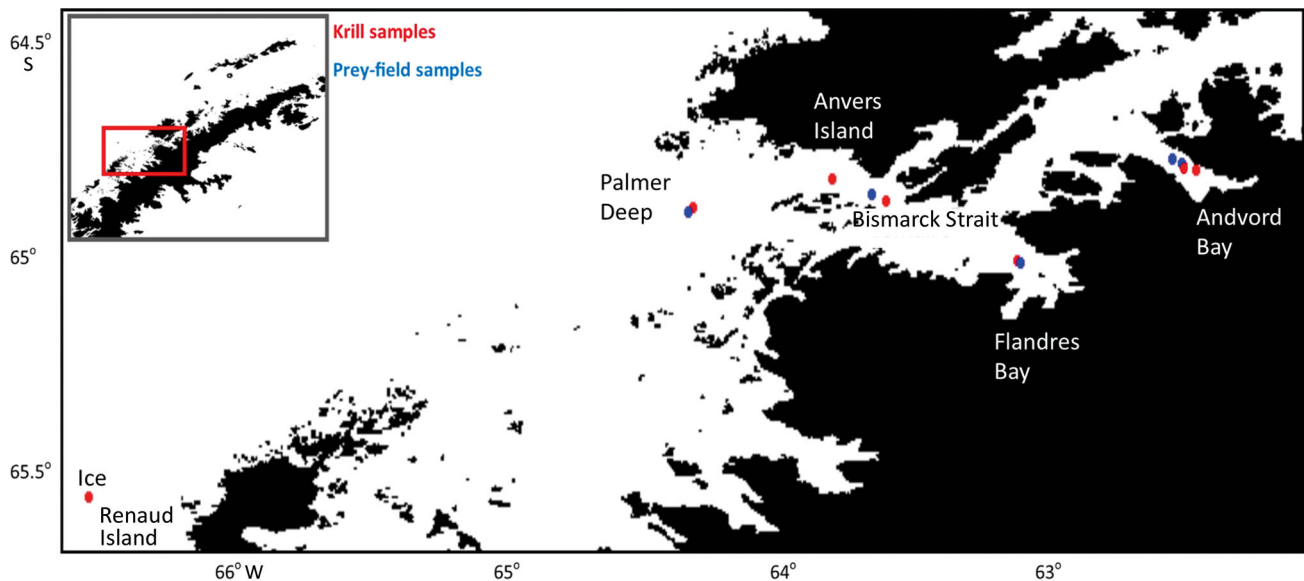
Fig. 1. Sampling locations. Latitude is degrees south, longitude is degrees west. Land is indicated in black

upon landing the net to minimize digestion and potential net feeding. In order to capture sufficient krill for analysis in such short tows, tows were targeted on acoustically observed krill aggregations based on hull-mounted multifrequency acoustics (Simrad) and acoustic Doppler current profiler (ADCP; Teledyne RDI) backscatter. An additional krill sample was collected from the sea ice. Krill associated with pieces of sea ice which were overturned as the vessel broke a path were collected with a 1 l metal can attached to a long pole while the vessel was underway at low speed (approximately 1.5 knots). All krill were preserved immediately upon collection in 95 % reagent grade ethanol. To ensure sufficiently high concentrations of ethanol, krill made up no more than one-third of the total volume of each sample, and the initial preservation ethanol was replaced with fresh ethanol 12 to 24 h after sample collection.

To place krill feeding into an environmental context, water column parameters were measured with vertical profiles from the surface to 10 m above the seafloor using a SeaBird 911+ CTD. The CTD was equipped with dual salinity and temperature sensors, a WET labs AFLT fluorometer and a rosette of 12 l Niskin bottles. Water samples were collected at 10 m depth for DNA analysis of the community composition. Duplicate subsamples of 100 ml of water from each of the sampled areas were filtered onto 0.2 μm pore size, 25 mm diameter membrane filters under gentle vacuum pressure. One sampling location in the western Bismarck Strait was used for comparison with the krill samples collected both in the eastern Bismarck Strait and near the coast of Anvers Island (Fig. 1). Water filters for DNA analysis were preserved at −80°C, and were maintained frozen until DNA extraction. Extracted chlorophyll was measured as per Jespersen & Christoffersen (1987) at approximately 5, 10, 20, 50 and 100 m and near the bottom in each area. Additional information on the distribution of chlorophyll in the sampling region was provided by continuous surface water measurement with a WET labs FLRTD fluorometer. The availability of mesozooplankton prey for krill was assessed with vertical tows from 100 m depth to the surface in each sampled area with a ring net (0.5 m^2 diameter, 64 μm mesh). Ring net catches were preserved in a final concentration of 4 % sodium-borate buffered formalin, and later counted under 40 to 100× magnification. The distribution of krill biomass was measured continuously throughout the cruise with hull-mounted multifrequency acoustics (Simrad) as per Warren et al. (2009). Although sampling methods for potential prey (phytoplankton, microzooplankton, macrozoo-

plankton) were designed to provide a representative snapshot of the available prey field, they are not an exact measure of the prey available to krill, due to krill movements and potential biases of the sampling gear.

A feeding experiment was conducted to investigate prey selectivity. Krill were collected in a dedicated MOCNESS tow and gently transferred to filtered seawater immediately upon landing the net on deck. Krill handling was minimized, and all transfers of individuals were done in water (with small beakers or soup ladles) to minimize damage to appendages, as per King et al. (2003). Krill were maintained for 2 d in large flowing seawater tanks to acclimate to laboratory conditions and to recover from collection. A total of 30 krill individuals which exhibited active swimming behaviour and had no visible injuries were carefully transferred to a 20 l polycarbonate bucket of filtered seawater fitted with a rigid mesh liner to evacuate their stomach contents. After 24 h, krill were transferred in the mesh liner to a new 20 l bucket containing a high density of natural prey. Natural prey were collected with the ring net described above in Flandres Bay. A sub-sample of this prey assemblage was filtered and preserved as described for environmental DNA filters above. The bucket was floated in a 1000 l tank of flowing seawater to maintain ambient temperature. Krill fed for 1 h at 22:20 local time on 23 December, during which they were actively compressing their filtering baskets, their guts rapidly filled with visible food and they produced faecal pellets. After the hour of feeding, krill were preserved as described for wild krill. Five of the individuals in this experiment were analysed for diet.

Laboratory processing

Krill were selected at random from amongst the adult individuals within each sample for analysis. Krill were each dissected in a fresh disposable plastic weigh boat, with forceps ethanol-flame sterilized between each step, and particularly between contact with the krill's exterior and its interior. Each krill was measured to the nearest 0.5 mm for standard 1 length (Everson 2000). The front of the carapace was removed, and the stomach was isolated under 40× magnification. Passmore et al. (2006) suggested that concentrations of ethanol above 80 % make krill brittle and difficult to dissect, but we did not find this to be the case. Gut fullness was estimated visually to the nearest 10 %, and krill stomachs were kept on ice

until DNA extraction, which was started within 3 h of dissection.

DNA was extracted both from krill stomachs and water filters with the DNeasy blood and tissue kit (Qiagen) as per manufacturer's directions. All lysis steps were carried out overnight. Krill stomachs were first broken open with a sterilized toothpick to ensure the contents would fully lyse. For water filters, double volumes were used of lysis buffers to ensure the filter was submerged and thus fully lysed. DNA extraction and preparation of the first round of PCRs was carried out in a separate clean hood from all post-PCR work to minimize potential contamination. DNA from krill and water filters were never extracted on the same days.

A nested PCR approach was used to amplify the v7 region from all non-krill 18S rDNA within the krill gut DNA extracts. This nested approach was used as preliminary tests indicated that PNA probes did not effectively block amplification when used with the long adaptor primers necessary for illumina sequencing. We believe this is due to the high melting temperatures of these long primers, allowing them to outcompete the probe. In the first PCR, krill stomach DNA extracts were amplified with universal 18S primers, while krill's own DNA was blocked with a krill-specific PNA probe. These reactions included 1× GoTaq Green Master Mix (Promega), 0.5 µM each forward and reverse primers (forward: 5′-GGG CAT CAC AGA CCT G-3′, reverse: 5′-GGC TYA ATT TGA CTC AAC RCG-3′; modified from Gast et al. 2004 as per Cleary et al. 2016), 20 µM krill-specific PNA (from 100 µM stock re-suspended in 2.5 % trifluoroacetic acid, 5′-CGT CGG GTT GTC TTG-3′; Cleary et al. 2012), and 20 % by volume stomach DNA extract. DNA was used at extracted concentrations. In a few reactions, no amplification was observed, and those reactions were re-run with DNA diluted to 10 % of its original concentration. All krill amplified under one or the other of these conditions. Thermocycling consisted of 95°C for 30 s, followed by 30 cycles of 94°C for 30 s, 67°C for 30 s, 58°C for 30 s, 60°C for 30 s, with a final extension at 60°C for 5 min and then immediate cooling of the reactions to 4°C. In the second PCR, the stomach contents amplicons were re-amplified for only a few cycles with the primers containing the illumina adaptors, in order to add these adaptor sequences onto the ends of the amplicons. These reactions contained 1× GoTaq Green Master Mix, 0.2 µM each forward and reverse primers and 20 % by volume amplicons from the first round of PCR. Illumina primers were reversed, which is to say the Read1 adaptor (traditionally forward)

was put on the reverse primer, and the Read2 adaptor on the forward primer (Read1 primer: 5′-CGT CGG CAG CGT CAG ATG TGT ATA AGA GAC AGG GCT YAA TTT GAC TAA CRC G-3′, Read2 primer: 5′-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGG GCA TCA CAG ACC TG-3′). Thermocycling for these reactions consisted of 94°C for 30 s, followed by 10 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 30 s, with a final extension of 72°C for 5 min and then immediate cooling to 4°C. Krill were amplified in batches of 22 samples with a negative (no template DNA) control included in each batch, which was carried through both of the nested PCRs.

Because water filters are not expected to have an overwhelming amount of krill DNA, no blocking probe was needed and PCR was done in a single round. This difference is likely to have very little effect on the resulting amplicons, as previous results have shown minimal effect of the krill PNA on the amplification of non-krill targets (Cleary et al. 2012), while saving time and cost, as the PNA probe is the most expensive reagent of the PNA-PCRs. These reactions contained 1× GoTaq Green Master Mix, 0.2 µM each forward and reverse illumina primers and 20 % by volume DNA extract at extracted concentration. Thermocycling consisted of 94°C for 30 s, followed by 30 cycles of 94°C for 30 s, 58°C for 45 s, 72°C for 30 s, and a final extension at 72°C for 5 min with immediate cooling to 4°C. Agarose gels with ethidium bromide were used to confirm all sample reactions had produced amplicons of the expected size, and that no amplification was visible in any of the negative controls.

DNA sequencing was conducted at the University of Rhode Island Genomics and Sequencing Center. A final PCR of 5 cycles was performed by the sequencing center to attach further sequencing adaptors and individual tags, and samples were combined into 2 sequencing pools. Attaching sequencing adaptors and sample identifying tags in a short final PCR has been recommended to minimize any potential biases related to the identification tags (Deagle et al. 2013). Krill individuals from each of the sampling locations were divided between the 2 pools; all captive krill were placed in the same pool. Each pool was sequenced in one lane on 2 separate Illumina MiSeq runs, with V2 chemistry for 200 bp in each direction.

Bioinformatics

Bioinformatic analysis was conducted on the Center for Computation and Visualization cluster at

Brown University, using Qiime version 1.9 (Caporaso et al. 2010), minimum entropy decomposition (Eren et al. 2015) and custom Bash scripts. The complete annotated pipeline script is available in the Supplement at www.int-res.com/articles/suppl/m595p039_supp.pdf.

The forward and reverse sequences for each amplicon were merged, and reverse primers and any trailing sequence were trimmed. The sequences were then reverse-complimented, and the forward primer and any upstream sequence were removed. Any sequence reads which failed the forward and reverse merging criteria, as well as any sequence reads which did not contain both of the correct primers were discarded as low quality.

We took a nested approach to clustering the sequences into operational taxonomic units (OTUs). This nested OTU picking approach significantly reduced the amount of computer memory required for data processing, and allowed for finer resolution of OTU picking and taxonomy assignment with the available computational resources. First, we did a rough clustering at 95% similarity with UCLUST, and removed singletons (Edgar 2010). We determined the taxonomy of the rough OTUs by automated Basic Local Alignment Search Tool (BLAST) comparison with the Silva database, and further removed all sequences which were identified as Malacostraca (Altschul et al. 1990, Morgulis et al. 2008, Quast et al. 2013). Preliminary analyses found no non-krill malacostracan sequences, so by filtering the OTUs at this class level we removed as many as possible of the reads derived from the krill's own stomach tissue. From this culled list of rough OTUs, we then retrieved all of the sequence reads belonging to the retained OTUs and created a new 'clean' file of sequences. This clean sequences file was then used to generate fine-resolution OTUs with minimum entropy decomposition, with a minimum substantive abundance of 100 for an OTU to be retained. Taxonomy was assigned to these final OTUs by UCLUST automated comparison with the Silva database with maximum accepts set to zero for an exhaustive search.

Chimeric sequences were detected with the blast fragments approach, and removed from the dataset. All sequences which were <200 bp in length were removed from the dataset as the expected length of amplicons for these primers is approximately 250 bp. OTUs which were high in abundance but whose taxonomy as determined in automated comparisons with Silva were ambiguous were further BLAST searched against the full National Center for Biotechnology Information (NCBI) GenBank database, and

taxonomy was refined if all of the hits within 3% sequence similarity were consistent. Sequences from krill themselves were again removed. Sequences which were identified taxonomically as belonging to known parasite or stomach or gut symbiont groups, regardless of known hosts, were removed and will be reported elsewhere. Sequences which were unable to be assigned taxonomy more refined than Kingdom were removed because it could not be determined if they represented true prey, parasites, or sequencing artefacts. The remaining sequence reads are considered to be representative of ingested prey, and will be subsequently referred to as 'prey sequence reads' or 'stomach contents sequence reads'.

Data analysis

CTD profiles were visualized with the Fathom toolbox for MatLab (Jones 2014) and chlorophyll concentrations were calculated as per Jespersen & Christoffersen (1987). Acoustic data were processed as per Warren et al. (2009), then visually inspected to remove noise from seafloor backscatter and binned into 'within fjords' and 'open water' for analyses. The spread or breadth of krill vertical distribution was calculated for each time point as

$$\frac{\sum_{i=1}^{\text{seafloor depth}} (\text{Depth}_i - \text{Peak Depth}) \times \text{Biomass}_i}{\text{Total Biomass}} \quad (1)$$

for i 1 m depth bins.

Prey sequence read counts were normalized across krill stomach samples. Multidimensional scaling (MDS) was conducted on Bray-Curtis distances between samples (Bray & Curtis 1957). A 1-way analysis of variance (ANOVA) was conducted on the percentage of sequence reads which were classified as *Chaetoceros* spp. in each sample within the following 5 categories: krill in fjords, krill in open water, water samples in fjords, water samples in open water, and captive krill.

Selectivity was analysed for the captive experimental krill by comparing the krill stomach contents with the available prey field. Only unicellular prey items were considered in the selectivity analysis because the small volumes of water filtered do not accurately reflect the mesozooplankton assemblage. Selectivity was measured using Ivlev's electivity index, calculated as $(r_i - p_i) / (r_i + p_i)$, where r_i is the proportion of prey i in the krill gut contents, and p_i is the proportion of prey i in the water samples, for the prey categories of Cercozoa, ciliates, *Chaetoceros* spp.

diatoms, other diatoms, dinoflagellates, fungi, non-diatom Ochrophyta and *Phaeocystis* spp. (Ivlev 1961). Because environmental samples (water filters) and krill were not collected from the same depths, selectivity results are not shown for *in situ* krill.

RESULTS

Environmental conditions

Throughout the study region, the water column exhibited salinity-driven stratification (Fig. 2). Mixed layers were shallowest (<10 m) in fjords, and showed the sharpest haloclines. In the open water stations, mixed layers were deeper (20 to 40 m), and haloclines, while still pronounced, were more gradual. Chlorophyll was concentrated within the surface mixed layer. In fjords, chlorophyll was strongly concentrated in the upper 10 m, with average extracted concentrations in these uppermost waters of 7.45 µg m^{-3}. CTD fluorescence profiles confirmed chloro-phyll was concentrated in the uppermost part of the water column, and declined sharply near the pycnocline (Fig. 2). Within fjords, very high chlorophyll was also observed in the surface-most layers, with flow-through fluorometer readings exceeding 30 µg m^{-3} at times. In the open water areas, chlorophyll was still concentrated in the upper water column, but chlorophyll peaks were broader, extending down to between 20 and 40 m, and much lower in magnitude, with an average extracted surface chlorophyll of only 0.94 µg m^{-3}.

Mesozooplankton communities were similar within fjords and in open water stations, though with generally higher abundances in fjords. Copepods dominated the assemblages, in particular *Metridia* spp. Across all samples, *Metridia* spp. copepods made up on average 65% of the zooplankton catch numerically. Other abundant copepods included *Oncaea* sp., *Euchaeta* sp., *Oithona* sp., *Rhincalanus* sp. and *Microcalanus* sp. Abundant non-copepod mesozooplankton included chaetognaths, ostracods, amphipods and polychaetes.
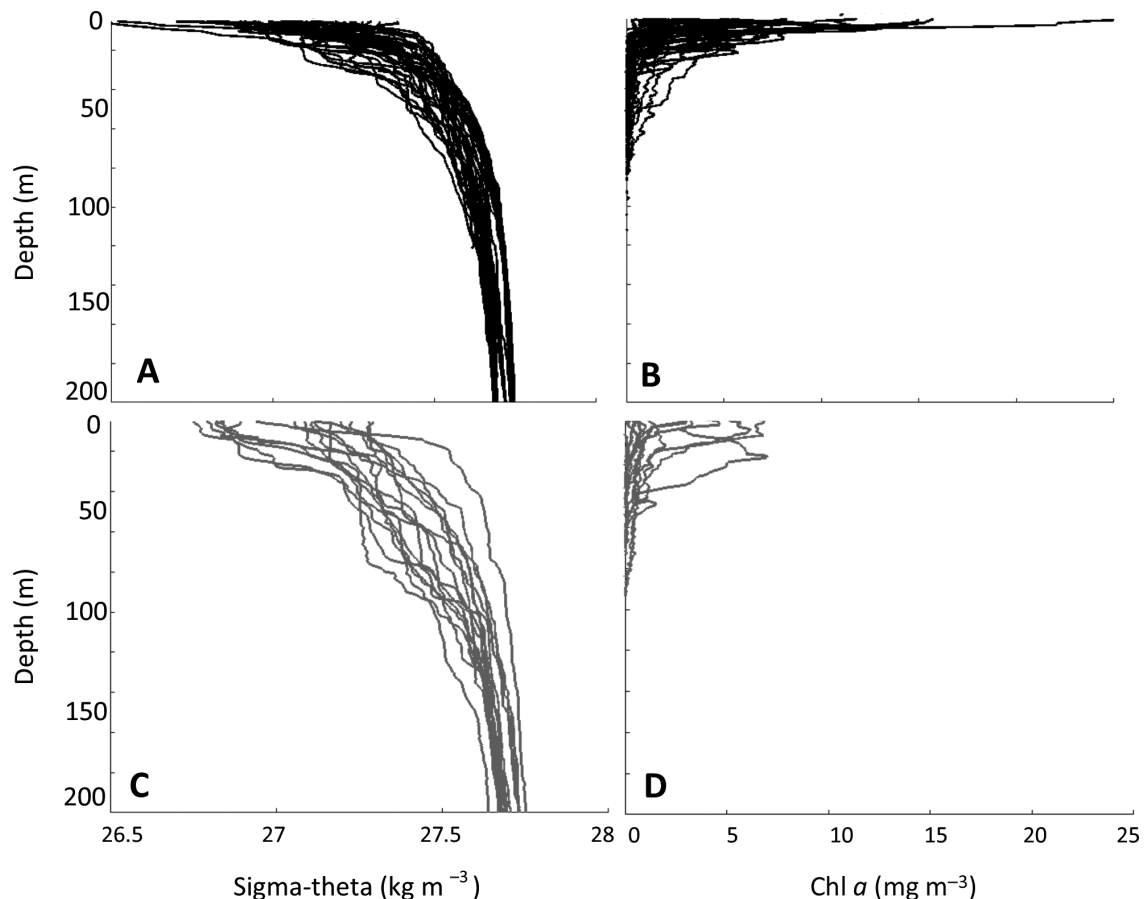

Fig. 2. Water column profiles of environmental conditions showing (A,C) potential density and (B,D) chlorophyll *a* within (A,B) fjords and in (C,D) open water areas during sampling

Krill within fjords were shallower than krill in open water, both in terms of the depth of peak biomass and the biomass weighted mean depth (peak: p < 0.0001, fjords = 81 m, open water = 93 m; weighted mean: p < 0.001, fjords = 80 m, open water = 96 m). Krill within fjords also showed a lower total water column biomass, and a lower maximum concentration than krill in more open areas (total: p < 0.001, fjords = 12, open water = 17; peak biomass concentration: p < 0.001, fjords = 2.4, open water = 4.3, in units of nautical area scattering co-efficient [NASC], which is proportional to krill biomass). Within fjords, krill were also more broadly distributed vertically (p < 0.001, fjords = 27, open water = 21).

Krill stomach contents

Krill ranged in length from 32 to 57 mm, with both a median and a mean length of 45 mm. Visually esti-mated gut fullness ranged from 0 to 100%, and was significantly higher in krill collected in fjords com-pared to krill from open water areas (*t*-test with un-

equal variance, p < 0.0001, mean in fjords = 65%, mean in open waters = 45%).

A total of 4 335 652 prey sequence reads were obtained. Of these, 3 561 275 reads came from the 169 wild krill individuals (mean ± SD: 21 073 ± 25 789), 4914 reads came from the 5 captive krill indi-viduals (983 ± 824), and the remaining 769 463 reads came from the 11 water filters (69 951 ± 18 933). Prey sequence reads clustered into 605 OTUs. Raw sequence data is available in the Short Read Archive (accession no. SRP131159), representative sequences from each OTU are available in GenBank (accession nos. KY863556 to KY864160), and counts of sequence reads for each OTU in each krill stomach are avail-able in the SCAR Southern Ocean Diet and Energetics Database (https://scar.org/data-products/southern-ocean-diet-energetics/). Prey OTUs included phyto-plankton (diatoms, chlorophytes, prymnesiophytes and others), microzooplankton (ciliates, Cercozoa, *Diplonema*, heterotrophic dinoflagellates and fungi), and mesozooplankton (copepods, chaetognaths, cteno-phores, siphonophores and pteropods) (Fig. 3).
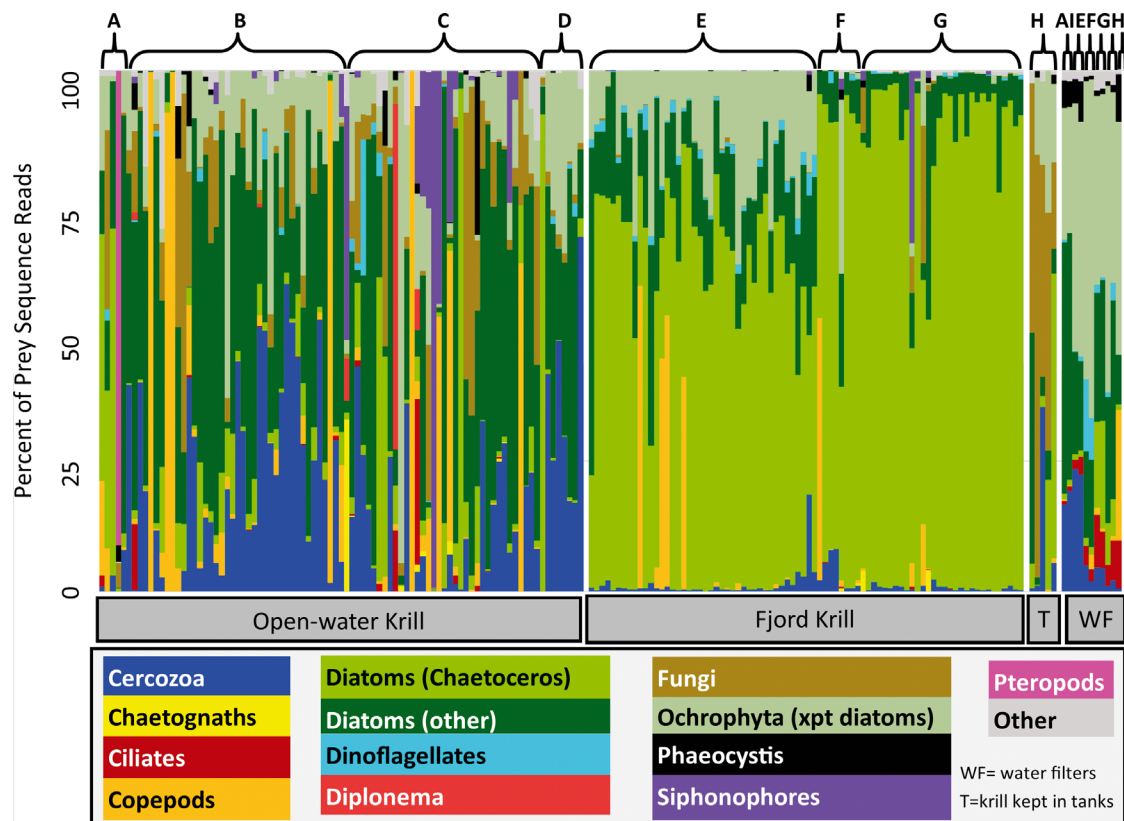

Fig. 3. Stomach contents sequence reads from all krill and water filters. Bar colour: prey sequence read taxonomic identity. Ochrophyta (xpt diatoms) includes all members of the Ochrophyta which are not diatoms. Grey bars below the bar graph: sam-ple type; letters above the bar graph: sampling locations (A: Palmer Deep; B: Bismarck Strait-East; C: Anvers Island; D: ice sta-tion near Renaud Island; E: Flandres Bay; F: Andvord Bay (early); G: Andvord Bay (late); H: tanks for captive krill; I: Bismarck Strait-West, located between Stns B and C)

Diatoms were the dominant prey sequence type, making up 75% of the total prey sequence reads recovered from wild krill. Other phytoplankton groups were observed only infrequently and at low abundance in krill stomach contents. Diatom genera included *Actinocyclus*, *Chaetoceros*, *Corethron*, *Coscinodiscu*s, *Eucampia*, *Porosira*, *Stellarima* and *Thalassiosira*. By far the most abundant of these were *Chaetoceros* spp. *Chaetoceros* spp. sequences were most closely related to *C. socialis* and *C. debilis*, although limited resolution within diatom genera make it impossible to assign precise species identity for diatoms from this gene fragment. *Chaetoceros* spp. sequence reads were particularly striking in abundance in krill stomach contents within fjords

(Figs. 3 & 4). ANOVA indicated that the percent of *Chaetoceros* was significantly different between krill in fjords and all other samples (water samples in fjords, water samples in open water, krill in open water and captive krill) with $p < 10^{-49}$. Other sample types were not significantly different from each other. Within fjords, *Chaetoceros* made up on average 71% of prey sequence reads, while in all other sample types *Chaetoceros* made up less than 17% of the prey sequence reads on average.

Microzooplankton prey sequence reads were mainly Cercozoa, and were more abundant in krill stomach contents from open water than from within fjords. The most abundant 2 of these Cercozoa OTUs were affiliated with *Cryothecomonas* spp. and *Ebria* spp.
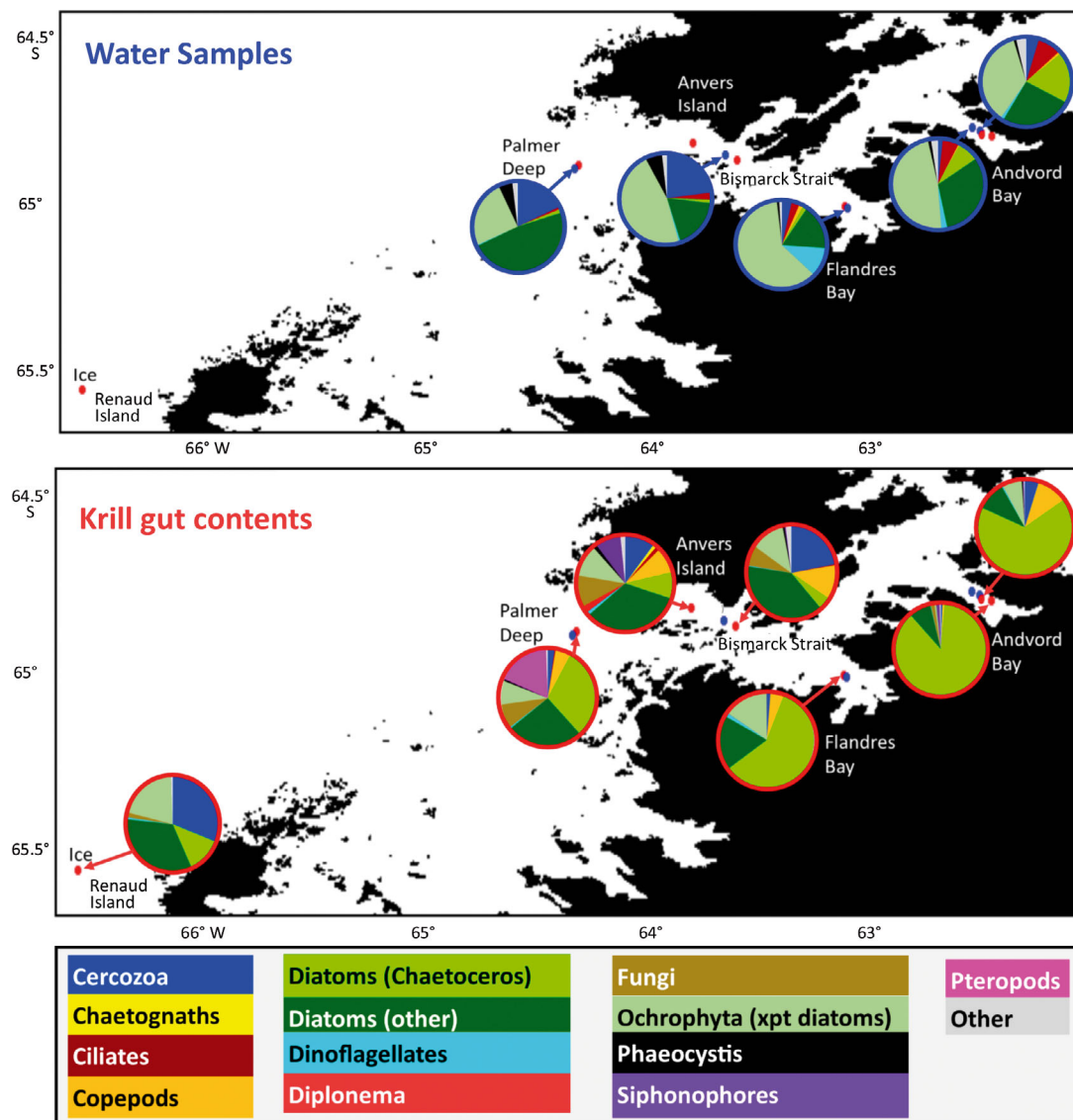

Fig. 4. Spatial differences in prey sequence assemblages in water filters (above) and krill stomach contents (below). Samples were normalized individually prior to combining within stations to account for variations in sequencing depth

Stomach contents of krill collected from the sea ice contained the highest average abundance of Cercozoa sequence reads (Fig. 4). The stomach contents sequences from one krill in the sample near Anvers Island were dominated by *Diplonema* sp., a poorly known protist within the Excavata. Fungi sequences were also observed in krill stomach contents, particularly from the captive feeding experiment krill (Fig. 3). Because fungal groups are poorly resolved by 18S sequences, it remains unclear whether these fungi represent true prey, symbionts/parasites, or trace contamination. No correlation was observed between fungal abundance and gut fullness (linear $r^2 = 0.04$) in krill from the water column.

Mesozooplankton consumed by krill were diverse, but were dominated by copepods. The percent of mesozooplankton within the stomach contents sequences of individual krill ranged from 0 to 99.9%, with a mean of 9.4% and a median of 0.08%. A total of 20% of individual krill guts (34 out of 169) contained greater than 5% mesozooplankton sequence reads, 10% of individuals (15) contained greater than 30% mesozooplankton sequence reads and 5% of the individuals (8) contained over 90% mesozooplankton sequence reads (Figs. 3 & 4). No correlation was observed between krill length and percent of mesozooplankton prey sequences. *Metridia* spp. copepods formed the largest component of the mesozooplankton reads in stomach contents, making up 30% of the total. The next most abundant mesozooplankter in the sequence reads was *Oithona* spp., making up 19% of the total.

Prey sequence assemblages from krill stomach contents and water filters clustered broadly into 2 groups: krill from fjords, and everything else (Fig. 5). Krill from fjords were clustered together, and within this overall pattern krill from different bays clustered together. Krill from 2 temporally separated collections in Andvord Bay clustered more closely together than either did with samples from the adjacent Flandres Bay, which was temporally between the 2 Andvord samplings. Krill from Palmer Deep were spread broadly in MDS space and did not cluster together particularly. Krill samples from Bismarck Strait and near Anvers Island clustered together. Most of the ice station krill clustered tightly together, and within the cluster formed by Bismarck Strait and Anvers Island krill. Captive krill also clustered in with these more open water krill. No clustering was detectible on the basis of krill length or sex or on the basis of sequencing run. All surface water samples, including water samples from within fjords, clustered with the open water krill stomach contents samples. Clustering of water samples also shows the high degree of similarity between the paired samples at each site, and of the pair of water filters from Flandres Bay with the vertically integrated (0 to 100 m) net tow sample from the same bay used as prey in the captive krill experiment.

Although the incubation experiment was limited in scope, it may provide indications of krill prey preferences to inform interpretation of the diets of wild capture krill. In the incubation, krill exhibited positive selection for *Chaetoceros* spp. diatoms, Cercozoa and fungi. Other groups (ciliates, other diatoms, dinoflagellates, non-diatom Ochrophyta, and *Phaeocystis* spp.) were avoided (negative selection).

Water filters
Captive fed krill
Andvord Bay krill (M8)
Andvord Bay krill (M24)
Flandres Bay krill (M18)
Palmer Deep krill (M15)
Ice station krill
Bismarck Strait krill (M16)
Anvers Island krill (M17)
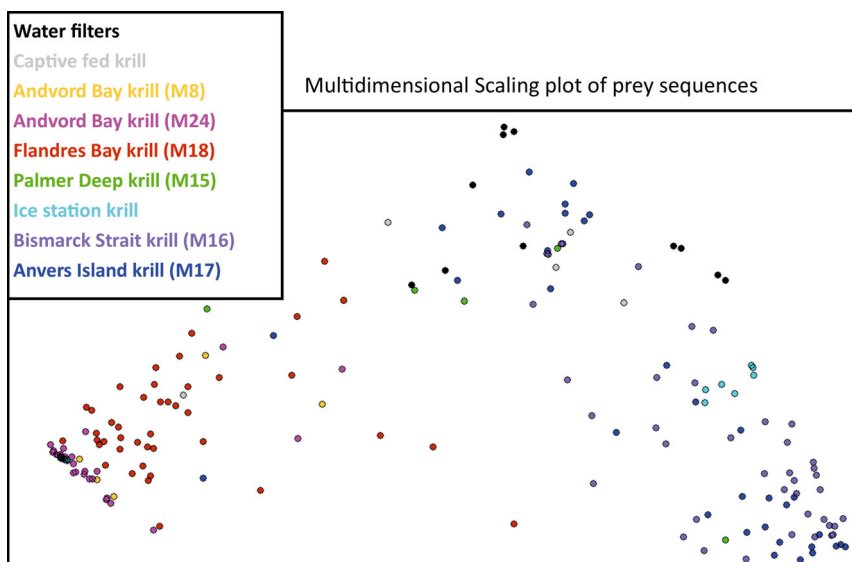
Multidimensional Scaling plot of prey sequences

Fig. 5. Multidimensional scaling plot of Bray-Curtis distances from operational taxonomic unit (OTU) read counts for all krill stomach and water filter samples. Note the general separation of krill guts from fjords from all other samples. The 3 water filter points which cluster together near the top of the plot are the 2 filters of surface water from Flandres Bay, and the 1 filter of net catch water from the captive krill experiment collected in the same fjord

DISCUSSION

The broad-scale pattern observed in krill stomach contents DNA, with a diatom-heavy diet supplemented by mesozooplankton, is consistent with previous work on *Euphausia superba* diet in this region. Diatom genera observed in krill gut contents have been previously noted in krill from the WAP (Hopkins 1985, Martin et al. 2006). Likewise, copepod genera consumed by krill in this study have been previously found in krill gut contents (Töbe et al. 2010, Schmidt et al. 2014). The consistency of the overall pattern observed here with what has been known from other approaches increases confidence that these DNA sequences provide a meaningful indication of krill diets. While these results provide information on the relative importance of different prey to krill in different habitats, they cannot be converted to exact biomass consumed without information on the digestion rate of the target DNA. Further controlled experiments will be needed to quantify DNA digestion rates for krill prey; it is hoped that future research, ideally with access to land-based krill aquarium facilities, can address this.

Differences between fjords and open waters

Striking differences were observed between the diets of krill collected within fjords and those collected in open waters (Fig. 3). *E. superba* are known to have different diets across their range (Perissinotto et al. 1997, Schmidt et al. 2014), but the extent of the dietary differences between our geographically quite close sampling locations was much greater than expected. Not only was the composition of krill diet different in fjords and open waters, but krill also showed differences in their feeding intensity between these 2 habitats. Krill gut fullness was over 40% higher in fjords than in open waters. Krill schools in fjords were also less dense, and spread more broadly over the water column. Less dense schooling behaviour has been observed for krill engaged in active feeding, whereas more compact schooling behaviour has been seen during area searching (Hamner 1984).

The differences in krill diet between fjords and more open waters are all the more surprising when taking into account that no significant differences were observed in the surface phytoplankton assemblages between these 2 habitat areas. Our sampling was relatively spatially and temporally restricted, and further research and more controlled studies will

be necessary to determine the broader applicability of these fjord–open water differences, and to pin down the precise mechanisms behind them. Possible explanations for these differences include differences in the water masses present, differences in vertical structuring of prey communities within the water column, differences in krill behaviour, or combinations of the above.

Some prey items may be associated with offshore water masses. Siphonophore sequences were surprisingly frequent and abundant in stomach contents from krill collected near Anvers Island. Eight of these krill had more than 10% of their prey reads derived from siphonophores, while one individual's prey sequence reads were 99% siphonophore (Fig. 4). Siphonophores are more typically associated with warmer and deeper waters, and are rarely found within the cold Antarctic surface waters (Pagès & Schnack-Schiel 1996). The Anvers Island sample was collected near the submarine Palmer Canyon, which transports warm deep waters, and the plankton found therein, up and onto the shelf (Schofield et al. 2013). Thus, it appears likely that krill in this area made use of advected siphonophores, or fragmented pieces thereof, as prey. Previous work has identified nematocysts in gut contents of *E. superba* in samples from the Scotia and Lazarev seas, but these cnidarians were not identifiable to lower taxonomic categories (Schmidt et al. 2014, Halbach 2015).

Fjords differ from open water habitats in many physical features which are relevant to the distributions of phytoplankton and other krill prey. Perhaps most obviously, fjords are shallower than adjacent, more open water areas. Fjords also showed strong salinity-driven water column structure, with sharp pycnoclines (Fig. 2). This salinity-driven stratification is typical of the very near coastal regions in the WAP, driven by summer melt of both glaciers and sea ice (Ducklow et al. 2013). Lastly, fjords are subject to sporadic strong and directional wind events. Katabatic winds descending from the mountains and glaciers of the WAP create sustained high wind speeds in a consistent direction. These winds funnel down the glacial valleys and over the fjords, but dissipate as they reach open water areas where they are no longer topographically constrained.

Because of these differences, fjords may have stronger vertical differences in phytoplankton assemblages. Phytoplankton can form layers, with thickness ranging from a few cm to a few dm, in which the assemblage of species can be very different from that observed in other areas of the same water column, with some observations of nearly monospecific layers

(Rines et al. 2002). Stronger density stratification in the water column is known to promote the formation of such layers, with phytoplankton observed to accumulate on pycnoclines (Rines et al. 2002). Similarly, directional sheer within the water column, such as that created by episodic katabatic winds, promotes the formation of layers by elongating phytoplankton patches horizontally (Durham & Stocker 2012). Both of these factors would tend to lead to more vertical structure in phytoplankton communities within fjords compared to more open water areas. The observed greater vertical changes in chlorophyll fluorescence in CTD profiles supports the idea of greater vertical phytoplankton structure within fjords.

Seasonal succession of phytoplankton species and groups over the brief Antarctic summer season can also lead to layering in phytoplankton, as senescent cells and newly formed resting spores slowly sink to deeper depths. While succession is likely to occur in both open water and fjord habitats, this process may have created differences in the deep water phytoplankton communities, which we did not sample, as a result of differences in surface communities prior to our sampling period, such as might be driven by physical factors including those mentioned above. *Chaetoceros* species closely related to our *Chaetoceros* spp. OTU, such as *C. socialis*, are known to form resting spores, and have previously been observed in early spring and associated with the initiation of the spring bloom (McMinn & Hodgson 1993, Brown & Landry 2001, Garibotti et al. 2003). Thus, it would not be unexpected to find deep layers of the senescing *Chaetoceros* spp. cells or recently formed resting spores from these blooms in deeper waters.

Krill were collected between the surface and 150 m depth, and acoustic observations indicated that krill biomass was concentrated around a mean depth of 81 m in fjords and 93 m in open waters. Because the krill were significantly deeper in the water column than the analysed water samples, krill may have experienced different prey fields within fjords compared to in open waters, despite the similarities of the observed surface water phytoplankton assemblages.

Krill may also make use of different parts of the water column in fjords than in more open water areas. If, as discussed above, fjords exhibit stronger vertical structuring of phytoplankton, krill may take advantage of areas of high prey concentration, potentially such as patches or layers of *Chaetoceros* spp. Krill are known to be able to locate phytoplankton patches and aggregate to them. In mesocosms, *Thysanoessa raschii* rapidly aggregated to a phytoplankton patch, and maintained position in the patch by turning back when they encountered the edges of the patch (Price 1989). *E. superba* efficiently find plumes of chemical cues in aquaria (Hamner & Hamner 2000), and the sheer created by katabatic winds over the fjords would spread these chemical cues and facilitate krill locating phytoplankton patches. Krill are strong swimmers, with aggregations able to travel over 10 km daily (Kanda et al. 1982), allowing them to locate phytoplankton patches in fjords. Krill feeding on patches of phytoplankton in fjords would also be consistent with the observed higher stomach fullness and more dispersed feeding-type schools within fjords, while outside of fjords krill schools spent more of their time in dense schools searching for prey, with lower stomach fullness and a more diverse diet.

Krill could potentially feed more selectively within fjords. Krill can feed selectively (Opali sky et al. 1997, Haberman et al. 2003), and in general, optimal diet theory would predict that selectivity is strongest when there is a high abundance of food resources to choose from (Emlen 1966). Measured selectivity indexes were strongly positive for *Chaetoceros* spp. suggesting this is a preferred prey item. Chlorophyll was over 5 times higher in fjord surface waters compared to surface waters at the open water stations (Fig. 2), potentially allowing krill to be more selective in their feeding. However, captive krill in buckets were fed at quite high concentrations of phytoplankton collected from a fjord, and their stomach contents contained relatively low levels of *Chaetoceros* sp. sequences, more consistent with open water krill than fjord krill. This suggests that while feeding within fjords may have been more selective, this higher selectivity alone is unlikely to explain the observed high proportions of *Chaetoceros* spp. sequence reads in the stomachs of krill collected in fjords.

Krill may also have fed on sediments, as has been observed in other areas (Clarke & Tyler 2008, Schmidt et al. 2011). Sediments are more accessible to krill in fjords due to the shallower depths compared to the farther off-shore open water stations. DNA extracted from sediments previously collected in these fjords was dominated by *Chaetoceros* spp. sequences (Cleary 2015), and these sequences showed 97% percent identity to the *Chaetoceros* sequence observed here in krill stomach contents. However, neither acoustic data nor *in situ* camera observations (E. G. Durbin unpubl. data) found any indications of krill aggregations in proximity to the seafloor during this summer field season, suggesting the seafloor is unlikely to have been the source of this dominant prey item for krill in fjords.

The difference between fjords and open water in the proportion of krill's diet composed of *Chaetoceros* spp. highlights the utility of DNA in stomach contents as a measure of krill feeding *in situ*. Incubation experiments cannot capture spatial variability such as phytoplankton patches and layers, stomach contents microscopy tends to underestimate *Chaetoceros* spp. due to their lightly silicified valves (Passmore et al. 2006) and stable isotopes and fatty acids lack the resolution to distinguish *Chaetoceros* spp. from other diatom species. However, these other approaches have other benefits, such as integrating over longer time periods. Combining stomach DNA with some of these other approaches has the potential to improve our understanding of krill feeding in the future.

Conclusions and implications

In terms of overall diet, DNA analysis of krill stomach contents was consistent with our existing understanding of krill diet from traditional methods. Diatoms made up the majority of prey sequence reads, and this herbivory was supplemented with consumption of common copepod species. The most striking result of our study was the difference between the stomach contents of krill collected in fjords and those collected in nearby open waters. Within fjords, krill fed almost exclusively on *Chaetoceros* spp. diatoms. These diatoms are likely to be a preferred food item, as krill in an incubation experiment actively selected for them. Higher gut fullness of krill in fjords additionally suggests fjords may have provided good feeding opportunities for krill. It appears likely that krill were able to exploit some form of patches or layers of *Chaetoceros* spp. within fjords, which they were not able to feed on in the more open water stations.

These results suggest that fjords may be important habitat for *E. superba*, in contrast to the classical paradigm of *E. superba* as a shelf and slope species (Atkinson et al. 2008). The WAP region is under increasing pressure from human activities, changing climate, tourism and fisheries (Ducklow et al. 2007, Bender et al. 2016, Nicol & Foster 2016). The observed differences in krill feeding between fjords and adjacent open waters suggests that these fjord regions may be worthy of special consideration in the spatial management of the WAP region, particularly with respect to the krill fishery. NGS of krill stomach contents offers a new tool, complimentary to the suite of existing measures of krill feeding. Applying this approach over broader temporal and spatial scales, ideally in combination with measures of the available prey field, may offer new insights into the trophic roles of this keystone species in Antarctic ecosystems.

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