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Population structure and production of four sibling species of *Pseudocalanus* spp. in the Chukchi Sea

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Copepods of the genus *Pseudocalanus* are important members of zooplankton communities in temperate and polar shelf regions, but few studies have focused on their species-specific biology due to the very subtle morphological differences between the species. We assess the distribution, population structure and production of four co-occurring species of *Pseudocalanus* across the Chukchi Sea during 2004, 2009 and 2012. Our approach used a combination of microscopic identification and species-specific polymerase chain reaction to discriminate between the species. Currently, the arctic *P. acuspes* dominates the genus (50–90%), with the relative distribution of species closely linked to water mass distribution and variations in physical properties, making *Pseudocalanus* important indicators of water mass origin. Although the temperate *P. newmani* had a significant presence throughout the Chukchi Sea, its stage distribution suggests that they recruit poorly in cold waters. Direct temperature-manipulation experiments further suggest that the reproductive activity of the two temperate species is inhibited at low temperatures, while the arctic *P. acuspes* exhibits reduced fitness and lower reproductive capacity when temperatures are increased to 10°C. Our results suggest that shifting oceanographic patterns and climate warming will have unequal impact on this genus, arising from species-specific differences in life histories and tolerance to environmental conditions.

KEYWORDS: *Pseudocalanus* spp.; Chukchi Sea; zooplankton; ssPCR

INTRODUCTION

The Chukchi Sea shares many features with other shallow Arctic Shelf seas, including ice-cover throughout a large portion of the year. However, in the summer months (June–September), it is dominated by advective processes and becomes largely Pacific in character. The

unique hydrography of the Chukchi Sea, as well as its role as both an Arctic and seasonally subarctic habitat, results in unique patterns within the zooplankton communities (Hopcroft *et al.*, 2010; Eisner *et al.*, 2012; Questel *et al.*, 2013; Ershova *et al.*, 2015a). Copepods of the genus *Pseudocalanus* are common members of planktonic ecosystems of the Chukchi Sea, as they are in temperate, subpolar and polar seas throughout the northern hemisphere. Despite a comparatively small body size, their extremely high abundance together with high production rates (Liu and Hopcroft, 2008; Hopcroft and Kosobokova, 2010) makes them one of the most important components of shelf zooplankton communities. Within the Chukchi Sea, *Pseudocalanus* spp. can contribute up to 50–90% of mesozooplankton abundance and 10–15% of zooplankton biomass (Ershova *et al.*, 2015a). Five of the seven species of this genus are reported from the Pacific Arctic region. *Pseudocalanus acuspes* and *P. minutus* are circumpolar species, shared with all Arctic and subarctic shelf seas; they are also common in the northern Bering Sea, which is oceanographically similar to the Chukchi Sea. *Pseudocalanus newmani* and *P. mimus*, however, are temperate species that are common throughout the North Pacific and are considered expatriates in the Arctic. While some studies have also reported *P. major* in the Chukchi Sea (Matsuno *et al.*, 2011), its occurrence has never been confirmed by taxonomists or other research groups with more intensive efforts in the region; even if present, this species likely plays a very small role in the overall community.

Closely related species can often share a very similar morphology, but differ substantially in their biology, thus playing distinctive roles within an ecosystem. There are numerous examples among high-latitude planktonic copepods, including *Pseudocalanus* spp., of congeneric species exhibiting distinct behavior and life history and contributing unequally to secondary production (e.g. Conover, 1988; Miller and Clemons, 1988; Renz *et al.*, 2008). Studies on species-specific biology of high-latitude organisms become particularly important in light of the rapid climate-related changes in their environment, which may shift conditions to favor some species while being detrimental to their close relatives. With longer ice-free seasons (Wood *et al.*, 2015), increasing water temperatures (Luchin and Panteleev, 2014) and observed shifts in phytoplankton communities (Arrigo and van Dijken, 2011), the Chukchi Sea is adapting to a "new normal" climate (Wood *et al.*, 2015), forcing changes in the pelagic system (Hopcroft *et al.*, 2010; Eisner *et al.*, 2012; Questel *et al.*, 2013; Ershova *et al.*, 2015a, b). One expectation of climate-related change is that there will be shifting prominence of closely related species. As Chukchi Sea conditions become more "boreal" for longer periods of the season cycle, the advected warm-water species, such as *P. newmani* and *P. mimus*, may have an opportunity to play an increased role in their communities. This may occur at the expense of the "resident" arctic *P. acuspes* and *P. minutus*, whose life history is thought to be closely linked to seasonal ice retreat and ice-associated production.

Despite the wide distribution and the fundamental role of *Pseudocalanus* spp. in pelagic ecosystems, comparatively little is known about their species-specific biology and ecology. Species-specific studies are generally hampered by very subtle morphological differences between the species, particularly in the juvenile stages, when they are virtually indistinguishable (Frost, 1989). Molecular studies on this genus suggest that even adults are systematically misidentified (Aarbakke *et al.*, 2011; Bucklin *et al.*, 2015). The mitochondrial cytochrome oxidase I (COI) gene is a commonly used barcoding tool to identify cryptic species. In recent years, multiple molecular protocols have been developed using COI to discriminate between several coexisting *Pseudocalanus* species, shedding some light on their distribution, abundance and population genetics (Aarbakke *et al.*, 2011; Holmborn *et al.*, 2011; Aarbakke *et al.*, 2014; Bucklin *et al.*, 2015). However, with the exception of a handful of recent studies (Matsuno *et al.*, 2011; Bailey *et al.*, 2015; Cleary *et al.*, 2015; Questel *et al.*, 2016), most of this research has been restricted to the Atlantic sector (Bucklin *et al.*, 2001; Grabbert *et al.*, 2010; Holmborn *et al.*, 2011; Aarbakke *et al.*, 2014; Bucklin *et al.*, 2015), while the majority of researchers still only focus on adult females, which may poorly represent the entire species population.

In this work, we examined species-specific aspects of *Pseudocalanus* spp. in the Chukchi Sea during summers of 2004, 2009 and 2012 within the framework of the Russian-American Long-term Census of the Arctic (RUSALCA) program. The present study is the first to examine the species-specific distribution, population structure and production of all four species of *Pseudocalanus* spp. in the Pacific Arctic using a species-specific polymerase chain reaction (ssPCR) to discriminate between species.

METHOD

Physical environment

Three expeditions occurred within the RUSALCA framework: 4–25 August 2004, 4–27 September 2009 and 2–24 September 2012 (Fig. 1). The thermal characteristics of the region, distribution and properties of the water masses and overall patterns in zooplankton

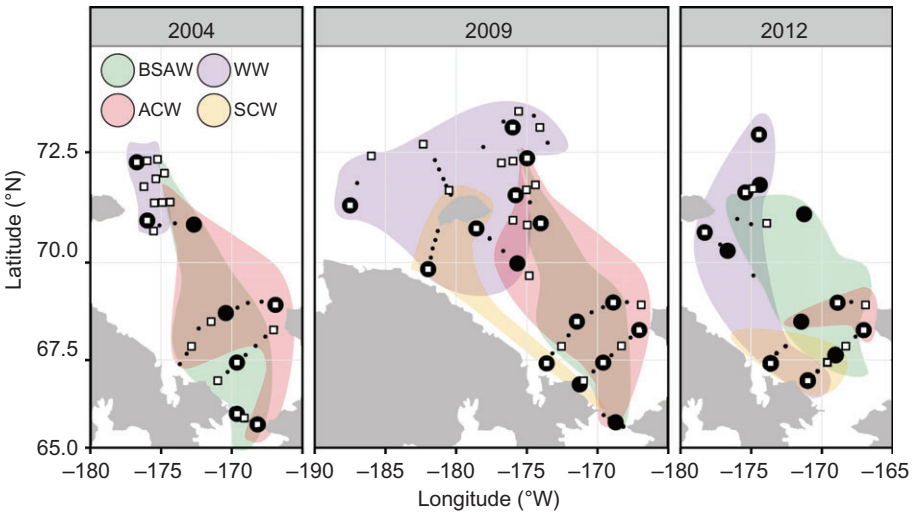

Fig. 1. The Chukchi Sea region with station locations in 2004, 2009 and 2012. Small black dots indicate stations where quantitative zooplankton samples and CTD data were collected; large black dots indicate stations where molecular identification of *Pseudocalanus* spp. was obtained; white squares indicate stations where egg production experiments were carried out. Color overlays indicate water mass types present at the stations (based on Pisareva *et al.*, 2015a). BSAW, Bering Sea Anadyr Water; WW, Winter Water; ACW, Alaska Coastal Water; SCW, Siberian Coastal Water.

communities in the Chukchi Sea during the three expeditions are described in detail elsewhere (Pickart *et al.*, 2010; Ershova et al., 2015a; Pisareva *et al.*, 2015a, b). Overall, 2004 was the warmest of the three study years, with an average sea surface temperature (SST) of 6.3°C over the sampled stations, and with surface waters as warm as 10–12°C present along the Alaska Coast and the entrance of the Herald Valley region. The years 2009 and 2012 were markedly colder, with the coldest SST observed in September 2012, averaging only 3.4°C. The warm and fresh water of the Alaska Coastal Current (Alaska Coastal Water, ACW) was constrained to the eastern shelf in 2012, resulting in a strong temperature gradient from east to west. In 2004, this water mass type also occupied the surface waters of a number of stations in the southwestern Chukchi (Fig. 1). During the 2009 expedition, the ACW was diverted by northerly winds onto the western shelf region and into Herald Valley (Pisareva *et al.*, 2015b). Bering Sea Anadyr Water (BSAW), characterized by colder temperatures and oceanic salinity, was found on stations through the central Chukchi and on the eastern side of Herald Canyon. Cold and fresh Siberian Coastal Water (SCW) was present in 2009 and 2012 near the coast of Siberia and around Wrangell Island. Cold and saline resident Chukchi Winter Water (WW), usually overlain by ice Melt Water (MW), was constrained to the northern Chukchi region in 2004 and 2009; in 2012, it was also observed in the southern sampling domain at the stations approaching the Siberian coast (Pisareva *et al.*, 2015a) (Fig. 1).

Plankton collection and processing

Zooplankton samples were collected using 150-μm double ring nets of 60 cm mouth diameter. The nets were hauled vertically over the entire water column from within 3–5 m of the seafloor to the surface. The average depth over the entire sampling area was 40–50 m. The total volume of water filtered through the nets was measured using General Oceanics or Sea-gear flow meters, which were positioned at the mouth of each net, and rigged not to spin during descent. Upon retrieval, one of the samples was preserved in 10% formalin and the other in 95% molecular-grade ethanol.

The formalin-preserved samples were processed in the laboratory to determine community composition, abundance and biomass (see Ershova *et al.*, 2015a). *Pseudocalanus* spp. adult females were identified to the species level; all juveniles and adult males were simply classified as *Pseudocalanus* spp. All individuals were separated into copepodite stages (C1–C5/AF/AM) and measured using a computer measurement system (ZoopBiom software, Roff and Hopcroft, 1986). Typically, a minimum of 80–100 individuals were enumerated per station. The dry weight (DW) of each organism was predicted from a length–weight regression relationship for this genus (Liu and Hopcroft, 2008). Total abundance and biomass were calculated by scaling the subsample counts of each copepodite stage with the total volume filtered by the net. *Pseudocalanus* nauplii were excluded from analysis because they are generally extruded by the 150-μm nets.

Oceanographic data was collected at each station with a Seabird 911+ CTD equipped with an oxygen sensor, transmissometer and fluorometer (e.g. Pickart *et al.*, 2010), with all physical data binned into 1-m intervals during post processing. Due to the frequent layering of distinct water masses at a single location, surface (averaged for 0–10 m), bottom (averaged for 10 m above seafloor) and mid-water temperature (10–10 m above seafloor) and salinity values at each station were examined as separate variables. Chlorophyll samples were collected by Niskin bottles on the CTD rosette every 5 m from the surface to bottom, filtered at low pressure onto GF/F filters and analyzed fluorometrically (Lee *et al.*, 2007; Yun *et al.*, 2014; 2016). Mean mixed-layer as well as maximum chlorophyll values (mg m^{-3}) were used in the analysis.

ssPCR identification

Species-specific primers were designed for each of the four species of *Pseudocalanus* spp. using 710-bp COI consensus sequences obtained from Questel *et al.* (2016). The forward primer was common for each species (PseudoF, 5′-TTCGAATAGAGYTAGGHMVAGY-3′) (Questel *et al.*, 2016); the reverse primers were selected from different sites along the COI gene at regions that were conserved within a species, yet allowed enough sequence variability between them (a minimum difference of 2 bp). Primers were selected using CodonCode Aligner (www.codoncode.com/aligner) and were examined for annealing temperature compatibility and primer dimer formation using online tools from Fisher Scientific. The species-specific primers were as follows, with numbers in the primer names indicating the location along the consensus sequence:

- acuspes238R; 5′-AGAGGAGGGTATACAGTTCACC-3′
- newmani522R; 5′-CACCCCCACCAACATCRTAG-3′
- minutus398R; 5′-CGCAAACARAGGTATTTGGTCT-3′
- mimus93R; 5′-ACYAGCCAGTTACCAAAACCC-3′.

The resulting amplification products for each species were of different lengths and were thus distinguishable using gel electrophoresis (Fig. 2).

Species-specific abundance and population structure was examined at 27 stations (Fig. 1). Stations were selected to represent the different water mass and community types that we observed during the three study years (Ershova *et al.*, 2015a). Between 100 and 200 individuals of *Pseudocalanus* spp. were randomly selected

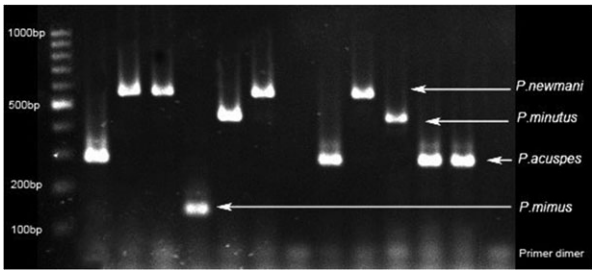

Fig. 2. Example of ssPCR results with all four species of *Pseudocalanus* spp. present.

from each station for molecular identification, with a minimum of 15 and typically 20–30 individuals of each copepodite stage selected per station. Insufficient numbers of adult males occurred within most samples to reach such thresholds. The stage, sex (for C5's and adults) and prosome length of each individual was recorded. To evaluate the accuracy of routine visual identifications, adult females were keyed to species based on prosome length and head shape (Frost, 1989). The copepods were soaked in distilled water for 20–30 min, and then transferred to individual wells on a 96-well plate, containing 16 µL of distilled water. The plates were microwaved for 2 min to remove any remnants of ethanol. PCR master mix, including a mix of the five primers, was added to bring the total reaction volume to 25 µL. No DNA extractions were done, and the PCR reactions were run with intact copepods in tubes (Grabbert *et al.*, 2010). The amplification protocol was: 94°C (40 s); 60°C (40 s); 69°C (50 s) for 35 cycles. The amplified DNA was electrophoresed on a 2% agarose gel, soaked in ethidium bromide solution and visualized under UV light. One negative and four positive controls for each species, confirmed by sequencing, were run with each 96-well plate. After confirming the morphological and morphometric differences between adult females of different species by obtaining results on ~300 females, for expediency we only ran PCR on those females that had ambiguous characteristics. The abundance of each species at each station was calculated by overlaying the relative contribution of each species and stage from molecular identification onto the quantitative data obtained from the formalin-preserved samples.

Egg production experiments

Pseudocalanus spp. carry egg clutches attached to their first abdominal segment until hatching. We measured egg production rates (EPRs) at 20 stations in 2004, 28 stations in 2009 and 12 stations in 2012 (Fig. 1) using established methods (Hopcroft and Kosobokova, 2010). For each experiment, 50–120 females were placed

individually into 70 mL polystyrene culture flasks containing 50-μm filtered seawater collected at the same station, then incubated at temperatures close (within 1–2°C) to ambient for 48 h. Females were monitored every 24 h for newly produced egg clutches; all reproducing individuals were removed and preserved individually. The remaining females were preserved together at the end of each experiment.

In the laboratory, preserved females were identified to species using morphological characteristics (Frost, 1989) and the eggs were counted from each clutch. Prosome length of each female was measured, and its DW was estimated from length. Eggs were assumed to have a constant weight within each species, as predicted from mean diameters known for each species in this habitat (Hopcroft and Kosobokova, 2010). EPR was calculated for each species as the total number of eggs produced per day by all the females of that species in the experiment; EPR was calculated for a minimum of eight individuals of each species. In 2004, production rates declined during the second 24 h period and were therefore significantly lower when averaged over 2 days than when calculated for only the first 24 h (see Hopcroft and Kosobokova, 2010); in 2009 and 2012 no decline was observed over the second day. Despite the potential bias of using a 48-h incubation due to bottle effects, EPR was averaged over 2 days because for the rarer species sample sizes were often quite small (~10 individuals), and 24 h was not enough to register any reproductive activity. During all 3 years, variability was lower and correlation of EPR to female body size was better when averaged for 2 days of the experiment; for these reasons, a 2-day mean EPR rate was used for analysis. To standardize egg production to female size, we calculated specific egg production (SEP) for each species, dividing the weight of the eggs produced (assuming 0.14 pg C μm^{-3}; Kiørboe and Sabatini, 1994) by the female's body weight for each female within each experiment. Production rates were adjusted for temperature by standardizing SEP to 0°C using a Q_{10} value of 1.43 for *in situ* sac-spawning adult copepods (Hirst and Bunker, 2003).

Secondary production

Secondary production was calculated as $P_i = g_i \times B_i$, where P_i was the production (μg DW m^{-3} day^{-1}), g_i was the growth rate and B_i was the biomass (μg DW m^{-3}) of copepodite stage i. Growth rate (g_i) for copepodite stages C1–C5 was calculated at each station as $g_i = \log(W_{i+1}/W_i)/D_i$, where W was the mean DW (μg) obtained from length–weight regression relationship, and D was the developmental time in days from i to $i + 1$ for this group of species (Liu and Hopcroft, 2008), adjusted for temperature using a Q_{10} of 2.6 for food-satiated sac-spawing juveniles (Hirst and Bunker, 2003). While other estimates for developmental times of different species of *Pseudocalanus* exist in the literature, those selected best reflect the conditions and species composition of the Pacific Arctic. For adult females, g_i = SEP obtained at each station (or neighboring stations when experiments were not carried out at a location). Overall production for each species at each station was obtained by summing the production for each stage. This overall production estimate excludes production by naupliar stages, which can be significant during recruitment pulses (Renz *et al.*, 2007).

Temperature-controlled egg production experiments

We conducted temperature-controlled egg production experiments to assess the responses of the different species of *Pseudocalanus* using collections from the Gulf of Alaska in mid-September 2013 and from the northeast Chukchi Sea in early October 2013. A minimum of 60–90 females from each location were placed into 500 mL flasks (20–30 females per flask) containing filtered seawater and a food mixture and incubated at temperatures of 0, 3, 7 and 10°C. The food mixture consisted of frozen cultures of *Pavlova* spp. and *Isochrysis galbana*, and a live culture of *Thalassiosira weissflogii* (cultured at 20°C in F/2 medium on a 12 h light/dark cycle). They were provided at a cell count ratio of 5:5:1, with a final phytoplankton concentration of ~0.4 μg C mL^{-1} that was found to be above the limiting food concentration for *Pseudocalanus* (Corkett and McLaren, 1978).

After allowing the females to acclimatize to the new conditions (~5–7 days), they were transferred to individual 70 mL flasks containing filtered seawater and the phytoplankton mixture and incubated for an additional 7–10 days. The flasks were mixed by inversion 2–3 times a day to keep the algal cells suspended; every 72 h the water was replaced with fresh seawater and food mixture. Females were examined twice a day for new clutches; once a female produced a clutch she was removed from the experiment. Prior to female preservation in 4% formalin, a subset of full egg clutches (30–80% of total clutches produced, with lower numbers incubated at lower temperatures due to long hatching time) was gently removed from the female with a probe, with experimental capacity dictated by incubator space and handling time. These eggs were counted and left to hatch in 10 mL of filtered seawater at the temperatures that they were produced. Eggs were monitored every 12 h for hatching with the number of successfully

hatched nauplii, stillborn nauplii and unhatched eggs recorded. At the conclusion of the experiment, all non-reproducing females were preserved in formalin.

Processing of females and egg clutches in the laboratory was similar to that described in the previous section. Females were identified to species using morphological characteristics (Frost, 1989). EPRs were calculated from the average number of eggs produced by all the living females in the experiment per day; rates were obtained for each 2-day period of the experiment. SEP rates were adjusted to 0°C using a Q_{10} of 2.6 for food-satiated sac-spawning adults (Hirst and Bunker, 2003).

Statistical analysis

All statistical analyses were carried out in R. Differences in abundances, population structure (mean developmental stage) and EPRs between years and species were compared using two-way Analysis of variance (ANOVA), with station-region used as a blocking factor. Significant interactions ($P < 0.05$) between categories were examined using the Tukey HSD Test. Mean stage of each species at each station was calculated when a minimum of 10 individuals of this species was identified at the station. For this reason, we do not show population structure of *P. mimus*, as there were very few stations where this criterion was met. When assumptions were met, multiple linear regression was used to examine the correlations between species abundances, size distributions, and EPRs to physical parameters.

The distribution patterns of *Pseudocalanus* species complex were explored using cluster analysis and non-metric parametrical scaling (nMDS) using the R package "vegan" (Oksanen *et al.*, 2013). We investigated abundance matrices for pooled stages of four species (four categories), as well as for each species divided into three groups: "early juveniles" (C1–C3), "subadults" (C4–C5) and "adults" (adult females and males) (i.e. 12 categories). As most researchers consider adult female abundances representative of the entire subadult population, we also investigated the patterns observed if only adult female abundances were employed (four categories). Abundances were log-transformed and the Bray–Curtis similarity index was calculated for all stations during each year. Hierarchical cluster analysis using average linkage was carried out and qualitative separation of groups was established by overall similarity (~65–70%). These resulting groups were superimposed on 2D plots of nMDS plots and spatial plots of study area. We established relationships between species abundance and environmental factors by examining correlations of the nMDS ordination to species abundances and environmental factors (bottom and SST and salinity). Additionally, we implemented the BIOENV routine (Clarke and Ainsworth, 1993), which establishes the best set of correlations between a matrix containing environmental data and an abundance similarity matrix. Significance of these correlations was established using a permutation test ($n = 10\,000$) at $P < 0.05$.

RESULTS

Molecular identification

A total of 4300 individuals were identified using ssPCR. Failure rate (the number of individuals that failed to produce a distinct band) ranged from 0% to 50% per station, and was highest in the oldest (2004) samples. Some reactions resulted in double banding (bright *P. acuspes* and faint *P. mimus* bands); sequencing these individuals always confirmed that they were *P. acuspes*.

All four species of *Pseudocalanus* were identified successfully using this method. The results of molecular identification revealed that within adult females ~95% of *P. minutus* and 90% of *P. acuspes* and *P. newmani* were identified correctly based on morphology only. Within the misidentified individuals, small *P. minutus* were usually mistaken for *P. mimus*, and small *P. acuspes* and large *P. newmani* were confused where their size ranges overlap (Fig. 3). Of the 12 *P. mimus* females that were identified using ssPCR, only 5 were identified correctly using morphology only, the remaining 7 were mistakenly identified as *P. minutus* or *P. acuspes*.

The estimated probability function (Fig. 3) revealed that each species, with the exception of *P. mimus*, has a distinct size mode at each stage. However, the size classes overlapped significantly, particularly between *P. acuspes* and *P. newmani*, and increasingly so at younger stages. Pooling the species at each stage produced an indistinctly bimodal distribution for the later-stage individuals (C4 copepodites to adults); however, at younger stages, it resulted in a near-normal distribution without any distinct peaks.

Abundance and distribution of *Pseudocalanus* spp.

Pseudocalanus acuspes had highest abundance and biomass during the 3 years, comprising 50–90% of all *Pseudocalanus* in the region (Fig. 4, Table I). Mean abundance and biomass of *P. acuspes* was over three times higher in 2009 and 2012 than in 2004 ($P < 0.05$). Despite the similar values observed for this species in 2009 and 2012, *P. acuspes* played a much larger role

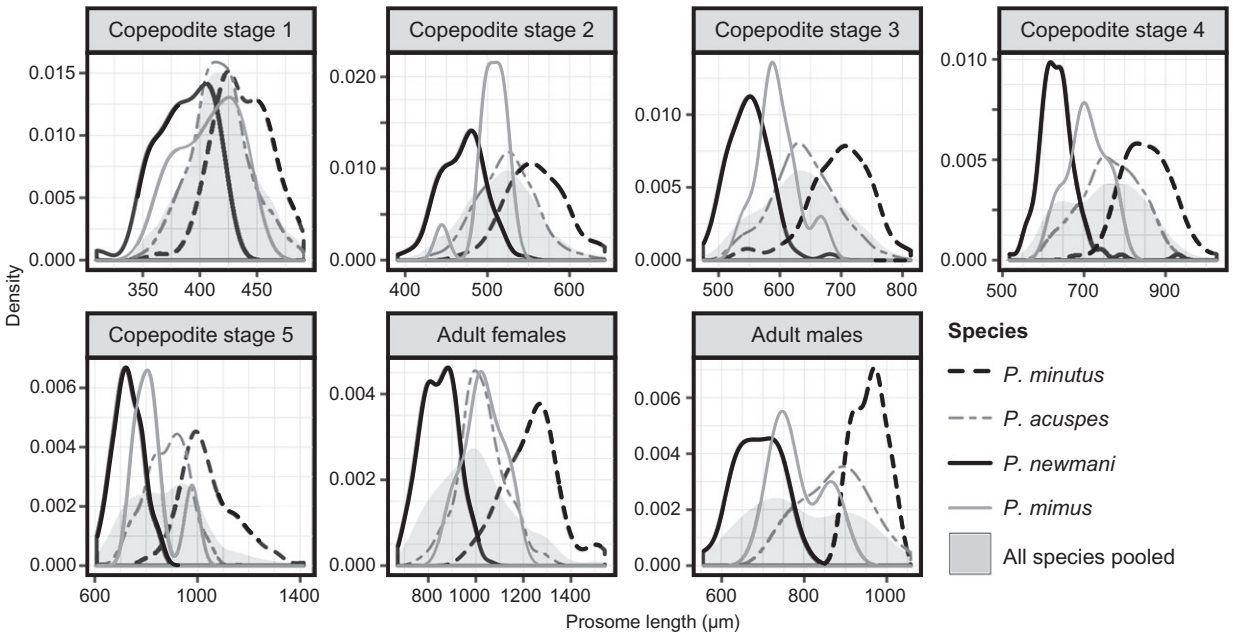

Fig. 3. Density distribution of size frequencies for all developmental stages (C1-Adults) of four species of *Pseudocalanus* spp. in the Chukchi Sea. Shaded curve represents the density plot for each stage with all species pooled.

relative to other species in 2012, nearly 90% of the *Pseudocalanus* population. However, abundance and biomass of *P. newmani* were highest in 2009 and drastically lower in 2012 ($P < 0.05$), accounting on average for only 4% of the *Pseudocalanus* observed during 2012 (Fig. 4, Table I). Although overall occurrence of this species was lower in 2004 than in 2009 (NS), its relative contribution to abundance and biomass of *Pseudocalanus* was comparable. There were no significant differences between years in abundance of *P. minutus*; however, in 2004, its proportional contribution was ~2 times greater than in 2009 or 2012. *Pseudocalanus mimus* was the rarest of all species during all 3 years, but showed a significantly higher presence in 2004 than in 2009 and 2012 (Fig. 4, Table I).

Species-specific population structure

No spatial or inter-annual differences in population structure were observed for the *P. acuspes* population, which generally consisted of all developmental stages, with the mean stage being 2.9–3.1 (Fig. 5). Adults rarely exceeded 10% of the population. However, the mean stage of the *P. newmani* population was significantly higher than that of *P. acuspes* during all 3 years, especially in 2004 and 2012, when it was 3.8–4.0. No significant differences in stage distribution were observed between the years. The *P. minutus* population was heavily skewed toward later-stage juveniles (C4–C5's) in 2009 and 2012, with a mean stage of ~3.5; in 2004, there was a sharp contrast between the southern stations, which were dominated by adults and later-stage juveniles, and the northern stations, which were almost entirely composed of early stages. The stations near the Siberian coast in 2009 and 2012 were notable in their almost complete absence of adults and later stages for both *P. acuspes* and *P. minutus* (Fig. 5).

Community structure and relationship to physical factors

Abundances of *P. newmani* and *P. mimus* showed a high positive correlation to temperature and salinity, with surface measurements producing the strongest relationship ($P < 0.01$, $R^2 = 0.46$ and 0.65, respectively). Abundance of *P. minutus* correlated with bottom salinity ($P < 0.01$, $R^2 = 0.35$), the relationship to temperature was statistically insignificant. When these environmental factors were incorporated into the multiple regression model with year as a categorical variable, differences in abundance between years became insignificant (with the exception of *P. mimus*, where 2004 > 2009). In contrast, overall abundance of *P. acuspes*, while significantly different between years, was not significantly correlated to physical factors. The results were somewhat different when population structure was taken into account. Abundance of young copepodites (C1–C4) of *P. acuspes* showed significant relationships to both temperature

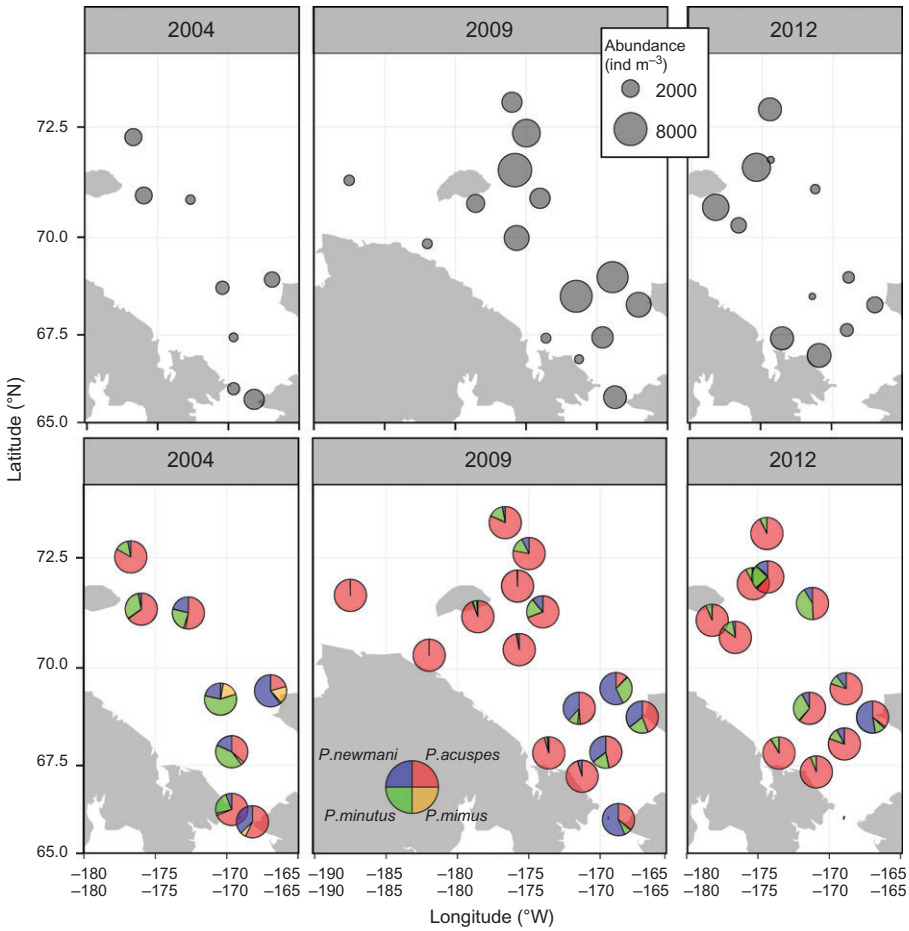

Fig. 4. *Pseudocalanus* spp. abundance (**a**) and relative contribution of species at each station (**b**) for four species of *Pseudocalanus* spp. in 2004, 2009 and 2012.

and salinity ($P < 0.05$, $R^2 = 0.25$), with higher abundances occurring at lower temperatures and higher salinities; but no such relationship was observed for adults plus subadults of this species (Supplementary data, Fig. S1). While overall abundance of *P. minutus* was not significantly related to temperature, the abundance of juveniles of this species (C1–C4) correlated negatively to bottom temperature, as well as bottom salinity; separating population into adults/subadults and juveniles resulted in an increased R^2 of 0.42 for the juveniles. Separating the data by copepodite stage did not improve the models for *P. newmani* or *P. mimus*.

When relative abundances of species were examined together (each species split into three categories: juveniles C1–C3, C4–C5 and Adults (C6)), the stations clustered into five distinct groups (with one outlier), with most groups containing stations from all 3 years. This separation was confirmed by nMDS ordination (2D stress = 0.1) (Fig. 6). Among the four species, abundance of *P. acuspes* was strongly and significantly correlated with MDS Axis 1, while *P. newmani* and *P. minutus* were driving Axis 2. Axis 2 was strongly driven by bottom temperature ($r = 0.88$, $P < 0.05$), while bottom salinity was the main factor driving Axis 1 ($r = 0.85$, $P < 0.05$). The groups identified by cluster analysis and nMDS corresponded exactly to the water mass types present at these stations (Fig. 1), confirming that the relative abundance of *Pseudocalanus* species was shaped by physical parameters and water mass distribution. The BIOENV routine predicted that the relative *Pseudocalanus* abundance was best correlated to all four variables (bottom and SSTs, bottom and surface salinities), resulting in a Spearman's correlation of 0.48. Using overall species abundance (four categories), produced similar, but slightly less robust results. However, when only adult female abundance was used and the copepodite stages were grouped as *Pseudocalanus* spp., no spatial structure or correlation to physical variables was observed.

Table I: Abundance (individuals m^{-3}), biomass (mg DW m^{-3}) and overall contribution (%) of four species of Pseudocalanus *spp. in the Chukchi Sea in 2004, 2009 and 2012*

		2004	2009	2012	*P*-value	Significant interactions
P. acuspes	Mean abundance	630	1998	1784	0.08	2009 > 2004
	Mean biomass	1.6	5.1	4.7		
	%Total abundance	51	64.4	87		
P. minutus	Mean abundance	232	372	171	0.15	None
	Mean biomass	0.7	1.2	0.6		
	%Total abundance	18.8	12	8.3		
P. newmani	Mean abundance	295	714	92	0.01	2009 > 2012
	Mean biomass	0.8	2.7	0.3		
	%Total abundance	23.9	23	4.5		
P. mimus	Mean abundance	79	15	3	0.01	2004 > 2009, 2012
	Mean biomass	0.2	0.1	<0.1		
	%Total abundance	6.4	0.3	0.2		
Total abundance		1236	3099	2050	0.03	2009 > 2004
Total biomass		3.3	9.1	5.6		

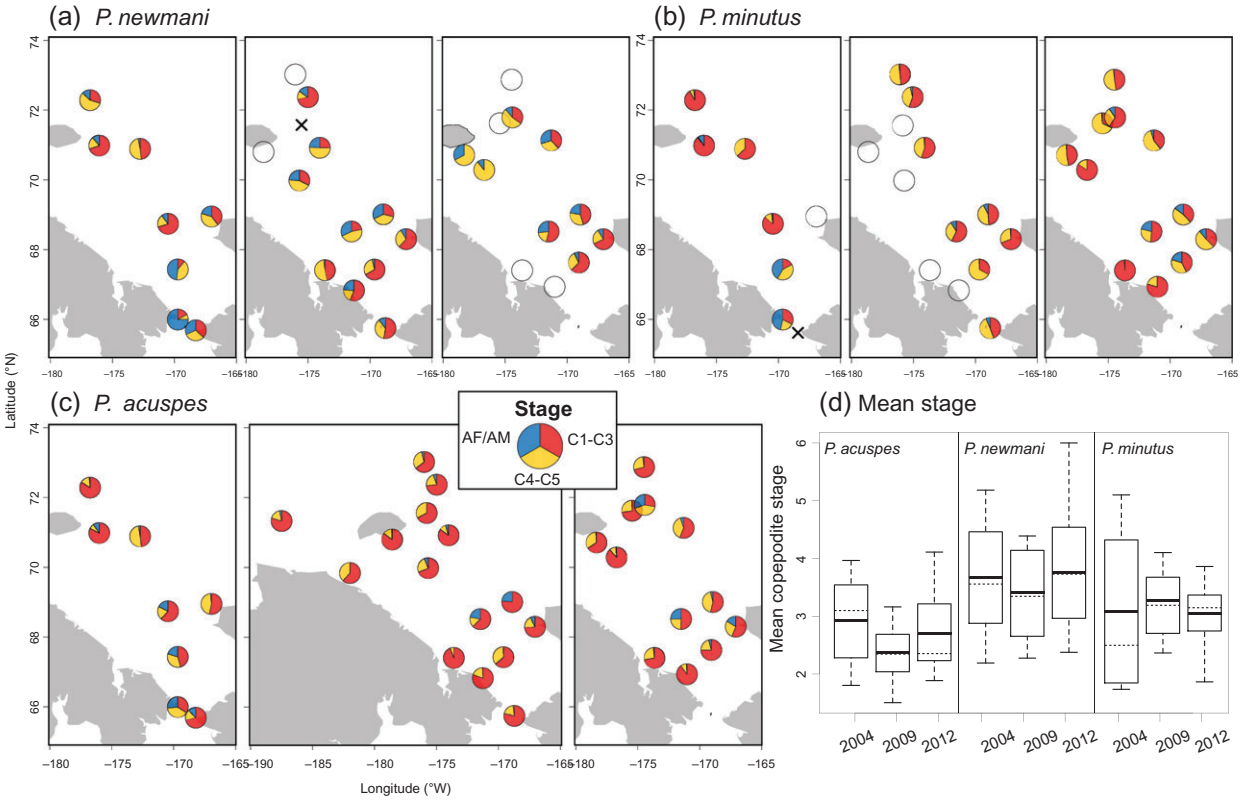

Fig. 5. Relative contribution of early stage juveniles (C1–C3), late stage juveniles (C4–C5) and adults (AF/AM) for three *Pseudocalanus* species in the Chukchi Sea in summer during (**a**) 2004, (**b**) 2009 and (**c**) 2012; (**d**) shows mean (solid black line) and median (dashed line) stage of each species per cruise. Box margins indicate 25/75% quartiles and whisker caps indicate 10/90% percentiles.

Size structure

Since size is one of the main features routinely used to identify *Pseudocalanus* spp., we examined the distribution of size variability and its relationship to physical parameters (Supplementary data Fig. S2). In *P. acuspes* and *P. newmani*, prosome length of each stage negatively correlated to water temperature ($P < 0.01$, $R^2 = 0.25$–0.6), except for C1 and C2 of *P. newmani* where the number of observations was low. In *P. minutus*, the relationship was significant only for C2; no relationship could be

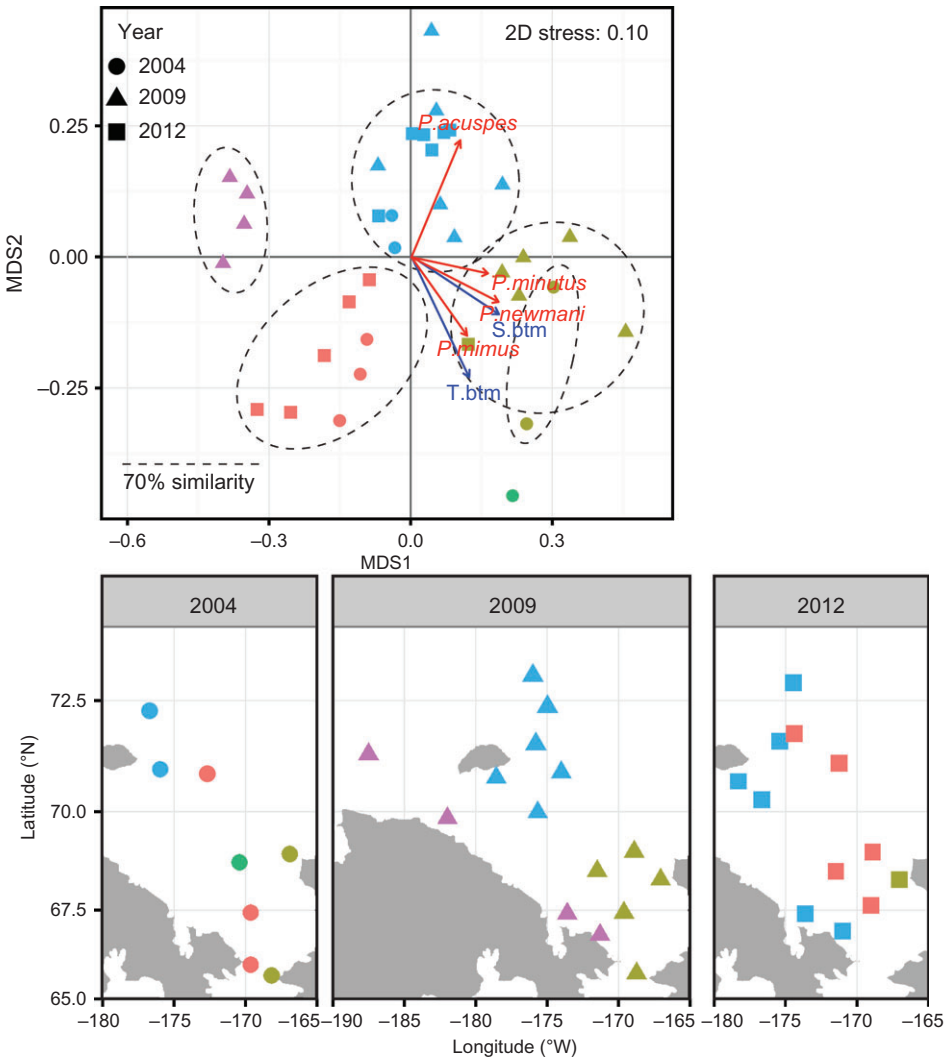

Fig. 6. Multivariate analysis of log-transformed abundance of four species of *Pseudocalanus* in the Chukchi Sea in 2004, 2009 and 2012; (**a**) nMDS plot with vectors indicating correlations of species and variables to axes; colors represent station groupings at 65% similarity; dotted line indicates clusters at 70% similarity; (**b**) station clusters overlaid on map of study area.

established for C1 or older stages (C3-adults). No relationships were observed to salinity or chlorophyll for any species.

Egg production rates

Observed daily specific EPRs (SEP) (Fig. 7) were significantly higher for *P. acuspes* in 2004, when they averaged 16%, than in 2009 and 2012, when they were 8–9% (ANOVA, $P < 0.05$). For *P. newmani*, production rates were significantly higher in 2004 (14%) than in 2009 (7%); the differences between 2004 and 2012 (9%) were insignificant. Insufficient observations were obtained for *P. minutus* in 2009 and 2012 to compare their production between years. No significant differences in production rates were found between species; *P. newmani* displayed comparable SEP values to *P. acuspes* and *P. minutus* at all stations where they co-occurred.

SEP of *P. newmani* showed a strong positive correlation to log-transformed chlorophyll-a ($P < 0.001$, $r^2 = 0.43$); a weaker relationship was observed for *P. acuspes* ($P < 0.01$, $r^2 = 0.31$) and *P. minutus* ($P < 0.5$, $r^2 = 0.19$) (Fig. 8). Standardization of SEP to 0°C using a Q_{10} value of 1.43 (Hirst and Bunker, 2003) improved the model for *P. newmani* ($r^2 = 0.48$); however, no significant improvement was observed for *P. acuspes*. For *P. minutus*, standardizing the SEP values to temperature made the relationship to chlorophyll non-significant; on the contrary, SEP values of this species showed a slight negative relationship to temperature (including temperature in the model improved R^2 to 0.21).

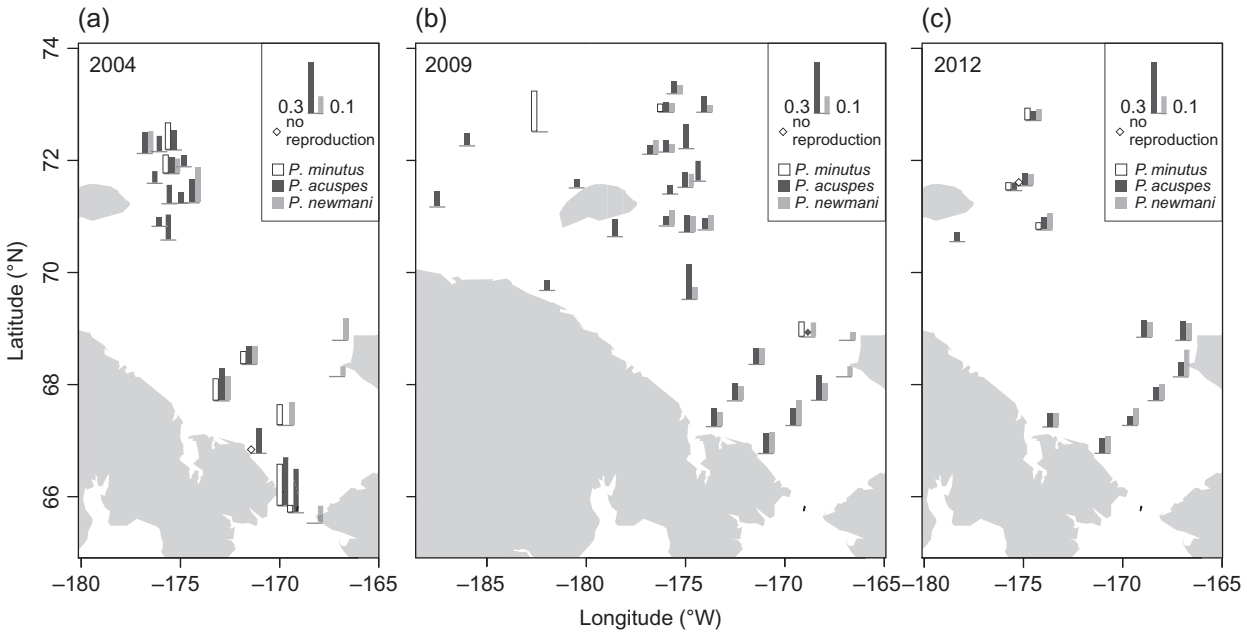

Fig. 7. Daily SEP of *P. acuspes*, *P. minutus* and *P. newmani* in the Chukchi Sea in (**a**) 2004, (**b**) 2009 and (**c**) 2012. Diamonds represent at SEP of zero; empty bars indicate no experimental data for this species at this location.

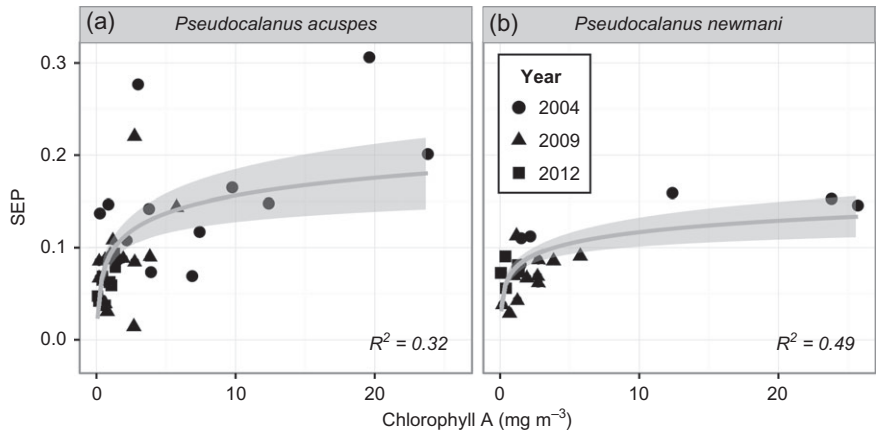

Fig. 8. Daily SEP in relation to maximum *in situ* chlorophyll for (**a**) *P. acuspes* and (**b**) *P. newmani*. Shaded area indicated 95% confidence interval.

The differences in production rates between years for *P. acuspes* and *P. newmani* remained significant even after accounting for chlorophyll and temperature.

Temperature-controlled egg production experiments

Within the Chukchi Sea, the females were mainly identified as *P. acuspes* and *P. newmani*, with insufficient *P. minutus* obtained to estimate EPRs. Clutch size for *P. acuspes* was significantly lower (by ~5 eggs) at 10°C than at 0 and 3°C ($P < 0.01$). No significant differences in clutch size were observed for *P. newmani*. Average Q_{10}-adjusted SEP of *P. acuspes* remained fairly constant at 0–7°C; but was slightly lower at 10°C, mainly due to smaller clutches produced by females. In contrast, SEP of *P. newmani* was lowest at 0°C, and highest at 3°C, with a small decrease in production observed at the highest temperatures (Table II). Hatching success rates were variable within both *P. acuspes* and *P. newmani*, ranging from ~70% to 100%. Significantly lower hatching rates were observed for *P. newmani* at 0°C (Table II), but no significant differences were observed between the other temperatures or for *P. acuspes*.

The females obtained from the Gulf of Alaska belonged primarily to *P. mimus*, with a smaller presence of *P. newmani*. Reproduction of *P. mimus* was extremely low at the coldest temperatures (0 and 3°C); at 0°C females nearly ceased reproducing. *Pseudocalanus newmani* exhibited lower reproduction rates at 0°C than individuals of this species collected in the Chukchi Sea; however, rates were higher than those observed for *P. mimus*. At 3 and 7°C, reproductive rates of *P. newmani* from the Gulf of Alaska were comparable to those obtained for *P. newmani* and *P. acuspes* from the Chukchi Sea, with highest rates observed at 10°C (Table II).

Secondary production

Total daily secondary production (i.e. mg DW m^{-3} d^{-1}) by *Pseudocalanus* spp. was estimated to be ~1.3 times higher in 2009 than in 2004 and 2012; however, differences were not statistically significant (Table III). It is notable that although the biomass of *Pseudocalanus* spp. in 2012 was almost double that of 2004, production was nearly equal due to warmer temperatures and significantly higher daily production rates (ANOVA, Tukey HSD Test, $P < 0.05$) observed in 2004 (Table III). Similarly, differences in secondary production observed between 2009 and 2004 were much less pronounced than the differences in abundance or biomass. Of the three species, contribution to secondary production was highest by *P. acuspes* during all three expeditions (ANOVA, Tukey HSD Test, $P < 0.05$). However, *P. newmani* had significantly higher daily production rate than the other two species (ANOVA, Tukey HSD Test, $P < 0.05$) during all three study years.

DISCUSSION

This study is the first to detail species-specific production and distribution of all four species of *Pseudocalanus* in the Pacific Arctic using a molecular method to discriminate between species. While other studies have implemented molecular methods, including ssPCR, to distinguish between co-occurring species of *Pseudocalanus* in the North Atlantic and in the Baltic Sea (Bucklin *et al.*, 2001; Grabbert *et al.*, 2010; Aarbakke *et al.*, 2011; Holmborn *et al.*, 2011; Bucklin *et al.*, 2015), this is the first to provide a method to discriminate between the sympatric species found in the Pacific Arctic. Previously developed ssPCR

Table II: (a) Daily SEP of Pseudocalanus *spp. in the Chukchi Sea and Gulf of Alaska at 0, 3, 7 and 10°C (Q_{10}-corrected to 0°C), mean ± SD (number in parentheses indicate number of females in experiment) and (b) hatching success rate of* P. acuspes *and* P. newmani *in the Chukchi Sea at 0, 3, 7 and 10°C, mean ± SD (number in parentheses indicate number of clutches and total number of eggs incubated)*

	0°C	3°C	7°C	10°C	*P*-value	Significant interactions
(a) Daily SEP, % (mean ± SD (number of females))						
Chukchi Sea						
P. acuspes	9 ± 4 (38)	9 ± 4 (30)	9 ± 2 (31)	6 ± 1 (29)	NS	None
P. newmani	3 ± 2 (53)	7 ± 3 (39)	6 ± 1 (42)	7 ± 2 (46)	<0.01	0 < 3, 7, 10°C
Gulf of Alaska						
P. newmani	2 ± 0 (26)	6 ± 2 (21)	7 ± 1 (22)	9 ± 1 (18)	<0.01	0 < 3, 7, 10°C; 10 > 3, 7°C
P. mimus	0 ± 0 (36)	3 ± 1 (50)	6 ± 1 (41)	6 ± 1 (31)	<0.01	0 < 3, 7, 10°C; 3 < 7, 10°C
(b) Percent live hatched nauplii (mean ± SD (number of clutches/total number of eggs))						
P. acuspes	77 ± 23 (8/151)	72 ± 24 (8/152)	90 ± 10 (14/196)	91 ± 7 (7/153)	NS	None
P. newmani	60 ± 22 (9/100)	72 ± 28 (23/289)	88 ± 12 (24/324)	89 ± 11 (35/436)	<0.01	0, 3 < 7, 10°C

Table III: Secondary production, P *(mg DW m^{-3} day^{-1}) and productivity,* P/B *(production/biomass; day^{-1}) for populations of the three species of* Pseudocalanus *in 2004, 2009 and 2012, mean value (25–75% quartiles)*

	P. acuspes		*P. newmani*		*P. minutus*	
Year	*P*	*P/B*	*P*	*P/B*	*P*	*P/B*
2004	0.09 (0.03–0.16)	0.061	0.06 (0.01–0.09)	0.075	0.04 (0.01–0.06)	0.057
2009	0.17 (0.07–0.31)	0.031	0.10 (0.003–0.1)	0.037	0.03 (0.01–0.05)	0.027
2012	0.13 (0.05–0.21)	0.025	0.012 (0.003–0.011)	0.040	0.02 (0.01–0.02)	0.033

protocols have been usually used to separate two co-occurring species (Bucklin *et al.*, 2001; Grabbert *et al.*, 2010; Aarbakke *et al.*, 2011; Bucklin *et al.*, 2015); we present the first effective protocol to include four different species. The method proposed here is relatively inexpensive and can be done routinely on large numbers of animals of any developmental stage, as it does not require DNA extractions (e.g. Holmborn *et al.*, 2011) or sequencing (e.g. Bailey *et al.*, 2015). Our results confirm the morphological ambiguity and the high variability in body size at all developmental stages of *Pseudocalanus* spp., making size alone a poor predictor of species identity.

All four species were found within the Chukchi Sea; all but *P. mimus* were important contributors to zooplankton abundance and biomass. The arctic *P. acuspes* was the dominant species during all years and at most stations in terms of abundance, biomass and production. However, abundance of its younger stages declined in warmer waters, and its reproductive output was slightly reduced at 10°C compared to lower temperatures, suggesting that this species could be negatively affected by warming of the region. The temperate *P. newmani* was present at nearly every station sampled and was more abundant in 2009, than 2004, despite the colder temperatures observed during 2009. Its reproductive activity was also comparable to that of *P. acuspes* and *P. minutus* at all but the lowest temperatures, and overall daily production rates were highest among the three species. Despite its southern origin, *P. newmani* seems to be well adapted to the Chukchi environment in the summer months. The temperate *P. mimus*, however, was practically absent from the communities in all years but 2004. This species is common in the Gulf of Alaska and the outer domain of the Bering Sea (Napp *et al.*, 2005; Bailey *et al.*, 2015), where it is the dominant *Pseudocalanus* species. Their near absence in the Chukchi, and by inference, in the rest of the Pacific Arctic during most years suggests they have a lower tolerance for cold temperatures; this is also inferred by their elevated presence in the oceanographically warmer summer of 2004 and by the extremely low reproductive rates observed for this species in the Gulf of Alaska at 0 and 3°C.

Even when data on seasonal dynamics of a species are not available, examining the developmental stage composition of a copepod population can provide important insight into the life history of a species. A continuously reproducing and growing population will be expected to have a lower mean stage than a population whose recruitment is paused or inhibited, due to the latter being dominated by longer living later stages and adults. Although the production of the temperate *P. newmani* is comparable to that of *P. acuspes* and *P. minutus*, the prevalence of older copepodite stages of *P. newmani* suggests that recruitment may be arrested compared to the two Arctic species. This is further reinforced by the higher egg mortality of this species observed at 0°C temperatures that are typical for the Arctic environment. Additionally, our study emphasizes the importance of incorporating all life stages of a species into ecosystem studies, rather than just the adults. Distribution and abundance of adult females, the only *Pseudocalanus* developmental stage identified to species in most zooplankton community studies, failed to reveal the same strong patterns of association to water masses that we observed for the entire population (Fig. 7). The same species may also respond differently to environmental factors over the course of its life cycle. Varying tolerance over different ontogenetic stages to changing temperatures, salinity and CO_2 levels has been observed for several groups of marine crustaceans (Byrne, 2011; Miller *et al.*, 2013). The negative correlations to temperature that we observed for earlier stage juveniles of *P. acuspes* and *P. minutus*, but not adults and subadults, suggest that the earlier stages may be more vulnerable to climate-related increases in temperature.

The EPRs that we observed in our study, particularly during the two colder years (2009 and 2012), are in the lower range or significantly lower than most of those observed at lower latitudes for *Pseudocalanus* spp. in the North Pacific (Lee *et al.*, 2003; Napp *et al.*, 2005; Halsband-Lenk *et al.*, 2005). However, spring-time estimates of *Pseudocalanus* spp. reproduction in the southeastern Bering Sea (Vidal and Smith, 1986) report SEP rates of around 4–5.5%, which are substantially lower than we observed in the Chukchi Sea in the summer at comparable temperatures. EPRs and daily secondary production rates in 2009 and 2012 were similar to values obtained for *P. acuspes* in the Baltic Sea at substantially higher temperatures but lower salinity (Renz *et al.*, 2007), where SEP of females was 1–13% and maximum daily production rates were estimated to be around 3% day^{-1}. It should be noted that our estimates of production are snapshots in time, and assume consistent isochronal development across all species present, which is likely not the case. A study on the life cycles and population dynamics of temperate *P. elongatus* and arctic *P. acuspes* in the North Sea has shown that these two species differ dramatically in their life history, with the faster growing *P. elongatus* completing up to four to five generations per year, and the larger and slower growing *P. acuspes* never exceeding one to two generations (Renz *et al.*, 2008). This resulted in an almost 8-fold higher secondary production by *P. elongatus* despite comparable abundance values (Renz *et al.*, 2008). The smaller-bodied *P. newmani* may also have faster developmental times than *P. acuspes* and *P. minutus*, as

has been demonstrated for some populations of *P. newmani* off the coast of Japan (Lee *et al.*, 2003), which suggests a likely underestimation of production rates for this species in our study. Alternatively, if the advected population of *P. newmani* experiences arrested development in the Arctic environment, as their population structure suggests, then our production estimates may be too high.

Closely related and morphologically similar co-occurring species can often differ significantly in their biology and life history. Within the North Pacific system, a notable example is the ecologically important sibling species pair *Neocalanus plumchrus* and *N. flemingeri*, which were only recently separated into two species (Miller, 1988). Later studies demonstrated that these two species differ significantly in their reproductive strategy and life history, capacity to store lipids and vertical habitat (Miller and Clemons, 1988). Few studies that have focused on species-specific life history of *Pseudocalanus*, although the example of *P. elongatus* and *P. acuspes* in the North Sea (Renz *et al.*, 2008), shows that the differences between species may be dramatic. Recent findings further suggest that different species of *Pseudocalanus* may have different feeding strategies, with *P. acuspes* diet consisting significantly of heterotrophic flagellates, and *P. minutus* and *P. newmani* feeding predominately on pelagic diatoms (Cleary *et al.*, 2015). The much stronger relationship of reproductive activity to *in situ* chlorophyll concentrations for *P. newmani* compared to *P. acuspes* supports these observations. *Pseudocalanus acuspes* and *P. minutus* are also believed to depend on sea ice production (Conover *et al.*, 1986; Runge and Ingram, 1991); the common occurrence of these species in the Bering Sea (Bailey *et al.*, 2015) is likely a direct consequence of the Arctic-like ice dynamics that occur in that region. *Pseudocalanus acuspes* and *P. minutus* are also generally much larger in size, and richer in lipids, than their temperate counterparts (McLaren *et al.*, 1989), which has direct implications for higher trophic levels which rely upon these organisms as a food source.

Distribution of *Pseudocalanus* spp. in the Chukchi Sea is closely related to oceanography and water mass distribution, making them important indicator organisms of water mass types, as well as potential markers of climate-related change in the communities. Patterns of *Pseudocalanus* distribution (Fig. 7) are remarkably similar to those observed for the entire zooplankton community (Ershova *et al.*, 2015a), as well as the water mass types present at these stations (Fig. 1). It is notable that despite strong inter-annual variability, the stations grouped by water mass types, rather than by year. Nevertheless, the overall distribution, abundance and production patterns were markedly different during the oceanographically warm summer of 2004 from the colder summers of 2009 and 2012. Daily rate of production by *Pseudocalanus*, driven by higher temperatures and a favorable food environment, was highest during 2004, despite the lowest overall abundances observed during that year. However, not all inter-annual differences observed can be attributed simply to thermal conditions. For example, despite ~50% of the variability in *P. newmani* abundance being accounted for by temperature and salinity, with higher abundances observed at warmer temperatures, this species was most abundant during 2009 rather than the warmer 2004. It is likely that the spreading of the Alaska Coastal Current over the western Chukchi shelf and into Herald Valley during the time of the 2009 expedition (Pisareva *et al.*, 2015a) resulted in the increased presence of this species. The lower abundances observed during 2004 may reflect a number of different factors, such as a higher mortality due to predation or lower abundances and recruitment in the Bering Sea during that year. Alternatively, 2004 may simply reflect an earlier seasonal state, since the 2004 expedition occurred in August, while the years 2009 and 2012 were sampled in September.

Pseudocalanus respond very rapidly to the surrounding environment, as seen from their instantaneously increased reproductive rates associated with higher food availability, as well as the strong relationship of body size to water temperature. Overall, temperature appears to be the main factor influencing *Pseudocalanus* spp. production and distribution (McLaren and Corkett, 1978; Liu and Hopcroft, 2008). This is manifested through direct effects, such as temperature-dependent growth and reproduction, as well as indirectly, through temperature-controlled sex ratios (Lee *et al.*, 2003), growth inhibition through temperatures outside of the species' preferred thermal range (Klein Breteler *et al.*, 1995; Lee *et al.*, 2003) and effects on the overall pelagic ecosystem.

The on-going reduction in sea ice extent and timing of coverage in the Arctic Ocean has been particularly pronounced within the Pacific sector (IPCC, 2013; Wood *et al.*, 2015). As the ice-free summer becomes longer, and waters become warmer within the Chukchi Sea region, we expect to observe a shift in the biological communities. A significant increase in overall zooplankton biomass has already been observed over the past century (Ershova, *et al.*, 2015b), and several Pacific species within the plankton (Ershova, *et al.*, 2015b) and fish communities (Wassmann *et al.*, 2015) may have extended their ranges northward. Since life cycles of *P. acuspes* and *P. minutus* may be highly dependent on sea ice algae production, reduction in winter ice extent in

the Bering Sea may result in a range contraction of these species, decreasing their advected biomass into the Chukchi Sea. Furthermore, a longer ice-free summer in the Chukchi may continue to negatively impact these two arctic species, which may be stressed by the increasing temperatures, while benefiting the advected Pacific species. However, at present *P. acuspes* successfully inhabits many temperate seas in the north Atlantic and does not appear to be particularly stressed by temperatures up to 13°C in some locations (Ershova *et al.*, 2016). The reduced ability of *P. newmani* and *P. mimus* to accumulate and store lipids (McLaren *et al.*, 1989; Aarbakke *et al.*, 2011; Bucklin *et al.*, 2015) will likely prevent them from establishing self-sustaining populations in the Arctic due to seasonal ice-cover and limited primary production during the winter months, thus restricting them to the summer months. Most likely, a combination of abiotic, biotic and oceanographic factors will determine who will be the "winners" and "losers" in the Chukchi Sea under climate change scenarios.

Conclusions

The distribution of the four species of *Pseudocalanus* in the Chukchi Sea is tightly linked to water mass distribution and thermal regimes in the region, making this assemblage important indicators of water mass origin, as well as potential sentinels of climate change. The contribution and northward penetration of the two temperate species, *P. newmani* and *P. mimus*, is expected to increase with warming temperatures and increasing inflow from the Pacific during the summer months, although it is unlikely that they will establish self-sustaining populations in the Arctic. Shifting oceanographic and climate patterns may have unequal impacts on the different species, as determined by their individual life histories and tolerance to environmental conditions. Studying species-specific biology of closely related species, such as *Pseudocalanus* spp., may provide important insights on ecosystem shifts under climate change scenarios.

SUPPLEMENTARY DATA

Supplementary data can be found online at *Journal of Plankton Research* online

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