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A Time Series Investigation of the Cryptic Copepods Pseudocalanus spp. on the NW Atlantic Continental Shelf

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A Time Series Investigation of the Cryptic Copepods *Pseudocalanus* spp. on the NW Atlantic Continental Shelf

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ABSTRACT

Zooplankton occupy pivotal positions in pelagic ecosystems and have direct impacts on trophic and fisheries dynamics on continental shelves. Time-series records of zooplankton species diversity, distribution, and abundance are critically needed to understand, document, and predict impacts of environmental conditions on pelagic ecosystems. This study focuses on species of the calanoid copepod genus *Pseudocalanus* on the NW Atlantic continental shelf. Time-series analysis was conducted on the distribution and abundance of *Pseudocalanus* spp. and of two co-occuring cryptic species from 1977-2012 and 1995-2012 respectively, based on data from Ecosystem Monitoring (EcoMon) surveys by the NOAA-NMFS Northeast Fisheries Science Center and the US GLOBal Ocean ECosystem Dynamics (US GLOBEC) surveys on Georges Bank. Analysis and mapping of abundance anomalies during May-June showed that depth-averaged temperature and salinity were not strongly correlated with *Pseudocalanus* spp. abundance in the Georges Bank region, although correlations were significant for annually-averaged abundance and environmental data. Real-time quantitative PCR (qPCR) was used to discriminate the cryptic species, *P. moultoni* and *P. newmani*, in samples collected on Georges Bank from 1995 to 2012. Time-series analysis of the individual species abundances and abundance anomalies, with comparative analysis of depth-averaged temperature, salinity, and other environmental variables, provided evidence of species-specific differences. This study demonstrated that closely-related, cryptic species may have different responses to environmental variation and change. Time-series analysis and mapping of species-specific abundances yielded new understanding of the underlying causes of time/space variation in these populations. Accurate resolution of species diversity is needed to improve predictions of the responses of zooplankton populations and pelagic ecosystems to variability and climate change.

I. Introduction:

I.A. *Pseudocalanus* Background:

Species of the genus *Pseudocalanus* are among many types of copepods that dominate the zooplankton assemblage on the NW Atlantic Shelf. This pelagic ecosystem is made up of the Gulf of Maine (GOM), Georges Bank (GB), Southern New England (SNE), and the Mid-Atlantic Bight (MAB) (Davis, 1984; Kane, 2007). Two of the seven known *Pseudocalanus* species, *Pseudocalanus moultoni* and *P. newmani*, occur exclusively and sympatrically throughout much of these NW Atlantic shelf regions (Frost, 1989). The two species have been consistently grouped together in scientific studies aimed at understanding zooplankton responses on seasonal and interannual time scales, because they are acknowledged to not be not readily morphologically distinguishable (Frost, 1989; Hare and Kane, 2010; Kane, 2014; Mountain and Kane, 2010; Pershing et al., 2005). Due to the importance of *Pseudocalanus* spp. in larval cod and haddock diets and as possible indicators of climate change, investigations are needed that discriminate the cryptic species in order to gain a true understanding of their species-specific dynamics in the NW Atlantic Shelf regions (Friedland et al., 2013; Mountain and Kane, 2010; Munk, 1997; Richardson, 2008).

I.A.1. *Pseudocalanus* Population Drivers:

Pseudocalanus and Physical Population Drivers:

Environmental drivers, like temperature, can cause increases or decreases in populations. Such changes may lead to shifts in biogeographic ranges of species and assemblages which may occur in marine communities at scales from 4 to 6 km/year in the GB region under future conditions; these range shifts can occur faster than those occurring in terrestrial environments

(Pereira et al., 2010). The rate at which the temperature changes in a given region can play a role, and species may vary. It is not always the case that ranges move poleward under warming due to variability of response among assemblages and taxa (Burrows et al., 2011). Shifts can be species-specific, but can also occur across the entire assemblage in a given area and in a specific direction, or within the depth of the water column in response to top or bottom temperature (Pinsky et al., 2013). Temperature is therefore an important environmental factor to be considered when examining species distributions and abundances over a long time-series and in order to make future predictions.

Stressor analysis involving temperature change conducted by Stegert et al. (2010) showed that *Pseudocalanus* spp. exhibit positive population growth at temperatures less than 18˚C, but higher temperatures will cause negative population growth associated with increased mortality. In addition, a temperature increase will result in the increased abundance of another copepod, *Centropages typicus*, a species typical of warmer water (Stegert et al., 2010). Such species oscillations and changes in relative abundances occur even though the species are exposed to the same environmental forcing; varying responses could have been due to climate forcing on bottom-up or top-down processes (Stegert et al., 2012). Phenological changes in communities caused by environmental drivers may impact entire trophic webs (Richardson, 2008). Understanding these relationships is important as sea surface temperatures are expected to be between 2 and 4˚C warmer by 2100 in the NW Atlantic continental shelf, (http://nefsc.noaa.gov/ecosys/climate_change/projected.html). It is therefore crucial to understand relationships between temperature and species-specific abundances over extended time periods to accurately assess and understand reactions to environmental change.

***Pseudocalanus* Predator and Prey Dynamics:**

Pseudocalanus spp. are herbivorous, and various phytoplankton taxa (including diatoms and dinoflagellates) have been found to be correlated with *Pseudocalanus* spp. abundance in some regions of the NW Atlantic, which has been considered to indicate potential control caused by climate forcing (Davis, 1987; Ji et al., 2013; Kane, 2014). Ji et al. (2013) showed through a modeling study that when phytoplankton concentration was increased and decreased by 20 percent, a relatively uniform spatial increase and decrease in *Pseudocalanus* spp. abundance occurred across the GOM and GB.

Pseudocalanus spp. of all life stages are preferred prey items for juvenile cod, haddock, and herring compared to other copepods of similar size; they are therefore particularly important for the continuation and stability of these commercial fisheries (Friedland et al., 2013; Munk, 1997). Ecosystem based approaches to fisheries management highlight the need for an understanding of environmental factors as well as biodiversity impacting commercial fish stocks in the NW Atlantic shelf region. Among these is the incorporation of lower level trophic dynamics, as changes in the zooplankton assemblage in relation to commercial fisheries are very important for sustainability (Johnson et al., 2011; Pershing et al., 2005).

Competitors, predators, and prey items for *Pseudocalanus* spp. have been studied. Local factors like predation, which drive biodiversity, play important roles in zooplankton abundance in the coastal NW Atlantic shelf region (Johnson et al., 2011). Predators or competitors of *Pseudocalanus* spp. are known to include siphonophores, salps, chaetognaths, and possibly omnivorous copepods, and significant correlations have been found between the abundances of competitors/predators and *Pseudocalanus* spp. (Davis, 1984b; Kane, 2014). Similarly, in a modeling study where *Pseudocalanus* spp. mortality rates were increased and decreased by 20 percent, mimicking increased and decreased predation, *Pseudocalanus* spp. abundances in the

GOM and GB regions changed between 1 and 3-fold and abundances were more sensitive to changes in mortality than to phytoplankton concentration changes (Ji et al., 2013). This indicates that competitor or predator relationships may play an important role in *Pseudocalanus* spp. abundances. Predator and prey dynamics indicating top-down and bottom-up control on species of *Pseudocalanus* have yet to be evaluated seasonally and annually.

I.B. Time-series Analysis of *Pseudocalanus* spp. Abundances:

Time-series analysis is important for understanding long-term trends in zooplankton abundance, since population fluctuations may both reflect and be used to predict responses to climate change. Due to their sensitivity to environmental variables (e.g. temperature) and short life spans, copepods can act as indicator species for climate variability (Richardson, 2008).

Pseudocalanus spp. have been observed over long time-scales in the GOM, GB, SNE, and MAB revealing population oscillations that occur both seasonally and interannually. In the 1970s, 1980s and 2000s, *Pseudocalanus* spp. abundance was low while an increase in abundance occurred in the 1990s (Hare and Kane, 2012; Kane, 2007; Pershing et al., 2005). Interestingly, abundances in the early 2000s were 10-fold lower than in other low-abundance years included in the time-series beginning in the 1980s; this decline was potentially a response of *Pseudocalanus* spp. to observed temperatures during these years, which included cooler winter months and warmer spring months than the long-term mean, resulting in habitat restrictions that may also have been directly or indirectly influenced by predator or prey abundance (Friedland et al., 2013; Kane, 2007; Kane, 2014; Pershing et al., 2005). These interannual abundance patterns were also observed in other species of zooplankton, *Centropages typicus*, *Oithona* spp., and *Metridia lucens*, but were opposite to the patterns for *Calanus finmarchicus* and euphausiids (Pershing et

al., 2005). These multi-species patterns are indicative of community shifts in the zooplankton assemblage of the GOM, which could occur through biogeographic or seasonal changes in timing of peak abundances of a variety of species (Johnson et al., 2011; Pershing et al., 2005).

I.C. Cryptic Species Discrimination:

Pseudocalanus spp. species present in the NW Atlantic continental shelf regions are *P. moultoni* and *P. newmani*. They are morphologically indistinguishable, but can be discriminated using genetic techniques based on the mitochondrial cytochrome oxidase I (COI) gene for which genetic divergence of 18% exists (Aarbakke et al., 2014; Blanco-Bercial et al., 2014; Bucklin et al., 1998, 1999, 2001, 2003, 2011, 2015). Morphologically, there are subtle differences between species, e.g., *P. moultoni* is observed to be larger in size, but size is not a diagnostic characteristic for species identification or discrimination (Frost, 1989). The species may also exhibit different habitat preferences. Both species occur in the NW Atlantic shelf regions, yet *P.moultoni* is thought to be more coastal and *P. newmani* more oceanic (Aarbakke 2011, 2014; Frost, 1989). *Pseudocalanus moultoni* has been found to exist in the coastal waters of the New York Bight to Chedabucto Bay in Nova Scotia, Canada, while *P. newmani* has been described in temperate-boreal waters of the NW Atlantic with continued distribution through the Canadian Arctic (Frost, 1989).

Pseudocalanus spp. life histories, egg production, and distribution are known to differ among species and across regions (Frost, 1989; Napp et al., 2005; Hopcroft and Kosobokova, 2010). *Pseudocalanus* spp. in the GB region have a developmental time of approximately two months and there are approximately four generations each year, beginning with the initial increase in abundance in December, through peak abundance in May and June, and decline in

abundance between July and September (Davis, 1987; Kane, 2014). Within this generational and developmental time frame, studies have shown that multiple species are present in differing abundances and at different but overlapping times (McGillicuddy and Bucklin, 2002; McGillicuddy et al., 1998). Species-specific abundance partitioning on regional scales was observed in samples collected from Browns Bank, where *P. moultoni* was found to be more abundant in winter and spring, while *P. newmani* was more abundant in spring and summer (McLaren et al., 1989). Similarly, Bucklin et al. (2001, 2015) reported that *P. moultoni* and *P. newmani* had different patterns of distribution and abundance in the GB region, through studies that discriminated the species in samples from 1997 and 1999. In particular, *P. moultoni* was present on the northern edge of GB in late spring/early summer, while *P. newmani* was more dominant off the Bank, presumably due to different source regions of each species. Vertical distributions of both *P. moultoni* and *P. newmani* were also found to change across seasons, although at the time of peak abundance (May and June), both species were evenly spread across GB in the upper 100 m (Bucklin et al., 2015). Differences between the species have been hypothesized to result from variable responses to environmental parameters, differences in micro-habitat preferences, and/or differences in abundance in stratified layers of frontal regions (Bucklin et al., 2015).

No time-series observations have been made that discriminate the cryptic species of *Pseudocalanus*, so differential species-specific responses are unknown. Almost certainly, environmental conditions contribute to the different source regions and habitat preferences for *P. moultoni* and *P. newmani*. Also unknown is how these species interact in the NW Atlantic shelf ecosystem, especially over longer timescales.

I.D. Introduction to the Study Area: Georges Bank:

Although *Pseudocalanus* spp. occur throughout the NW Atlantic continental shelf from the GOM to the MAB, Georges Bank was selected as the geographic region for sample selection for genetic analysis and discrimination and identification of the cryptic species. Georges Bank forms the southern boundary of the GOM region and is approximately 300 km by 150 km in size with variable depths ranging from 200 m to 5 m; the Bank represents a zoogeographic boundary that sets both north and south limits of species ranges (Wiebe et al., 2002). Waters across GB vary in temperature and salinity resulting from the mixing of source waters from the Scotian shelf and continental slope waters which are classified as being cold with low salinity, and warmer with higher salinity comparatively and respectively (Smith et al., 2001; Wiebe et al. 2002). Georges Bank is economically important for commercial fisheries, and in particular, as part of the NW Atlantic continental shelf, it is a critically important area for larval cod and haddock recruitment (Mountain and Kane, 2010). Zooplankton abundance trends (including *Pseudocalanus* spp.), have been found to be related to cod and haddock recruitment to GB fisheries as well as to larval survival based on prey capture efficiency and preference (Buckley and Durbin, 2006; Pershing et al., 2005; Petrik et al., 2009, 2014). In addition, Georges Bank historically has had year-round high amounts of phytoplankton, an important factor that may reduce stress from food limitation, and consequently increase the relative importance of physical factors as causes of species abundance shifts (Davis, 1984).

Additional reasoning for the focus on GB as the chosen study area was due to high sampling density during the US GLOBal Ocean ECosystem Dynamics Program (US GLOBEC) and the Ecosystem Monitoring of the Northeast Continental Shelf Program (EcoMon) surveys, which sampled the Bank from 1994 to 1999, and from 1977 to the present day, respectively. US GLOBEC cruises characterized the underlying physical and biological processes governing GB

through broad-scale surveys that sampled 40 set station locations on GB using $1m^2$ MOCNESS (Multiple Closing/Opening Net, Environmental Sensing System) equipped with 150 μm mesh nets (Wiebe et al., 1985, 2002). Stations were selected for sampling on EcoMon cruises using a random stratified method covering the NW Atlantic shelf region multiple times a year; zooplankton samples were collected with 61-cm Bongo nets with 333μm mesh (http://www.nefsc.noaa.gov/epd/ocean/MainPage/shelfwide.html).

I.E. Goals and Objectives of this Study:

This study had two related aims: 1) to document spatial and temporal patterns of *Pseudocalanus* spp. abundances from GOM to MAB through all seasons and from 1977-2011, utilizing location and abundance data from the EcoMon database (ftp://ftp.nefsc.noaa.gov/pub/dropoff/jhare/EcoMon_Data/); and 2) to characterize the species-specific abundances of the cryptic species of *Pseudocalanus*, *P. moultoni* and *P. newmani*, in time-series analysis from 1995 to 2012 based on samples collected during peak abundance months (May and June) from US GLOBEC and EcoMon surveys. These goals are explained in more detail hereafter.

Pseudocalanus spp.: A better understanding of *Pseudocalanus* spp. (*P. moultoni* and *P. newmani*) abundances in the NW Atlantic shelf regions over decadal time-scales will shed new light on interactions between environmental conditions and zooplankton abundances. Also, these may provide a proxy for patterns of *Pseudocalanus* spp. and species-specific interactions. Although Kane (2014) observed no shifts in *Pseudocalanus* spp. distribution from the 1970s to the 2000s, there were significant negative correlations between *Pseudocalanus* spp. abundance and salp abundance, as well as negative correlations with temperature and salinity. These

findings strongly indicate that species-specific patterns for the *Pseudocalanus* are needed to understand the driving forces determining species abundances and distributions.

Discrimination of Cryptic Species of *Pseudocalanus:* A species-specific approach was designed to differentiate the cryptic species using a quantitative real time PCR (qPCR) for detection and quantification of relative abundances of each species. The qPCR analyses were done for *Pseudocalanus* spp. individuals identified to the genus level. Proportions of the two cryptic species were determined from qPCR reaction results, and compared to archived data on species abundances. The methods used built upon species-specific PCR (SS-PCR) work by Bucklin et al. (2001, 2015) to discriminate the cryptic species, but made a significant advance in the use of pooled individuals identified to genus level. SS-PCR can reveal annual cycles and drivers, but requires examination of individual copepods (Bucklin et al., 2001). In contrast, qPCR provides an efficient tool for species identification and relative quantification. Both molecular protocols (SS-PCR and qPCR) offer new opportunities for rapid, accurate, and cost-effective discrimination of cryptic species of marine zooplankton. These studies are essential to allow accurate measurement, management, and prediction of changes in continental shelf zooplankton. In particular, cryptic species identification will shed light on differential species response to environmental change and suggest more general approaches to the analysis of marine zooplankton diversity, including *Pseudocalanus* spp.

II. Methods:

II.A. Visualization and Mapping of *Pseudocalanus* spp. Abundance 1977-2012:

The NOAA Northeast Fisheries Science Center (NEFSC) has carried out quarterly surveys of the pelagic assemblage and environmental conditions of the NW Atlantic continental

shelf through various programs since 1977. The NEFSC Ecosystem Monitoring Program (EcoMon) has yielded comprehensive zooplankton abundance data for four regions of the shelf: GOM, GB, SNE, and MAB (Figure 1).

Maps of *Pseudocalanus* spp. abundance for the entire EcoMon survey region were created for four seasonal snapshots each year based on abundances recorded for all cruises occurring during the following 2-month periods: January and February, March and April, May and June, and July and August. The maps were used to create animated videos in MATLAB (http://www.mathworks.com/products/matlab/?refresh=true) using linear interpolation to connect $\log_{10}$ transformed abundances at the station locations over the sampled region, with a changing image every year representing that year. This was done to provide a visualization of *Pseudocalanus* spp. abundances on the NW Atlantic continental shelf.

Pseudocalanus spp. abundance anomalies over time on GB were also calculated ($\log_{10}$(observed value) minus mean($\log_{10}$(values). Temperature and salinity data were extracted from NEFSC database for cruises over the same time period and spatial area. The environmental data were formatted for MATLAB specific to May and June for GB and averaged to create depth-averaged temperature and salinity data (ftp://ftp.nefsc.noaa.gov/pub/hydro/spool_hydro). Anomalies were calculated for both depth-averaged water column temperature and depth-averaged salinity.

Pseudocalanus spp. abundances from EcoMon on GB were compared to hydrographic data with Pearson Correlation and regression analysis for the May-June time period. *Pseudocalanus* spp. abundances from EcoMon for GB were also compared to the hydrographic data for the May-June time period using the online analytical interface COPEPODITE

(http://www.st.nmfs.noaa.gov/copepodite/). Additional environmental variables available on the COPEPODITE interface were compared to *Pseudocalanus* spp. abundances by regression analysis. Variables included Hadley sea surface temperatures (HadISST), Hadley EN Salinity at 5m depth, International Comprehensive Ocean-Atmospheric Data Set (ICOADS) sea surface wind, satellite chlorophyll-α, North Atlantic Oscillation (NAO) winter index, Arctic Oscillation (AO) index, and Atlantic Multidecadal Oscillation (AMO) index values.

II.B. Molecular Discrimination of *Pseudocalanus* Species:

Twenty female *Pseudocalanus* spp. were identified and picked from ethanol-preserved samples collected from EcoMon cruises GU1305, (Stn 80) and GU1302, (Stn 47). DNA was extracted using the Qiagen DNeasy kit (Qiagen, Valencia, CA) following manufacturer instructions with an incubation time of 1.5 hrs. DNA concentrations per extraction were measured using the Qubit 2.0 and the HS DNA kit (Life Technologies, Carlsbad, CA). Extracted DNA was used for Polymerase Chain Reaction (PCR) of a portion of the mitochondrial cytochrome oxidase subunit I (COI) gene using LCO1490 and HCO2198 primers (Folmer et al., 1994). The PCR reaction consisted of 12.5 μL molecular grade water, 5 μLGo taq 5x flexi buffer, 2.5 μL $MgCL_2$, 1 μL of each primer (10 μM) and of dNTPs (10 mM), 0.15 μL of Promega Gotaq® Flexi Polymerase (5 U μL^{-1}) and 2 μL of sample per reaction. The initial PCR reaction protocol was: 94˚ C (3 min), 35 cycles of 94˚C (45 sec); 45˚C (45 sec); and 72˚C (45 sec), and a final extension at 72˚ C for 15 min. A second PCR reaction was done with the protocol: 94˚ C (3 min), 35 cycles of 94˚C (45 sec); 45˚C (45 sec); and 69˚C (45 sec) and a final extension at 69˚ C for 15 min. The PCR products were electrophoresed on a 1% agarose gel. For samples showing a product of the correct size, a gel extraction was performed with the QIAGEN

Gel Extraction Kit following manufacturer instructions (Qiagen, Valencia, CA). After gel extraction, sequencing PCRs were performed using the BigDye® Terminator ver. 3.1 Cycle Sequencing Kit (Life Technologies, Carlsbad, CA) with 1.45 μL buffer, 0.7 μL RRP-100, 4.5 μL molecular grade water, 0.15 μL primer and 3 μL sample for each reaction (using both LCO1490 and HCO2198 primers). PCR products were sequenced on an Applied Biosystems Inc. (ABI) 3130 Genetic Analyzer (Life Technologies, Carlsbad, CA).

II.B.1. Design and Evaluation of Species-specific Primers for qPCR:

DNA sequences were aligned using Molecular Evolutionary Genetics Analysis (MEGA, ver. 6) (Tamura et al., 2013) together with sequences for *P. moultoni* and *P. newmani* available in Genbank (http://www.ncbi.nlm.nih.gov/genbank/). Species-specific COI primers were designed using PrimerQuest (https://www.idtdna.com/Primerquest/Home/Index?Display=SequenceEntry) based on the consensus DNA sequences for *P. moultoni* and *P. newmani* from environmental data and from Genbank. Primers were consistent with suitability requirements for qPCR amplification, which included: overall amplicon length of approximately 100 base pairs, primer lengths of 18-22 nucleotides, G C content of 40%-60%, and no hairpins or primer interactions. Primer sequences created in PrimerQuest were imported into MEGA and aligned with the consensus COI sequences for *P. moultoni* and *P. newmani* to evaluate their suitability for qPCR reactions and specificity for species-specific discrimination. A total of five5 *P. newmani* primers and three *P. moultoni* primers were designed and used for further testing.

All possible pairs of the selected primers were tested for specific-species amplification, amplification across primer sets, and across species, using PCR reactions with 2 μL of DNA

extracted from the female *Pseudocalanus* spp. and run at various annealing temperatures. Annealing temperatures were optimized within PCR reactions with species-specific primers; 62˚C was determined to be the optimal annealing temperature for primer pairs. Additional temperatures tested included, 63˚C, 61.5˚C and 61˚C, yet yielded less clear DNA bands following elecetrophoresis on a 2% agarose gel than those from PCR reactions with annealing temperature of 62˚C . The species-specific PCR protocol for *Pseudocalanus* spp. involved 94˚ C (3 min), 30 cycles of 94˚ C (30 sec), 62˚ C (30 sec), and 72˚ (30 sec); and a final extension step of 72˚ C (7 min). This was done even though the qPCR reactions would always contain isolated species-specific primers.

The best-performing primers were:

Newmani 5-F: 5'-GGATCATTGATTGGAGATGATCAGATT -3' and

Newmani 5-R: 5'-GGTACTAATCAGTTGCCAA ATCCC-3', which produced an amplicon of 118 base pairs, and

Moultoni1-F: 5'-CTCTAGGGAATTTACGAGTATTTGGA-3' and

Moultoni1-R: 5'-ACCAGCTAACACTGGTAAAGATAA -3', which produced an amplicon of 112 base pairs.

II.B.2. qPCR Protocol Development:

Annealing Temperature Selection:

Determination of a consensus annealing temperature for the qPCR protocols for each species was done using DNA extracted from specimens of *P. moultoni* and *P. newmani.* DNA

was identified using species-specific primers and the previously described PCR protocol. Additional tests were done to determine optimum template DNA concentration. DNA extracted from individual specimens was diluted to concentrations of $1/50^{th}$, $1/20^{th}$, $1/10^{th}$, and $1/4^{th}$ of their original concentration which ranged from 0.1 to 0.36 ng μL^{-1} of DNA. Annealing temperatures tested with the range of DNA concentrations for qPCR detection were: 60˚ C, 62˚ C, 63˚ C, and 64˚ C. These qPCR reactions to determine the optimum annealing temperature were comprised of 3 µL of sample, 4.6 µL of molecular grade water, 10 µL of SsoFast™ EvaGreen® supermix, 0.4 µL of ROX™ passive reference dye, and 1µL of each primer per well across 96- well optical plates. Thresholds, (i.e., manually set lines representing certain fluorescence) were set uniformly across all plates at 2.521049 for *P. newmani* and 3.906050 for *P. moultoni* for the entirety of the project making across plate comparisons possible. The qPCR annealing temperature of 63˚ C provided the lowest and most compact amplification at a given threshold cycle or Ct value (i.e., the number of PCR cycles at which the amplification curve crossed the threshold) and also showed the necessary species-specificity (Figure 2).

Sample DNA Concentration:

The optimum template DNA concentration was evaluated using DNA extracted from identified individual specimens, which was concentrated or diluted to yield a wide range of template concentrations for detection of both high and low species abundances. DNA tested for *P.newmani* included 7 sample dilutions ranging from 0.004 to 3.68 ng μL^{-1}. *Pseudocalanus moultoni* DNA was tested with 4 sample dilutions ranging from 0.005 to 3.7 ng μL^{-1}. Results were analyzed using Ct values versus DNA amount (Figure 3). The DNA concentrations chosen for further analysis included 0.5, 0.8 and 1.2 ng μL^{-1}, since they allowed clear discrimination of the species at all dilutions. Further tests were done with species-specific samples which were

concentrated and diluted to 1/50th and 1/100th of those concentrations (0.5, 0.8, and 1.2 ng μL^{-1}) to test for species-specific qPCR amplification. After testing, 0.8 ng μL^{-1} was determined to be the optimal initial DNA concentration for qPCR amplification and reliable detection of species.

Standard Curve Development:

A standard curve for calculation of species ratios was created using mixtures of DNA from previously identified *P. newmani* and *P. moultoni* specimens combined in these proportions: 100:0, 99:1, 98:2, 96:4, 80:20, 50:50, 20:80, 4:96, 2:98, and 0:100. The curve was created using ratios of species from the same samples for a variety of samples to account for variance in DNA integrity. Results were plotted as the $\log_{10}$ of the ratio of *P. moultoni* to the ratio of *P. newmani*, (equivalent to the ratio of *P. moultoni* to1 minus ratio of *P. moultoni*), versus the ratio of the reported threshold values ($Ct_{P.moultoni}/Ct_{P.newmani}$). All values were within the 95% confidence interval (Figure 4). Species ratios from *Pseudocalanus* spp. DNA can thus be calculated using the linear regression equation which was determined to be: Y=-0.1281x + 1.0475 where $x = \log\left(\frac{ratio\ Pmoultoni}{ratio\ Pnewmani}\right) = \log\left(\frac{ratio\ Pmoultoni}{1-ratio\ Pmoutonii}\right)$ and an $R^2 = 0.95$

II.C. Analysis of Species-specific Abundances on Georges Bank 1995-2012:

II.C.1. Selection of Samples for Analysis:

US GLOBEC samples to be used for analysis of species-specific patterns of abundance were selected based on collection during May and June, which is known to be a period of high *Pseudocalanus* spp. abundance (Davis, 1987). Samples were also selected based on station location within each of the five Georges Bank zones designated in the US GLOBEC grid (Figure 5) in order to give an accurate representation of *Pseudocalanus* spp. abundances across the entire

Bank. After zone determination using MATLAB, stations were selected with a stratified random method, resulting in one station per zone per year for a total of five selected stations per year from 1995-1999.

EcoMon samples to be used for analysis were collected during May and June 2002-2007 and 2009-2012. No samples were available in 2000, 2001, and 2008. Fewer ethanol-preserved samples were collected over GB during EcoMon cruises than during the US GLOBEC broad-scale surveys. All available samples collected during May and June from EcoMon cruises were therefore selected for analysis, although this was sometimes fewer than 5 samples in each year (Table 1).

II.C.2. Determination of Relative Species Abundances using qPCR:

Selection of Samples for Analysis:

From each of the selected samples collected over GB during cruises by US GLOBEC and EcoMon, 50 female *Pseudocalanus* spp. were identified. The first 50 specimens identified were picked in all cases. For the EcoMon samples, one-half splits were done using a box splitter prior to removal of the 50 specimens. For samples containing fewer than 50 individuals, a smaller number of specimens were used for analysis; these included: DE0905 Stn. 71 (21 copepods), Stn. 73 (32 copepods), HB1202 Stn. 72 (33 copepods), and AL9707 Stn. 7 (28 copepods). In all, specimens from 77 samples were identified to be used in analysis.

Extraction and qPCR:

DNA was extracted from pooled *Pseudocalanus* spp. samples with the Qiagen DNeasy blood and tissue kit. Prior to extraction, copepods were hydrated twice with molecular grade

water. Samples were incubated per extraction protocol for 3 hrs with vortexing every 30 min. Two aliquots of 100 μL Buffer AE were used in the final step for a total volume of 200 μL.

DNA concentrations per sample were measured using the Qubit 2.0 (using the HS DNA kit) (Table 1). For analysis, DNA concentrations were adjusted to 0.8 ng μL^{-1}. One qPCR reactions per sample per primer pair was run on the same optical plate. Threshold levels for each species were set to pre-established values consistently across plates at the end of each qPCR run (2.521049 for *P. newmani* and 3.906050 for *P. moultoni*). When necessary, the baseline was set manually to correctly account for background fluorescence.

Ct values for all samples were exported for analysis and the standard curve linear equation was used to determine the proportion of *P. moultoni* to *P. newmani*. The calculated ratios were applied to counts of *Pseudocalanus* spp. in EcoMon and US GLOBEC samples. Counts of US GLOBEC nets were summed to represent the whole water column; those US GLOBEC stations with counts available in the database for male and stage C5 were included for consistency as EcoMon counts include these life stages, although no males or C5s were picked for qPCR analysis.

II.D. Data Analysis:

The geometric mean of species-specific abundances for each year was calculated in order to reduce the impact of large outlier values. Spline interpolants were applied to fill the three gaps created by lack of samples in 2000, 2001 and 2008. Spline interpolants were calculated from $\log_{10}$ transformed abundance data using the Basic Fitting tool in MATLAB. For all variables, to highlight trends in annual data, a Gaussian function (adapted from http://imaging.mrc-cbu.cam.ac.uk/imaging/PrinciplesSmoothing) was used for data smoothing on $\log_{10}$ transformed

data with a window from x-2 to x+2 for each data point. In addition, a 3-year moving average of $\log_{10}$ transformed abundance data, including spline interpolants, was determined to highlight trends in annual data. Pearson Correlation coefficients and linear regressions were calculated from anomalies to determine the significance of relationships between all abundance data transformations and depth-averaged temperature as well as the North Wall Index (http://www.pml-gulfstream.org.uk/Data%20Web2014.pdf) and the North Atlantic Oscillation Index (http://www.cpc.ncep.noaa.gov/products/precip/CWlink/pna/norm.nao.monthly.b5001.current.ascii). A serial correlation between anomalies of abundance data and temperature data was calculated using Pearson Correlation Coefficients in 3 and 5 year increments to reveal any internal significance between anomaly abundance data for each species and temperature on smaller time scales. Additional analysis included species-specific abundances compared to environmental variables using the COPEPODITE interface, where anomalies of each variable were calculated and linear regression analysis was performed.

III. Results:

III.A. Visualization and Mapping of *Pseudocalanus* spp. Abundance 1977-2012

There was visible variation in *Pseudocalanus* spp. abundances on the NW Atlantic continental shelf across space (e.g., among MAB, SNE, GOM and GB regions) and over time (e.g., interannual). The MATLAB animation of seasonal snapshots of combined-species abundances showed consistently higher abundances in the north, with lower abundances in the south for all seasonal groupings and across time (Erikson et al., 2013). *Pseudocalanus* spp. abundance on Georges Bank during May-June varied from year to year between 1977 and 2012,

as did temperature and salinity (Figure 6). *Pseudocalanus* spp. abundance on GB was found to be weakly significantly negatively correlated with depth-averaged salinity for the time period of 1977 to 2012 ($R = -0.3750$, $p = 0.0412$, $R^2 = 0.1406$). *Pseudocalanus* spp. abundance on GB was not significantly correlated with depth-averaged temperature over this same time period ($R = -0.1059$, $p = 0.5775$, $R^2 = 0.0112$) nor with any environmental variable accessible through COPEPODITE

III.B. Analysis of Species-specific *Pseudocalanus* Abundances on Georges Bank 1995-2012:

III.B.1. Time-series Analysis of Species-specific Abundances

Pseudocalanus moultoni and *P. newmani* abundances showed year-to-year variation from 1995 to 2012, as well as variation within years, based on qPCR discrimination of the species and calculation of relative abundances based on counts of *Pseudocalanus* spp. in the archived samples (Figure 7). Data treatments to reveal underlying trends included untransformed data spline interpolants to fill gaps, data that had been smoothed with a Gaussian function, and a moving average (Figure 8).

III.B.2. Comparative Analysis of Species-specific Abundances and Environmental Parameters:

Significant correlations were found between *P. moultoni* abundance anomalies (untransformed and with various data transformations) and depth-averaged temperature anomalies, although no significant correlations were found for the same comparisons and data transformations with *P. newmani* abundance anomalies (Table 2; Figure 9). No significant correlations were found between the abundances of the two species over the observed period (1995-2012), based on untransformed data or data with spline interpolants, yet when the

abundance data were transformed with moving-average or Gaussian smoothing significant correlation between species was found (R = 0.6456, p = 0.0069, and R = 0.5058 p = 0.0322 respectively; see Table2). Both linear regressions and correlation coefficients were determined for each species in comparison to temperature data (Table 2).

Abundances anomalies for *P. moultoni* and *P. newmani* with or without spline curve interpolation or other data transformation (e.g., moving average, or Gaussian smoothing, etc.) showed no significant relationships to annual or seasonal Gulf Stream North Wall Index or North Atlantic Oscillation Index values (Table 3).

Serial correlations analyses were done with untransformed data that included spline interpolants. Significant correlations were found between *P. moultoni* abundance data and depth-averaged temperature anomalies for 1998 - 2000 (R = 0.9999, p = 0.0055), between *P. newmani* abundance and *P. moultoni* abundance anomalies for 3-year serial correlations between 2009-2011 (R = 0.9979, p = 0.0407) and 2007-2011 (R = 0.9187, p = 0.0275), and between *P. newmani* abundance anomalies and depth-averaged temperature anomalies for 5-year serial correlations from 1995-1999 (R = 0.9609, p = 0.0092), 1996-2001 (R = 0.9541, p = 0.0117), and 1997-2002 (R = 0.9608, p = 0.0092) (Figure 10).

Untransformed and spline interpolant abundances for each species were entered into the COPEPODITE online analytical interface and compared with depth-averaged temperature data for May-June and March-April, as well as other variables provided by the interface, in order to examine relationships between abundances and both current temperature and temperature over the prior 2 months, with the time-lag chosen to reflect delayed impacts on development and generation times. The resultant linear regressions showed significant relationships between

P.moultoni untransformed abundance data (with no spline interpolants) and the HADISST, and depth-averaged temperatures for both March-April and May-June (with $R^2 = 0.2892$, $R^2 = 0.2825$ and $R^2 = 0.3334$, respectively all with $p \leq 0.05$). Significant regressions were also found between *P. moultoni* abundance with spline interpolants and HadISST ($R^2 = 0.2676$, $p \leq 0.05$), as well as depth-averaged temperatures for May-June and depth-averaged temperatures from March- April ($R^2 = 0.3255$, $p \leq 0.05$ and $R^2 = 0.3588$, $p \leq 0.01$, respectively). No significant regressions between *P. newmani* abundances and any environmental variables were found.

IV. Discussion:

IV.A. Time-series Mapping and Visualization of *Pseudocalanus* spp. Abundances:

Time-series visualization of the abundance of *Pseudocalanus* spp. from 1977-2012 revealed marked variation interannually based on two-month groupings among years and among the sampled regions of the NW Atlantic continental shelf (Erikson et al., 2013). Higher abundances were observed in northern regions, which can be associated with known *Pseudocalanus* spp. biogeographical ranges for *P. moultoni* and *P. newmani*.

Time-series analysis of *Pseudocalanus* spp. abundance revealed trends within May and June in the GB region; abundances reached peak values in 2001 and 2010, which matched the positive temperature anomalies for the May-June season during these years. In general, abundances were low in the 1980s-2000s and high in the 1990s. Similar patterns of abundances were observed for other zooplankton species considering the GOM and GB regions. This general trend is consistent with the findings of Pershing et al. (2005), who reported a community shift in species dominance and abundance in the GOM over the same time period, with high annual mean abundance of *Pseudocalanus* spp., along with other small-bodied copepods in the 1990s,

followed by a dramatic decline during 2001 - 2002. Species of another copepod genus, *Calanus*, showed opposite trends (Pershing et al., 2005). The similarity in temporal patterns of variation between GB and GOM suggests high levels of connectivity between these adjacent regions of the NW Atlantic continental shelf including exchange among *Pseudocalanus* spp. populations. Similar oscillations in zooplankton species abundance were described throughout the NW Atlantic shelf highlighting the possible connectivity of all regions (GOM to MAB). This may reflect possible responses to environmental variation and/or changes in abundances in upstream source regions such as the Scotian Shelf (Kane, 2007, 2014).

Comparative analysis of species abundances and environmental variables has been used to gain insight into the underlying causes and drivers of community composition and change. A primary environmental factor that can impact zooplankton species abundance is temperature, which may play an especially important role at the biogeographic boundaries and physiological tolerance limits of the species. Water temperature rose by 1˚ C on the NW Atlantic continental shelf during the last century, and *Pseudocalanus* spp. are exposed in this region to temperatures ranging seasonally from 3˚ to 22˚ C (Friedland et al., 2013). *Pseudocalanus* spp. abundance data from the EcoMon surveys were analyzed in comparison with environmental data from the COPEPODITE database and no significant relationship (regression) was found for the GB region for the May-June period from 1977 to 2012, with the exception of a significant relationship to depth-averaged salinity. It should be noted that GB is within *P. newmani* and *P. moultoni* latitudinal ranges in the NW Atlantic, and the temperatures are likely within the usual physiological tolerance limits, especially within the typical 3˚ to 22˚ C range (Friedland et al., 2013; Frost, 1989).

Significant negative correlations have been found in other studies between annually averaged abundance of *Pseudocalanus* spp. and temperature on GB. Kane (2014) found a negative correlation between abundance and temperature, which may be due to inclusion of annual data transformed through spline curve smoothing to remove the seasonal variation. In contrast, this study used the single May-June annual snapshot, times of peak *Pseudocalanus* spp. abundance but not peak temperature, and no data transformations were done on *Pseudocalanus* spp.

Another environmental variable that did show a significant relationship (i.e., weak negative correlation) was salinity. Based on the findings of a significant negative correlation between zooplankton species abundance and salinity in the GOM during the early 1990s, Pershing et al. (2005) proposed that decreased salinity caused increased zooplankton abundance by driving increased stratification, which in turn increased primary production. When analyzed with different statistical techniques, over a longer time scale, and with chlorophyll as a proxy, this salinity correlation was found to not be related to *Pseudocalanus* spp. abundance (Hare and Kane, 2012; Ji, 2013).

In time-series analysis from the 1970s to the present, *Pseudocalanus* spp. peak abundances in the MAB, the supposed southern limit of the species range, varied temporally. Prior to the 1990s, peak copepod abundance was found during March-April; from the 1990s onward, there was a sharp decline in copepod abundance during spring, with highest numbers found in May-June (Kane, 2014). Annually averaged *Pseudocalanus* spp. abundances showed negative correlations with temperature and salinity at the southern limits of the combined species range (Kane, 2014). This correlation was not found in the May-June time period, but this could be due to the single annual snapshot used in this study. Thermal correlation and shifts in time of

the *Pseudocalanus* spp. abundance peak in the MAB suggests that *Pseudocalanus* spp. may be impacted by temperature, which may in turn impact limits of the species geographic range.

Temporal variation and shifts in zooplankton community composition and species abundances cannot be fully described or predicted without resolution of closely-related and cryptic species. Clearly, understanding interactions of *Pseudocalanus* spp. with environmental variation over extended time periods requires discrimination of the congeneric and co-occurring species, which are known to have different habitat preferences (Bucklin et al., 2001, 2015).

IV.B. Species-specific Analyses and Relationship to Environmental Variation:

Both *P. moultoni* and *P. newmani* inhabit overlapping regions in the NW Atlantic. The distribution of *P. moultoni* is known to be in temperate coastal waters of the NW Atlantic to regions as far north as Nova Scotia; distribution of *P. newmani* is similar, however the species is classified as an inhabitant of temperate and boreal waters, and is considered more oceanic (Frost, 1989).

Pseudocalanus moultoni and *P .newmani* have been shown to have different habitat preferences on GB and species may be more prevalent at different times. Bucklin et al. (2001) discerned differences between location and abundance in May and June in both sub-surface and surface waters. Species-specific depth distributions may be attributable to transport in currents over GB, as well as abundances of each species from source regions for GB populations (Bucklin et al., 2015). In this study, differences between the species in abundance anomalies were seen for a number of years from 1995 to 2012, with no consistent pattern in relative abundances (highs and lows). Nine of the 15 years for which species-specific patterns were determined showed higher abundances for *P. moultoni* than *P. newmani*. Furthermore, *P. moultoni* abundances were

found to be significantly positively correlated with depth-averaged temperature during 1995 to 2012 on GB, while *P. newmani* abundances were not. This analysis indicated that the two species may respond to temperature differently, and suggests that there may be other species-specific differences in responses to other environmental variables. Modeled *Pseudocalanus spp.* population growth was seen to decrease with warming North Atlantic waters especially in southern regions by 2080 (Stegert et al., 2010), and this visualization could be more species-dependent than is currently appreciated. It is possible that increases and decreases in *Pseudocalanus* spp. abundance over time may actually reflect non-uniform responses by the individual species to varying environmental conditions. An understanding of the underlying species-specific dynamics and causes of population variation could shed light on the larger picture of zooplankton responses to climate change.

A number of other environmental variables showed no significant correlation or regression either with *P. moultoni* or *P. newmani* abundance, including the NAO index and chlorophyll concentration. In the GOM, the NAO index was found to be correlated with abundance of the copepod *Calanus finmarchicus* and with temperature in longer time-series and using a variety of time lags; in contrast, over similar time frames on GB, the index was not correlated with salinity or temperature (Hare and Kane, 2012).

Over shorter timescales, serial correlation analysis did not reveal many significant relationships between abundances of *P. moultoni* versus *P. newmani* or between the species-specific abundances and temperature. Some significant correlations were found between *P. moultoni*, *P. newmani* and temperature over varying time periods, which highlights the possible differences between the species within longer time periods. This could mean that abundance dynamics are changing within the time-series in response to environmental variables, and that

there are times where temperature proved more favorable for species success. Serial correlation had been used to examine relationships between the NAO Index, species abundances, and temperature in the GOM using a 10-year window, which revealed correlation between some environmental variables and *Calanus finmarchicus* abundance (Hare and Kane, 2012).

Serial correlations used in this study to analyze shorter timescale relationships may not be accurate due to the small data sets used (i.e., very few 3 and 5 year groupings). In addition to inaccuracies in correlations due to small sample sizes, relationships may be deemed spuriously significant based on the incorporation of spline interpolants. Serial correlations could not be performed without interpolated data, since these trends would have been misinterpreted to reflect annual variation.

IV.C. Further Considerations of Time-series Analysis:

There are several important factors to consider regarding best practices for time-series analysis (Chatfield, 2009), including both the length of time examined and the frequency of observations. The lack of correlation between copepod abundance and other environmental variables, which are classically associated with plankton abundances, could have been due to analysis of a short time-series.

Correlations between abundance and environmental variables may have been strengthened or weakened with higher sampling density and increased spatial resolution. In this study, only 4 to 5 representative stations of 50 copepods accounted for the GB region. Future studies should use time frames that include more densely sampled regions of interest for better overall resolution. This is important because species ratios produced from qPCR results varied in

all samples and across GB, even when stations were physically close, therefore 4 to 5 samples may not be enough to capture the true species composition across the Bank.

IV.D. Importance of Discrimination of Cryptic Species and Usefulness of qPCR:

Quantitative real time PCR (qPCR) proved to be an extremely effective tool in differentiating the cryptic species of *Pseudocalanus* and estimating their relative abundances. The species-specific primers, protocol optimization, and determination of the standard curve for calculation of relative abundances yielded a reliable, accurate, and rapid technique that allowed characterization of the species abundances in an existing time-series ecosystem monitoring collection. There are numerous potential applications of qPCR for discrimination and quantification of cryptic species in zooplankton samples. Once qPCR protocols have been developed and optimized – which requires some investment of time and effort – qPCR analysis of pooled samples is much less time consuming and more cost-effective, especially when analyzing many samples, when compared to individual species-specific PCRs for species identification.

IV.E. Future Directions:

Future work could analyze species-specific abundances and correlations to environmental variables in the three other regions sampled by the EcoMon surveys of the NW Atlantic continental shelf, including MAB, SNE, and GOM. A full picture of the abundances and changes in *P. moultoni* and *P. newmani* and other *Pseudocalanus* species present in all four regions would allow both time-series analysis of past changes and predictions of future population shifts. The MAB and SNE are of particular interest, as *Pseudocalanus* spp. has been found there but has not been differentiated on a species level. If these species are *P. moultoni* and *P. newmani*, this

would represent the southernmost limit of their range in the NW Atlantic; composition of the zooplankton community in this region may be particularly subject to change over time. Relationships between species-specific abundances and temperatures in southern regions could act as predictors for what will occur in northern regions in the future as temperatures are expected to rise by 2-4˚ C by the year 2100 (http://nefsc.noaa.gov/ecosys/climate_change/projected.html).

Additional future work could seek to resolve smaller scale spatial patterns, including depth-stratification. Further analysis could also shed light on the abundances of *Pseudocalanus* species at all life stages, since immature stages are especially important as food for larval cod (Friedland et al., 2013). Work could also be done to look at different top-down and bottom-up controls on *Pseudocalanus* spp. in order to examine species-specific relationships with predators and prey.

IV.F. Conclusions:

Pseudocalanus moultoni and *P. newmani* appear distinct with regard to changes in abundance over time and response to environmental variables, particularly depth-averaged temperature, across the time frame studied. This response to environmental variables is different from what is observed when both species are grouped in combined analysis as *Pseudocalanus* spp. Both differences and similarities between species need to be further investigated using time-series analysis to develop an understanding of true *Pseudocalanus* spp. dynamics in the NW Atlantic shelf region. This understanding will allow for future responses to climate change to be discerned through predictions from past changes in *P. moultoni* and *P. newmani* abundances. This is valuable as abundances and population ranges will vary with changes in climate on

unknown scales. Comprehensive analysis of these cryptic species in the NW Atlantic through further time-series analysis using qPCR will help distinguish the *P. moultoni* and *P. newmani* species assemblage, species-specific preferences, and patterns of abundance across large timescales.

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Table 1. Collection information (year, cruise, station), and DNA concentrations for extracted bulk *Pseudocalanus* spp. samples used in qPCR analysis

Year	Cruise	Station	DNA Concentration (ng/μL)
1995	AL9505	**25**	13.7
		32	10.4
		28	10.4
		6	8.64
		16	9.28
1996	AL9605	7	17.6
		14	8.44
		18	10.2
		30	11.5
		39	16.2
1997	AL9707	40	4.4
		11	12.9
		17	14
		6	15.8
		7	14.6
1998	AL9806	25	3.79
		12	17.2
		18	18.8
		15	16.7
		7	14.6
1999	AL9906	40	6.68
		19	7.76
		21	11.4
		8	8.64
		7	10.2
2002	AL0206	93	14.2
		84	16.3
		85	13.1
		75	15.8
2003	DE0305	3	16
		6	4.88
		15	17.6
		28	17.8
2004	AL0405	81	17.5
		84	18.7
		53	19.9
		59	18
2005	AL0505	87	8.04
		81	11.8
		70	10.3
		69	10
2006	AL0605	65	6.16
		73	8.84
		81	12.4
		83	1.63
		98	12.8
2007	DE0706	96	22
		76	11.4
		93	18.2
		72	17.9
		70	14.8
2009	DE0905	71	0.04
		73	5.62
		75	0.872
		82	18.7
2010	DE1004	73	18.4
		93	15.4
		98	19.6
		77	13.9
2011	DE1105	77	5.04
		83	18.4
		90	18.4
		80	17.5
		86	0.36
2012	HB1202	85	14.4
		87	16.7
		71	16.4
		72	15.5

Table 2. Correlations, significance, and linear regressions relationships, (R, p, and R^2, respectively) between *P. moultoni*, *P. newmani*, and depth-averaged temperature anomalies on GB across time for all data transformations, significant correlations are labelled with an asterisk (*).

Combinations of Variables	R	p	R^2
P. moultoni without interpolant and temperature anomalies	0.5315	* 0.0415	0.2825
P. moultoni with interpolant and temperature anomalies	0.5705	*0.0134	0.3255
P. moultoni moving average and temperature moving average anomalies	0.8442	* 3.916E-05	0.7127
P.moultoni abundance smoothed and smoothed temperature data anomalies	0.82677	* 2.331E-05	0.6836
P. newmani without interpolant and temperature anomalies	0.2758	0.3197	0.0761
P. newmani with interpolant and temperature anomalies	0.3658	0.1354	0.1333
P. newmani moving average and temperature moving average anomalies	0.4095	0.1152	0.1677
P.newmani abundance smoothed with intepolant and smoothed temperature anomalies	0.32716	0.18510562	0.107
P. moultoni without interpolant and *P. newmani* without interpolant anomalies	0.2643	0.3411	0.0699
P. moultoni with interpolant and *P. newmani* with interpolant anomalies	0.3159	0.2016	0.0998
P. moultoni moving average and *P. newmani* moving average anomalies	0.6456	* 0.0069	0.4169
P.newmani abundance smoothed and *P.moultoni* abundance smoothedanomalies	0.5058	* 0.0322	0.2559

Table 3. Correlations, significance, and linear regressions, (R, p, and R^2, respectively) for relationships between *P. moultoni*, *P. newmani*, and North Wall Index and North Atlantic Oscillation Index, no correlations or regressions are significant.

Combinations of Variables	R	p	R^2
P. moultoni without interpolant and NAO winter index anomalies	0.1281	0.649	0.016420068
P. moultoni with interpolant and NAO winter index anomalies	0.0961	0.7046	0.009179134
P. moultoni and NAO winter index smoothed anomalies	0.1708	0.4979	0.029184418
P. moultoni and NAO winter index moving average anomalies	-0.1591	0.5563	0.001129078
P. moultoni without interpolant and annual NAO index anomalies	-0.0975	0.7296	0.009502312
P. moultoni with interpolant and annual NAO index anomalies	-0.0323	0.8988	0.000942833
P. moultoni and annual NAO index smoothed anomalies	0.0381	0.8808	4.61146E-05
P. moultoni and annual NAO index moving average anomalies	-0.0353	0.8968	0.001129078
P. newmani without interpland and NAO winter index anomalies	0.1484	0.5977	0.022015896
P. newmani with interpolant and NAO winter index anomalies	0.059	0.8162	0.003521815
P. newmani and NAO winter index smoothed anomalies	0.3415	0.1654	0.11662624
P. newmani and NAO winter index moving average anomalies	0.1483	0.5837	0.052605889
P. newmani without interpolant and annual NAO index anomalies	0.0188	0.9666	0.000139771
P. newmani with interpolant and annual NAO index anomalies	0.164	0.5155	0.026326235
P. newmani and annual NAO index smoothed anomalies	0.4605	0.0545	0.056873002
P. newmani and annual NAO index moving average anomalies	0.2286	0.3945	0.052605889
P. moultoni without interpolant and NW May and June index anomalies	0.2554	0.3583	0.0652115
P. moultoni with interpolant and NW May and June index anomalies	0.3326	0.1775	0.110608589
P. moultoni and NW May and June index smoothed anomalies	0.2741	0.271	0.075129894
P. moultoni and NW May and June index moving average anomalies	0.3414	0.1956	0.11656988
P. moultoni without interpolant and annual NW index anomalies	-0.01042	0.7117	0.010854773
P. moultoni with interpolant and annual NW index anomalies	0.0895	0.7241	0.008004417
P. moultoni and annual NW index smoothed anomalies	0.0101	0.9682	0.00010254
P. moultoni and annual NW index moving average anomalies	0.2918	0.2729	0.08512055
P. newmani without interpolant and NW May and June index anomalies	-0.0475	0.8664	0.002259256
P. newmani with interpolant and NW May and June index anomalies	0.262	0.2936	0.068649377
P. newmani and NW May and June index smoothed anomalies	0.3543	0.1491	0.125535411
P. newmani and NW May and June index moving average anomalies	0.2844	0.2857	0.080904076
P. newmani without interpolant and annual NW index anomalies	-0.0426	0.8803	0.00181121
P. newmani with interpolant and annual NW index anomalies	0.1846	0.4633	0.034089637
P. newmani and annual NW index smoothed anomalies	0.2383	0.341	0.056780284
P. newmani and annual NW index moving average anomalies	0.2266	0.3987	0.051341894

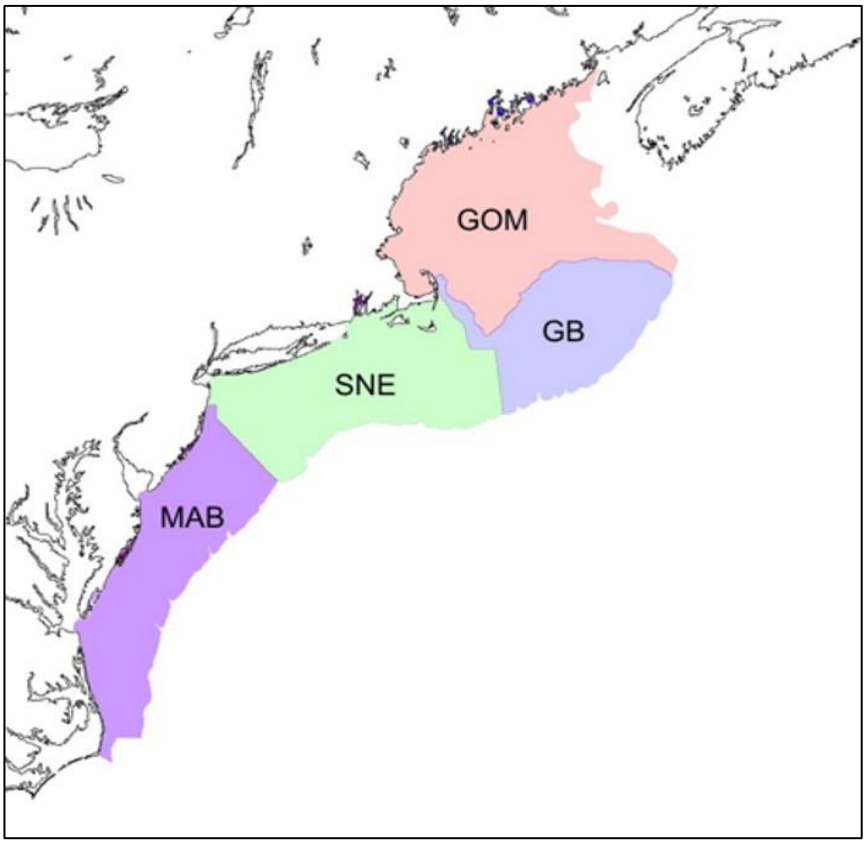

Figure 1. Map of regions covered by EcoMon cruises including the Gulf of Maine (GOM), Georges Bank (GB), Southern New England (SNE), and the Mid Atlantic Bight (MAB).

(http://www.st.nmfs.noaa.gov/plankton/time-series/site__northwest-nmfs-sne/html/origfig01.html)

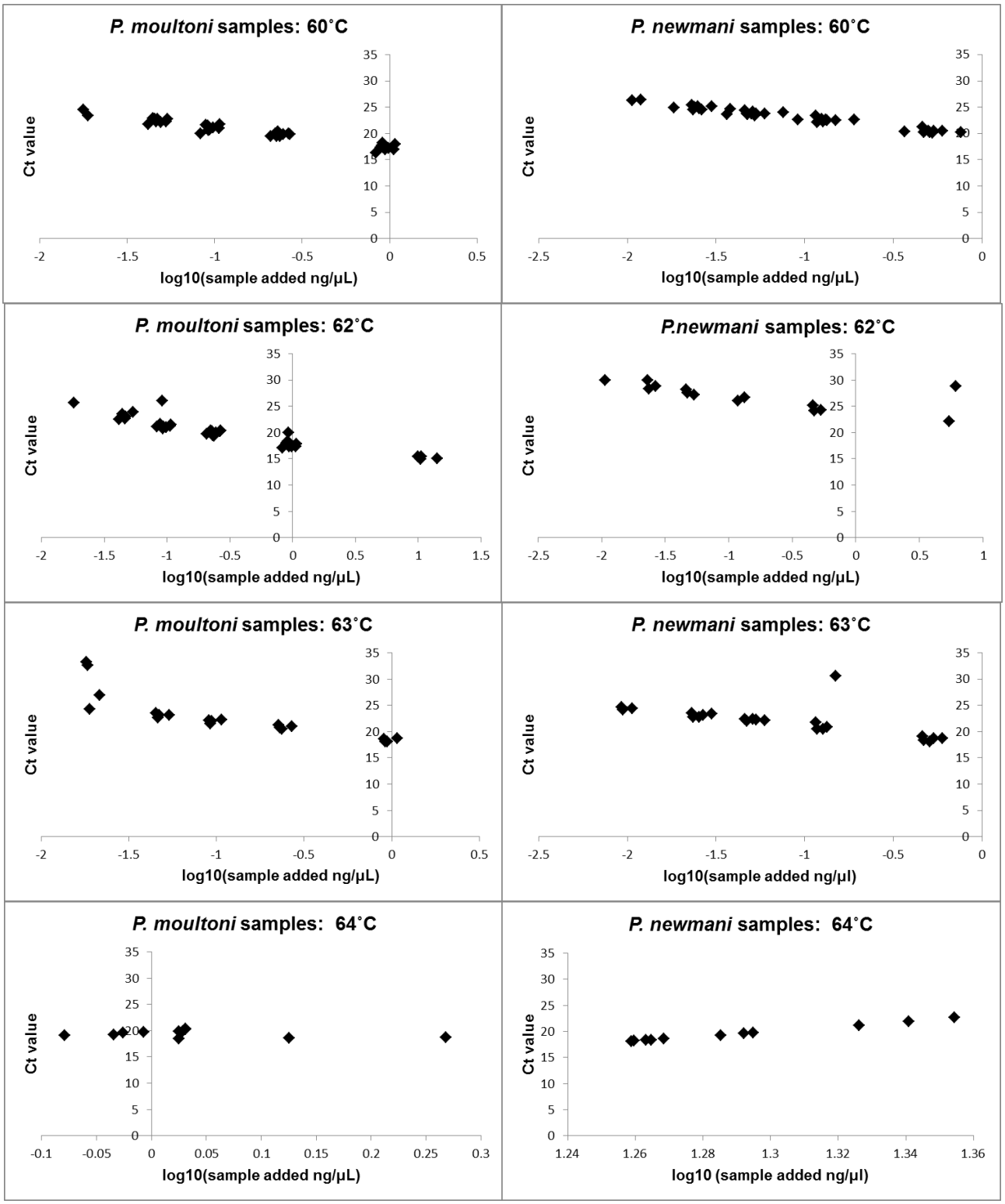

Figure 2. Tested annealing temperatures for the optimization of the qPCR reaction for both species and for both primer pairs for multiple samples

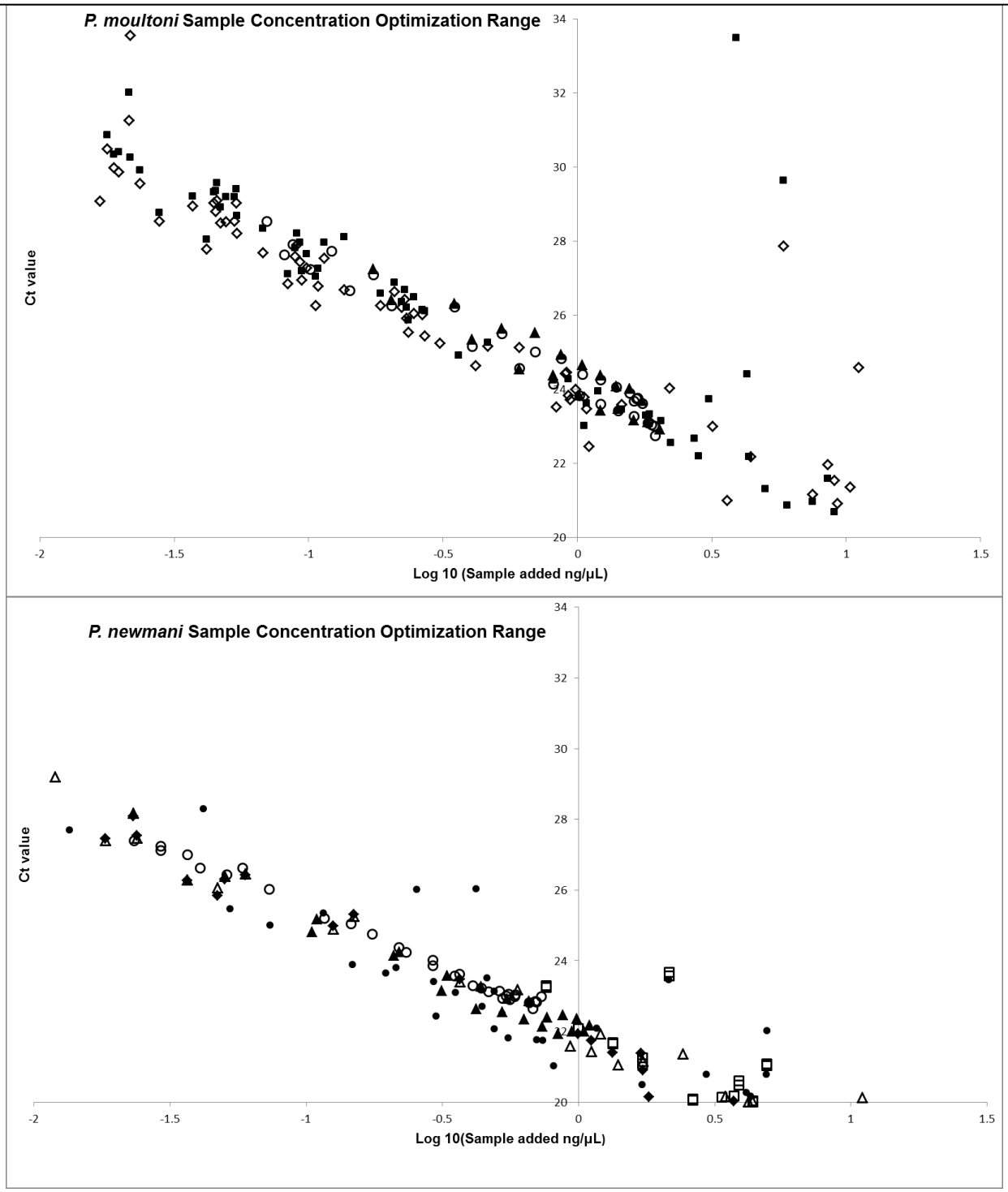

Figure 3. Range of concentrations tested from various samples used to determine range of qPCR detection for high and low *Pseudocalanus* spp. DNA concentrations

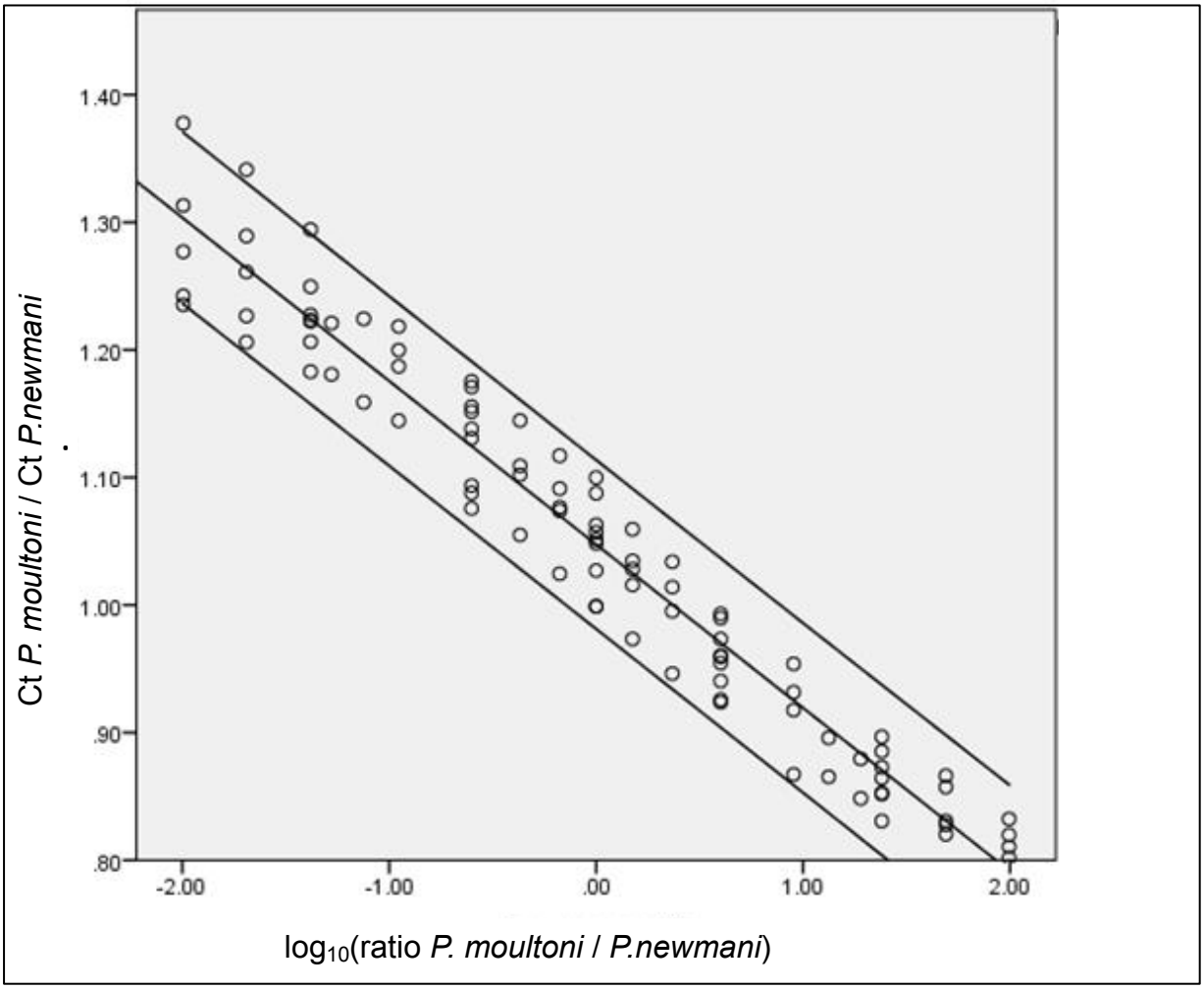

Figure 4. Standard curve used to determine proportions of cryptic species by solving the linear equation: $Y = -0.1281x + 1.0457$ where $x = \log(\frac{Pmoultoni}{Pnewmani})$ and $Pnewmani = 1 - Pmoultoni$; fits within a 95% confidence interval ($R^2 = 0.95$).

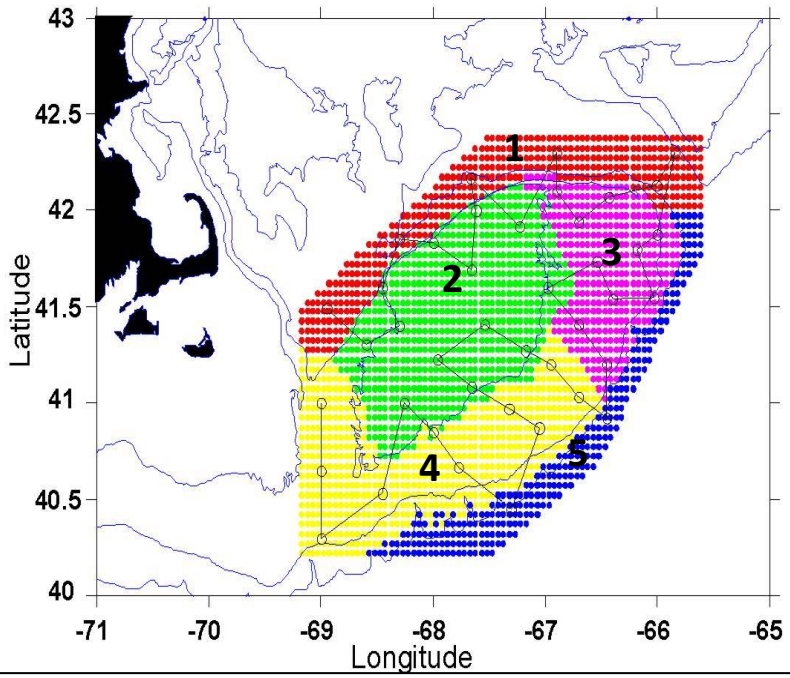

Figure 5. US GLOBEC zones; One station per region was selected unless full coverage of GB regions was not sampled or preserved in 95% ethanol.

Zones are indicated by: 1= Northern Flank (red), 2= Crest (green), 3=Northeast Peak (pink), 4=Southern Flank (yellow), 5= Slope Water (blue) (http://globec.whoi.edu/images/bsgridRev.jpg)

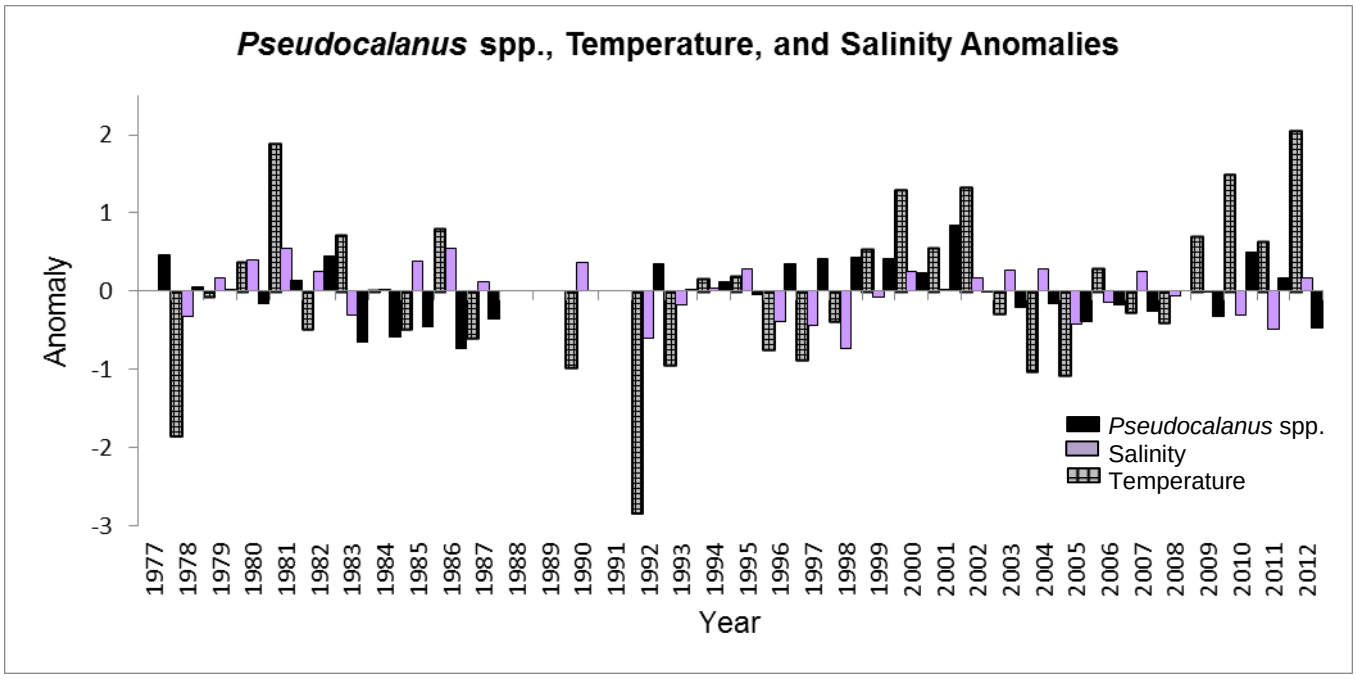

Figure 6. *Pseudocalanus* spp. abundance (black), depth-averaged salinity (purple), and depth averaged temperature (gray with grid pattern) anomalies from 1977-2012. Significant correlation was found between depth-averaged salinity and *Pseudocalanus* spp. abundance anomalies (R = -0.3750, p = 0.0412)

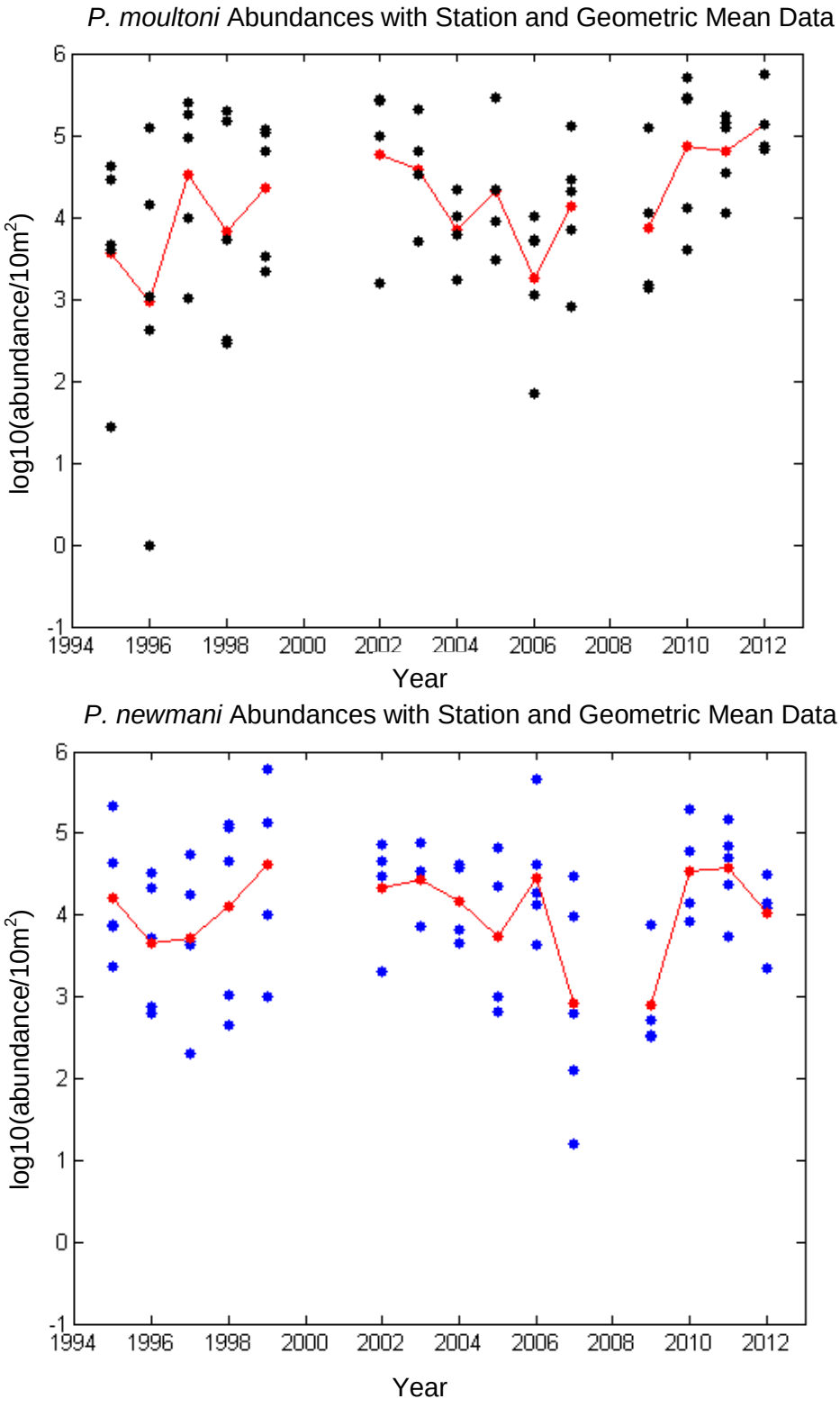

Figure 7. Species-specific abundances per station (circles) calculated from species-specific ratios and EcoMon and US GLOBEC abundance data; abundances were averaged annually using a geometric mean (line)

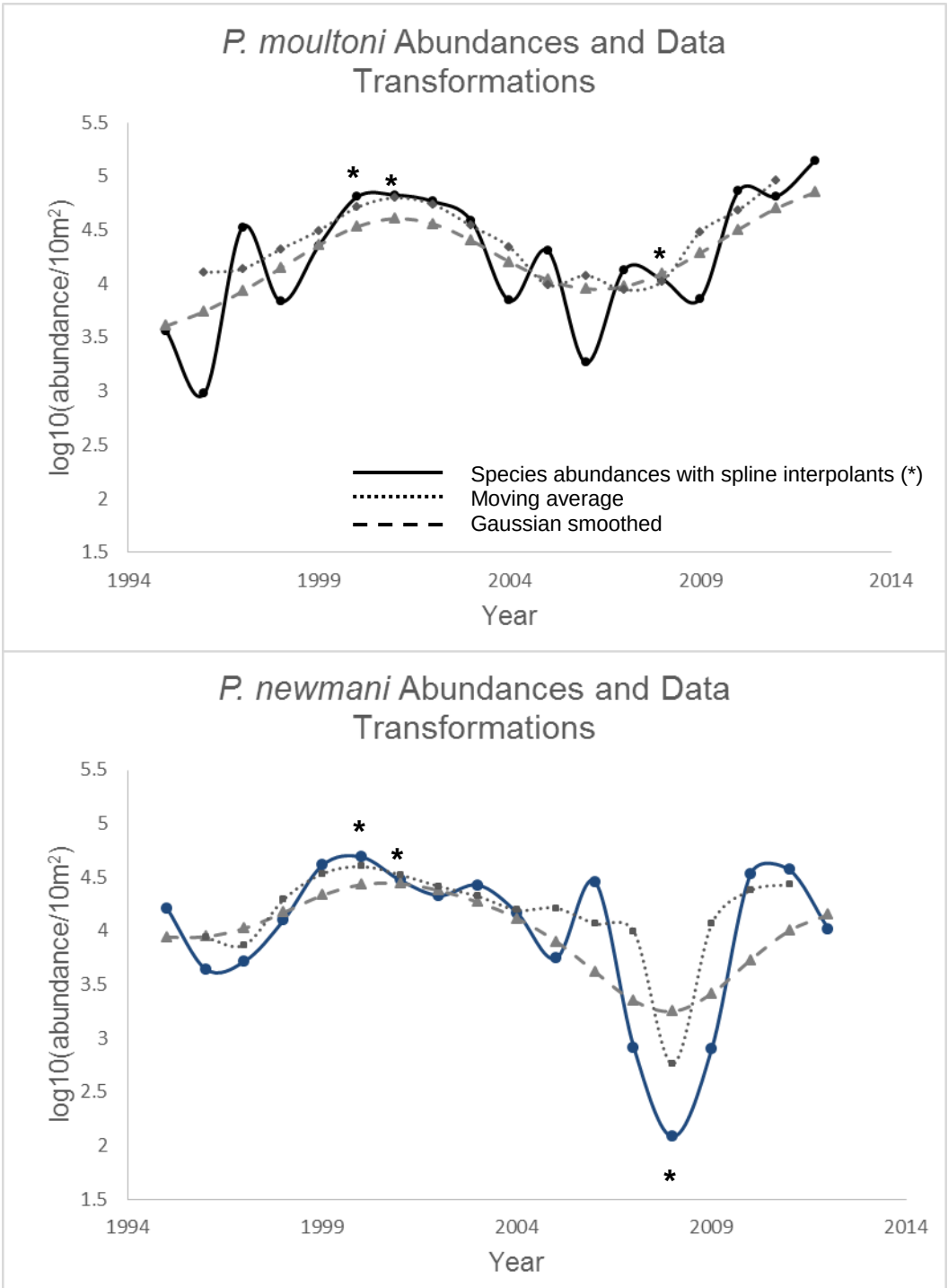

Figure 8. Different representations of *P. moultoni* and *P. newmani* abundance data; untreated data plus interpolated data are marked with a solid line and circle point (interpolated points marked with an asterisk, *); data transformed with Gaussian smoothing are marked with triangle points and dashed lines; data transformed with a 3-year moving average are marked with square data points and a dotted line. The key can be applied to both graphs.

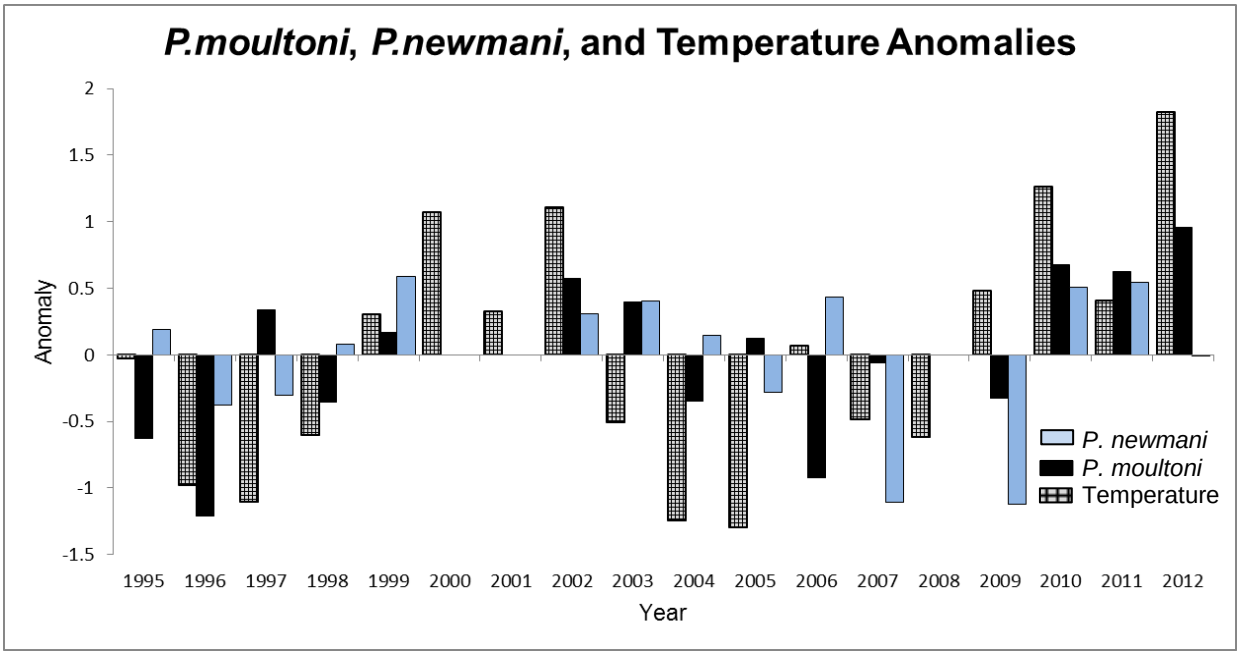

Figure 9. *Pseudocalanus moultoni* (black), *P. newmani* (blue), and depth-averaged temperature (gray with grid pattern) untransformed data anomalies in GB from 1995-2012. *P. moultoni* is significantly correlated with temperature (R = 0.5315, p = 0.0415)

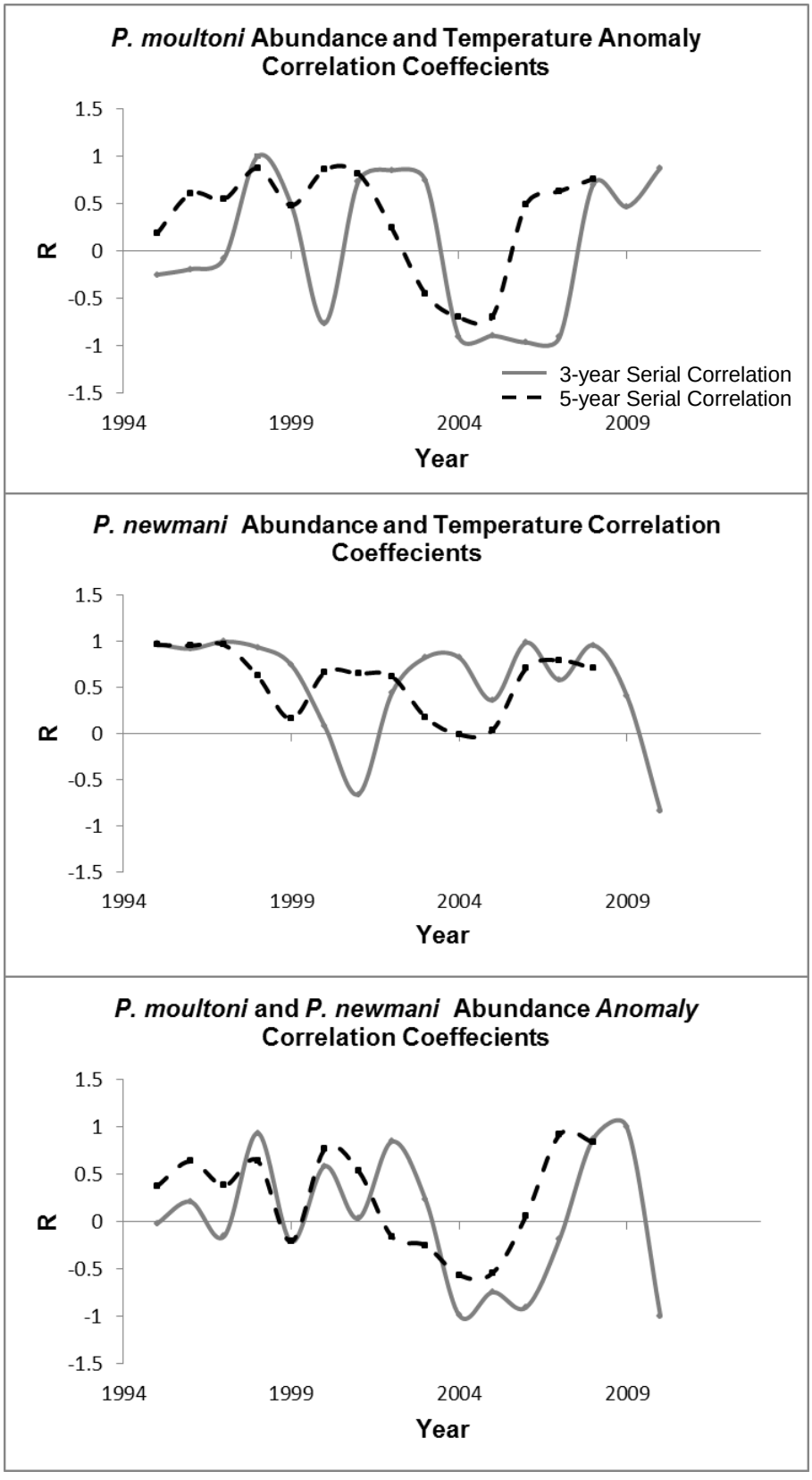

Figure 10. Serial correlation on 3-year (solid gray line) and 5-year (dashed black line) periods between *P. moultoni*, *P. newmani*, and depth averaged temperature anomalies on GB