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Species-specific patterns of distribution and abundance of the cryptic copepods *Pseudocalanus moultoni* and *P. newmani* on Georges Bank (NW Atlantic Ocean) during Spring 1995-2012

Kayla E. Crouch[1], Leocadio Blanco-Bercial[2], David E. Richardson[3], Nancy J. Copley[4], Peter H. Wiebe[4], and Ann Bucklin[1*]

[1]Department of Marine Sciences, University of Connecticut, Groton, CT, USA
[2]Bermuda Institute of Ocean Science, St. George's, Bermuda
[3]Oceans and Climate Branch, NEFSC/NMFS/NOAA, Narragansett, RI 02882 USA
[4]Biology Department, Woods Hole Oceanographic Institution, Woods Hole, MA 02543 USA

Abstract

Time-series analysis of zooplankton species diversity, distribution, and abundance are essential for ecosystem assessment and fisheries management on continental shelves. This study analyzed two morphologically cryptic species of the calanoid copepod *Pseudocalanus, P. moultoni* and *P. newmani*, in zooplankton samples collected during May-June 1995-2012 over Georges Bank, NW Atlantic Ocean. Samples were collected 1995-1999 by US Global Ocean Ecosystem Dynamics (US GLOBEC) and 2002-2012 by NOAA Northeast Fisheries Science Center Ecosystem Monitoring (EcoMon). The species were discriminated by real-time quantitative PCR (qPCR) based on DNA sequence variation of the mitochondrial cytochrome oxidase I (COI). Multivariate statistical analysis revealed significant positive correlation between the geometric mean abundance of *P. moultoni* and depth-averaged temperature at the collection locations; *P. newmani* abundances showed no relationship to temperature, suggesting different temperature niches and potential responses to environmental conditions. Interannual patterns of variation of the species-specific abundances of *P. moultoni* and *P. newmani* differed significantly from pooled *Pseudocalanus* spp. Nonmetric multidimensional (NMDS) and regression analyses confirmed significant interannual differences between *P. moultoni* and *P. newmani* geometric mean abundances during 1995 – 2012. This study demonstrates the need for discrimination of closely-related and cryptic zooplankton species to understand and predict impacts of environmental variation and climate change on marine ecosystems.

Keywords: Zooplankton, Cryptic species, *Pseudocalanus*, Quantitative PCR, Georges Bank, Time-series analysis

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*Corresponding Author Address:
Department of Marine Sciences
University of Connecticut
Groton, CT 06340 USA
Email: ann.bucklin@uconn.edu

1. Introduction

1.1. *Pseudocalanus* species on the NW Atlantic continental shelf

Species of the genus *Pseudocalanus* are among the copepods that dominate the zooplankton assemblage on the NW Atlantic Shelf. *Pseudocalanus* spp. life histories, habitat preferences, and distributions differ among species and across regions (Frost, 1989; Napp et al., 2005; Hopcroft and Kosobokova, 2010). Two of the seven known *Pseudocalanus* species, *P. moultoni* and *P. newmani*, occur sympatrically throughout much of the 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 readily morphologically distinguishable (Frost, 1989; Hare and Kane, 2010; Kane, 2014; Mountain and Kane, 2010; Pershing et al., 2005). In the Georges Bank (GB) region, *Pseudocalanus* spp. 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).

Pseudocalanus spp. of all life stages are preferred prey items for juvenile cod, haddock, and herring compared to other copepods of similar size; the species have been shown to directly impact fisheries recruitment and may therefore be particularly important for the stability of these commercial fisheries (Friedland et al., 2013; Munk, 1997; Murphy et al., 2018; Suca et al., 2018), albeit in association with a number of other factors (Bernreuther et al., 2018; Wilson et al., 2018).

Although the two species are difficult to distinguish morphologically, *P. moultoni* and *P. newmani* can be discriminated and identified using genetic techniques based on DNA sequence

divergence of the mitochondrial cytochrome oxidase I (COI) gene, which differs ~18% between the species (Aarbakke et al., 2014; Blanco-Bercial et al., 2014; Bucklin et al., 1998, 1999, 2001, 2003). There are subtle morphological differences between species, e.g., *P. moultoni* is observed to be larger in size, but size has been shown not to be a diagnostic characteristic for identification and discrimination of *P. moultoni* from other congeneric species in the NW Atlantic (Frost, 1989).

Studies that have discriminated the two species have shown that *P. moultoni* and *P. newmani* differ in patterns of distribution and abundance over a wide range of temporal and spatial scales (Bucklin et al., 1998, 2001, 2015; Manning and Bucklin, 2005; McGillicuddy and Bucklin, 2002). In the NW Atlantic, *P. moultoni* has been found in the coastal waters of the New York Bight to Nova Scotia, Canada, while *P. newmani* has been described in temperate-boreal waters, with continued distribution throughout the Canadian Arctic (Frost, 1989). The two species may exhibit different habitat preferences within NW Atlantic continental shelf regions, where *P. moultoni* is considered to be more coastal and *P. newmani* more oceanic (Aarbakke et al., 2014; Frost, 1989).

Vertical distributions of *P. moultoni* and *P. newmani* differed in a stratified water column over GB, although at the time of peak abundance (May and June), both species were similarly distributed in the upper 100 m across GB (Bucklin et al., 2015). In the Gulf of Maine, when the water column was stratified, *P. moultoni* was more abundant in deep water and *P. newmani* was concentrated at the surface (Manning and Bucklin, 2005).

1.2. Introduction to the Georges Bank study area

Georges Bank forms the southern boundary of the Gulf of Maine (GOM) region and is approximately 300 km by 150 km in size with variable depth ranging from 200 m to 5 m; GB

47 represents a zoogeographic boundary that sets both northern and southern limits of species
48 ranges (Wiebe et al., 2002). Waters across GB vary in temperature and salinity, resulting from
49 mixing of source waters from the Scotian Shelf and continental slope waters, which are classified
50 as being colder with low salinity, and warmer with higher salinity, comparatively and
51 respectively (Smith et al., 2001; Wiebe et al., 2002).

52 Georges Bank is within the latitudinal ranges of both *P. newmani* and *P. moultoni* in the
53 NW Atlantic, and water temperatures are within the documented physiological tolerance limits
54 of 3 – 22 ˚C range (Friedland et al., 2013; Frost, 1989). The species have been shown to have
55 differing patterns of distribution and abundance on GB that have been attributed to different
56 habitat preferences (Bucklin et al., 2015). Bucklin et al. (2001) described differences between
57 location and abundance in May and June in both sub-surface and surface waters. Species-specific
58 depth distributions may be attributable to transport in currents over GB (McGillicuddy et al.,
59 1998), as well as abundances of each species from source regions for GB populations
60 (McGillicuddy and Bucklin, 2002; Bucklin et al., 2015).

61 Seasonal differences in species-specific abundances were observed in samples collected
62 from Browns Bank, a potential source region adjacent to GB, where *P. moultoni* was found to be
63 more abundant in winter and spring, while *P. newmani* was more abundant in spring and summer
64 (McLaren et al., 1989). Bucklin et al. (2001, 2015) reported that *P. moultoni* and *P. newmani*
65 differed in patterns of distribution and abundance over GB, with *P. moultoni* present on the
66 northern edge of GB in late spring/early summer, while *P. newmani* was more dominant off the
67 Bank. Based on assimilation of species-specific abundance data from 1997 into a coupled
68 physical-biological model by McGillicuddy et al. (1998), the two species exhibited distinct
69 population centers in the late winter/early spring on GB, followed by species-specific patterns of

70 springtime evolution (McGillicuddy and Bucklin, 2002).

71 The wide range of biological and physical drivers of zooplankton species abundances on
72 GB and adjacent continental shelf regions have been studied over many years (Davis, 1984;
73 Friedland et al., 2015). The NOAA Northeast Fisheries Science Center has provided extensive
74 and detailed analyses of the NW Atlantic continental shelf ecosystem since 1977 (Davis, 1984;
75 Kane, 2007). The Ecosystem Monitoring of the Northeast US Continental Shelf Program
76 (EcoMon) and its predecessor, the Marine Resources Monitoring, Assessment, and Prediction
77 (MARMAP) program, have yielded comprehensive zooplankton abundance data for four regions
78 of the shelf: Gulf of Maine (GOM), Georges Bank (GB), Southern New England (SNE), and the
79 Mid-Atlantic Bight (MAB). Georges Bank was the primary focus during the US Global Ocean
80 Ecosystem Dynamics Program (US GLOBEC) from 1995 to 1999, yielding exceptionally high-
81 resolution sampling and environmental data collection during broad-scale surveys using the 1-m^2
82 MOCNESS or Multiple Closing/Opening Net and Environmental Sensing System (Wiebe et al.,
83 1985, 2002).

84 1.3. Time-series monitoring of the NW Atlantic continental shelf

85 Observations over multiple decades in the GOM, GB, SNE, and MAB regions have
86 revealed population oscillations of *Pseudocalanus* spp. that occur on both seasonal and
87 interannual time-scales (Kane, 2007). In the 1980s, *Pseudocalanus* spp. abundances were low in
88 GOM and GB, but generally higher in MAB, and mixed in SNE; during the 1990s, patterns of
89 abundance differed among the regions; and all regions showed decreased abundances during the
90 2000s (Hare and Kane, 2012; Kane, 2014). In fact, variation in the abundance of the combined
91 species has shown similar patterns throughout the NW Atlantic continental shelf, highlighting
92 the connectivity of the regions and the importance of environmental variation and/or changes in

93 abundances in upstream source regions, e.g., the Scotian Shelf (Kane, 2007, 2014).

94 Annual mean abundances of the combined species have shown exceptional departures
95 from the multi-decadal mean values for 1977 and 2012 (Kane, 2014). A sharp decline in 2002
96 may have been a response of *Pseudocalanus* spp. to unusually cool temperatures during winter
97 and unusually warm temperatures during spring, which may have restricted habitat and also
98 directly or indirectly influenced predator or prey abundances (Friedland et al., 2013; Kane, 2007,
99 2014; Pershing et al., 2005). These interannual abundance patterns were similar to those of other
100 copepod species, including *Centropages typicus, Oithona* spp., and *Metridia lucens*, but were
101 opposite to the patterns for *Calanus finmarchicus* and euphausiids (Pershing et al., 2005). Such
102 multi-species patterns are indicative of community shifts in regional zooplankton assemblages,
103 and may reflect biogeographical or seasonal changes in the timing of peak abundances of key
104 species (Johnson et al., 2011; Pershing et al., 2005; Richardson et al., 2010).

105 Time-series analysis is important for understanding long-term trends in zooplankton
106 abundance, since population fluctuations may both reflect and be used to predict responses to
107 climate change (Morse et al., 2017; O'Brien et al., 2013). Accurate characterization of species-
108 level diversity of zooplankton in time-series records is essential for ecosystem assessments and
109 fisheries management on the NW Atlantic continental shelf, which require integrative
110 understanding of how environmental factors and zooplankton biodiversity impact commercial
111 fish stocks (Friedland et al., 2013; Mountain and Kane, 2010; Munk, 1997; Richardson, 2008).
112 Studies have documented important functional linkages between biodiversity of the zooplankton
113 assemblage, lower level trophic dynamics, and the sustainability of commercial fisheries
114 (Johnson et al., 2011; Pershing et al., 2005). Georges Bank is an important region for larval cod
115 and haddock recruitment (Mountain and Kane, 2010; Richardson et al., 2010), which is known to

116 be related to GB zooplankton abundance trends (Buckley and Durbin, 2006; Pershing et al.,
117 2005; Petrik et al., 2009, 2014).

118 1.4. Importance of discriminating cryptic species

119 Accurate identification and discrimination of cryptic species, which cannot be
120 discriminated morphologically but are reproductively isolated, have important implications for
121 biodiversity assessments, ecosystem management plans, and conservation planning (Bickford et
122 al., 2006). Despite the lack of diagnostic morphological characters, cryptic species may differ in
123 their roles in the ecosystem, relationships to ecological and environmental variation, and long-
124 term responses to anthropogenic impacts, including climate change (Chenuil et al., 2019). A
125 number of studies that have examined and emphasized the importance of cryptic species in
126 biodiversity assessments, including impacts on measures of species richness (Fišer et al., 2018;
127 Gotelli and Colwell, 2001; Pfenninger and Schwenk, 2007). Our inability to discriminate
128 closely-related and cryptic species may prevent detection of their differing responses to
129 environmental variability and change, and we may thus not recognize ongoing change in ocean
130 ecosystems.

131 This study seeks to characterize patterns of variation in the distribution and abundance of
132 two species of *Pseudocalanus*, *P. moultoni* and *P. newmani*, on Georges Bank, NW Atlantic
133 Ocean, in Spring from 1995 to 2012. Quantitative PCR (qPCR) protocols were designed to allow
134 accurate measurement of species-specific abundances. Time-series and multivariate statistical
135 analyses were used to examine interannual patterns of variation in the abundances of the two
136 species and to evaluate the relationship to temperature and salinity. The overarching goal of this
137 study is to demonstrate the power of molecular techniques to discriminate cryptic species of
138 marine zooplankton, and the usefulness of this approach for advancing our understanding of the

139 responses of pelagic ecosystems to environmental variability and climate change.

140 **2. Methods**

141 2.1. Collection of zooplankton samples from Georges Bank 1995-2012

142 *Pseudocalanus* spp. were identified from a total of 65 samples collected during US GLOBEC
143 and EcoMon cruises between 1995 and 2012 (Table 1; Figure 1). US GLOBEC carried out
144 Broad Scale surveys that sampled pre-selected station locations on GB using a 1-m^2 MOCNESS
145 (Wiebe et al., 1985) equipped with 150 μm mesh nets. US GLOBEC samples used for analysis in
146 this study were collected during May and June 1995-1999 (see https://www.bco-
147 dmo.org/dataset/2334); stations were selected using a stratified random method to select one
148 sample from each of the five zones designated in the US GLOBEC Georges Bank grid (Bucklin
149 et al., 2015). EcoMon samples were collected with 20-cm Bongo nets with 150 μm mesh;
150 alcohol-preserved samples for genetic analysis were collected at a subset of EcoMon Survey
151 stations using a random stratified method
152 (http://www.nefsc.noaa.gov/epd/ocean/MainPage/shelfwide.html). EcoMon samples used for
153 analysis in this study were collected during May and June 2002-2012, although no samples were
154 available from 2008. Due to low sampling densities, fewer than five GB samples were available
155 in some years (Table 1). No samples were available from 2000 and 2001.

156

Table 1. *Pseudocalanus* spp., *P. moultoni,* and *P. newmani* abundances by station. *Pseudocalanus* spp. abundances are from morphological taxonomic analysis of samples collected during US GLOBEC cruises (1995-1999) and NEFSC EcoMon surveys (2002-2012). Individual species abundances were determined from relative proportions calculated based on qPCR reactions of the pooled samples of *Pseudocalanus* spp. Average values were calculated for each year. No samples were available from 2000, 2001, 2005, and 2008.

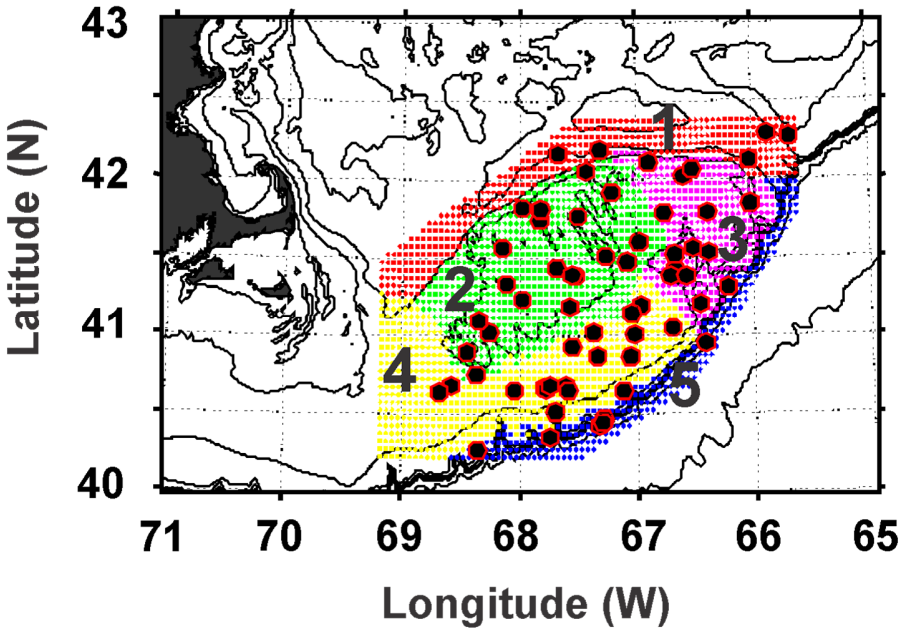
157

Year	Cruise	Station	Zone	Latitude (N)	Longitude (W)	Collection Date	Abundance (Females/10m^2)	*P. moultoni* (N/10m^2)	*P. newmani* (N/10m^2)	Average Total	Average *P.moultoni*	Average *P.newmani*
1995	AL9505	6	4	40.67	-67.79	10-May-95	47,898.1	4,082.2	43,815.9			
1995	AL9505	16	5	40.95	-66.42	12-May-95	7,203.4	27.6	7,175.8			
1995	AL9505	25	1	42.27	-65.68	14-May-95	221,454.1	4,616.1	216,838.0			
1995	AL9505	28	3	42.11	-66.90	14-May-95	44,727.8	42,386.8	2,341.0			
1995	AL9505	32	2	41.74	-67.83	15-May-95	36,858.0	29,382.9	7,475.1	71,628	16,099	55,529
1996	AL9605	7	5	40.46	-67.29	8-May-96	6,311.4	1,052.0	5,259.4			
1996	AL9605	14	4	41.20	-66.96	11-May-96	1,046.7	419.0	627.7			
1996	AL9605	18	3	41.42	-66.67	12-May-96	155,490.8	123,150.3	32,340.5			
1996	AL9605	30	2	41.92	-67.22	14-May-96	14,947.8	14,177.8	770.0			
1996	AL9605	39	1	42.12	-66.03	13-May-96	21,011.8	0.0	21,011.8	39,762	27,760	12,002
1997	AL9707	6	4	40.66	-67.77	20-Jun-97	14,561.4	9,850.8	4,710.7			
1997	AL9707	7	5	40.47	-67.29	20-Jun-97	1,215.2	1,017.4	197.8			
1997	AL9707	11	2	41.23	-67.98	21-Jun-97	112,228.8	94,408.0	17,820.8			
1997	AL9707	17	3	41.21	-66.46	22-Jun-97	181,101.1	176,760.5	4,340.6			
1997	AL9707	40	1	42.17	-67.67	25-Jun-97	306,741.7	250,789.0	55,952.7	123,170	106,565	16,605
1998	AL9806	7	5	40.43	-67.32	15-May-98	778.8	321.2	457.6			
1998	AL9806	12	2	41.39	-67.53	16-May-98	243,082.8	197,522.8	45,560.0			
1998	AL9806	15	4	41.05	-66.69	16-May-98	1,354.6	290.3	1,064.3			
1998	AL9806	18	3	41.39	-66.71	17-May-98	263,251.8	147,209.8	116,041.9			
1998	AL9806	25	1	42.29	-65.87	18-May-98	132,868.1	5,370.5	127,497.6	128,267	70,143	58,124
1999	AL9906	7	5	40.45	-67.30	16-Jun-99	3,213.1	2,197.4	1,015.8			
1999	AL9906	8	4	40.87	-67.06	17-Jun-99	13,656.2	3,344.2	10,312.0			
1999	AL9906	19	2	41.60	-66.98	19-Jun-99	196,983.2	62,861.3	134,121.9			
1999	AL9906	21	3	41.54	-66.39	19-Jun-99	246,121.1	108,170.3	137,950.8			
1999	AL9906	40	1	42.17	-67.67	22-Jun-99	737,138.0	117,179.2	619,958.8	239,422	58,750	180,672
2002	AL0206	75	4	41.02	-67.37	31-May-02	74,589.0	1,576.0	73,013.0			
2002	AL0206	84	3	41.79	-66.39	1-Jun-02	280,871.0	278,788.3	2,082.7			
2002	AL0206	85	3	41.79	-66.76	1-Jun-02	296,481.2	266,145.3	30,336.0			
2002	AL0206	93	2	42.05	-67.43	2-Jun-02	143,924.1	97,565.1	46,359.0	198,966	161,019	37,948
2003	DL0305	3	2	41.34	-68.11	25-May-03	12,292.1	5,031.6	7,260.5			
2003	DL0305	6	2	40.75	-68.37	25-May-03	98,930.9	63,837.2	35,093.7			
2003	DL0305	15	2	41.01	-67.02	26-May-03	235,292.6	208,354.7	26,938.0			
2003	DL0305	28	3	42.03	-66.60	27-May-03	108,593.0	32,459.8	76,133.2	113,777	77,421	36,356
2004	AL0405	53	4	40.68	-67.61	30-May-04	10,698.0	6,149.8	4,548.2			
2004	AL0405	59	4	40.69	-68.58	30-May-04	17,140.6	10,414.7	6,725.8			
2004	AL0405	81	3	41.31	-66.22	31-May-04	40,253.9	1,702.6	38,551.3			
2004	AL0405	84	3	41.85	-66.02	1-Jun-04	61,963.5	21,211.4	40,752.0	32,514	9,870	22,644
2006	AL0605	65	2	41.02	-68.26	8-Jun-06	24,221.9	5,247.0	18,975.0			
2006	AL0605	73	4	40.65	-68.05	8-Jun-06	451,845.4	71.6	451,773.8			
2006	AL0605	81	3	41.39	-66.58	9-Jun-06	42,260.2	1,102.1	41,158.2			
2006	AL0605	83	3	41.57	-66.52	9-Jun-06	14,244.2	10,002.7	4,241.5			
2006	AL0605	98	2	41.77	-67.50	9-Jun-06	18,207.7	5,025.4	13,182.3	110,156	4,290	105,866
2007	DL0706	70	5	40.27	-68.35	30-May-07	10,417.5	796.0	9,621.5			
2007	DL0706	72	4	40.65	-67.58	30-May-07	58,935.3	29,255.6	29,679.8			
2007	DL0706	76	2	41.19	-67.58	30-May-07	7,082.6	7,066.5	16.0			
2007	DL0706	93	3	42.07	-66.52	31-May-07	128,083.8	127,955.1	128.8			
2007	DL0706	96	1	42.19	-67.32	31-May-07	21,509.1	20,877.9	631.3	45,206	37,190	8,015
2009	DE0905	71	2	41.82	-67.98	5-Jun-09	11,431.0	11,097.1	333.9			
2009	DE0905	73	2	41.44	-67.68	5-Jun-09	2,006.2	1,498.5	507.7			
2009	DE0905	75	2	41.48	-67.09	5-Jun-09	123,017.3	122,697.2	320.1			
2009	DE0905	82	4	40.52	-67.70	6-Jun-09	9,054.9	1,334.8	7,720.1	36,377	34,157	2,220
2010	DE1004	73	2	40.90	-68.44	2-Jun-10	475,517.8	276,108.5	199,409.2			
2010	DE1004	77	4	40.65	-67.12	2-Jun-10	17,901.5	4,047.5	13,854.0			
2010	DE1004	93	3	41.52	-66.68	4-Jun-10	290,100.2	281,746.5	8,353.8			
2010	DE1004	98	2	41.56	-68.15	4-Jun-10	569,020.0	509,828.6	59,191.5			
2010	DE1004	110	2	41.81	-67.82	5-Jun-10	46,529.1	12,863.9	33,665.3	279,814	216,919	62,895
2011	DE1105	77	4	40.64	-68.68	10-Jun-11	219,415.4	168,913.7	50,501.7			
2011	DE1105	80	5	40.35	-67.75	10-Jun-11	17,047.9	11,489.4	5,558.6			
2011	DE1105	83	4	40.69	-67.75	10-Jun-11	272,710.1	121,611.4	151,098.8			
2011	DE1105	86	2	41.10	-68.35	11-Jun-11	103,479.0	34,950.5	68,528.5			
2011	DE1105	90	4	41.15	-67.05	11-Jun-11	163,804.9	139,723.1	24,081.8	155,291	95,338	59,954

159 2.2. Design and evaluation of species-specific qPCR protocols

160 Quantitative real-time PCR (qPCR) was used for detection and quantification of relative
161 abundances of each species in pooled samples of *Pseudocalanus* spp. individuals identified to the
162 genus level. Proportions of the two species were determined from qPCR reaction results, and
163 compared to archived data on species abundances. The protocols used were derived from

164



165

Figure 1. Map of Georges Bank and the 65 stations in five zones that were sampled during the US GLOBEC Program during 1995-1999 and the NOAA NEFSC Ecosystem Monitoring (EcoMon) Surveys during 2002 – 2012. Zones are indicated by number and color: Northern Flank (1, Red), Bank Crest (2, Green), Northeast Peak (3, Mauve), Southern Flank (4, Yellow), Slope Water (5, Blue).

166 species-specific PCR (SS-PCR) methods used for identification of individual specimens by
167 Bucklin et al. (2001, 2015). Details of protocol development and optimization are described in
168 Erikson (2015).

169 Pools of 20 female *Pseudocalanus* spp. were identified from ethanol-preserved samples
170 collected from two EcoMon cruises in 2013: GU1302 (Stn 47) and GU1305 (Stn 80). DNA was
171 extracted using the Qiagen DNeasy kit (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).

PCR amplification of the barcode region of the mitochondrial cytochrome oxidase subunit I (COI) gene used primers LCO1490 and HCO2198 (Folmer et al., 1994). The PCR reaction used the Promega Gotaq® Flexi Polymerase kit. 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 purified by gel extraction using the QIAGEN Gel Extraction Kit (Qiagen, Valencia, CA). DNA sequencing was done on an Applied Biosystems Inc. (ABI) 3130 Genetic Analyzer (Life Technologies, Carlsbad, CA) using the BigDye® Terminator (Ver. 3.1) Cycle Sequencing Kit (Life Technologies, Carlsbad, CA).

Species-specific COI primers were designed using PrimerQuest (https://www.idtdna.com/Primerquest) based on DNA sequences for *P. moultoni* and *P. newmani* available on GenBank (http://www.ncbi.nlm.nih.gov/genbank/). DNA sequences were aligned using Molecular Evolutionary Genetics Analysis (MEGA, Ver. 6; Tamura et al., 2013). Primers were consistent with suitability requirements for qPCR amplification were evaluated; a total of five *P. newmani* primers and three *P. moultoni* primers were designed and used for further testing to select primers, determine optimal DNA concentration, and design a qPCR protocol for species-specific amplification. The best-performing primers for *P. newmani* amplified a 118 base-pair product:

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

Newmani 5-R: 5’-GGTACTAATCAGTTGCCAA ATCCC-3’

195 The best-performing primers for *P. moultoni* amplified a 112 base-pair product:
196 Moultoni 1-F: 5'-CTCTAGGGAATTTACGAGTATTTGGA-3' and
197 Moultoni 1-R: 5'-ACCAGCTAACACTGGTAAAGATAA -3'
198 The optimum template DNA concentration was determined to be 0.8 ng-μL^{-1}. The species-
199 specific PCR protocol for *Pseudocalanus* spp. involved: 94˚ C (3 min); 30 cycles of 94˚ C (30
200 sec), 62˚ C (30 sec), and 72˚ (30 sec); and a final extension step of 72˚ C (7 min).
201 A standard curve for calculation of species ratios was created using mixtures of DNA
202 from previously-identified *P. newmani* and *P. moultoni* specimens combined in these
203 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
204 created using ratios of species from the same samples for a variety of samples to account for
205 variance in DNA integrity between samples. Results were plotted as the $\log_{10}$ of the ratio of *P.*
206 *moultoni* to *P. newmani* (equivalent to the ratio of *P. moultoni* to1 minus ratio of *P. moultoni*),
207 versus the ratio of the reported threshold values ($Ct_{P.moultoni}/Ct_{P.newmani}$). All values were within
208 the 95% confidence interval (Figure 2). Species ratios from *Pseudocalanus* spp. DNA were
209 calculated using the linear regression equation with R^2=0.95) determined to be:
210 $Y = -0.1281\ X + 1.0475$, where $X = \log\left(\frac{ratio\ Pmoultoni}{ratio\ Pnewmani}\right) = \log\left(\frac{ratio\ Pmoultoni}{1-ratio\ Pmoutonii}\right)$
211 2.3. Analysis of species-specific abundances based on qPCR
212 Female *Pseudocalanus* spp. were identified from each sample; initial microscopic
213 analysis was used to remove 50 specimens from each sample; the first 50 specimens identified
214 were used for analysis in all cases. The EcoMon samples were split in half using a box splitter
215 (Motoda, 1959) prior to removal of the 50 specimens. Some samples contained fewer than 50
216 individuals, requiring analysis of a smaller number of specimens: DE0905 Stn. 71 (N=21), Stn.
217 73 (N=32); HB1202 Stn. 72 (N=33); and AL9707 Stn. 7 (N=28).

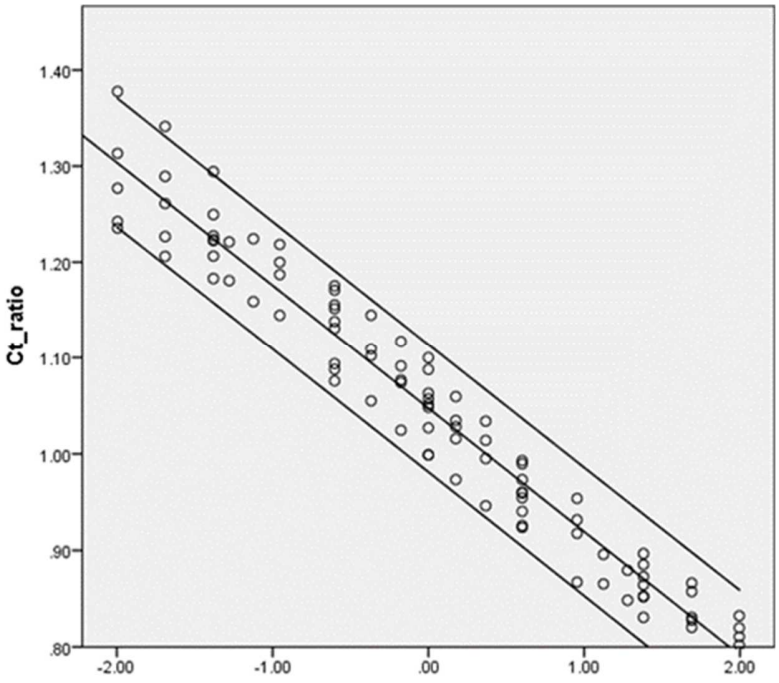


Figure 2. Standard curve to determine proportions of cryptic species by solving for the linear regression equation with 95% confidence interval (R^2=0.95).

DNA was extracted from pooled *Pseudocalanus* spp. for each sample using the Qiagen DNeasy blood and tissue kit (Valencia, CA). DNA concentrations per sample were measured using the Qubit 2.0 and the HS DNA kit (Life Technologies, Carlsbad, CA) and standardized to 0.8 ng-μL^{-1}. Replicate samples and pre-set threshold levels were used to ensure consistent results across multiple qPCR runs.

Critical threshold (Ct) values were used to calculate proportions of *P. moultoni* and *P. newmani* based on the standard curve equation. Abundances were determined using *Pseudocalanus* spp. counts from US GLOBEC and EcoMon data. Counts from all nets (depth intervals) at US GLOBEC stations were summed to represent the whole water column; counts for female, male, and copepodite stage C5 were included for consistency, since taxonomic counts in the EcoMon database do not discriminate these life stages.

229 2.4. Statistical and time-series analysis of species abundances and environmental variation

230 *Pseudocalanus* spp. abundance data were obtained from the EcoMon database (see
231 https://accession.nodc.noaa.gov/0187513). Calculations of the individual species abundances
232 were based up qPCR results (described above) using total counts of the combined species
233 reported as numbers per 10 m^2 for each of the sampled stations. Geometric mean abundance
234 anomalies were calculated as $\log_{10}$ of the observed geometric mean value for each year minus the
235 average $\log_{10}$ geometric mean value for 1995 - 2012. The use of geometric mean values for
236 species-specific abundances for each year reduces the impact of large outlier values.

237 Statistical analysis used functional regression analysis (Ricker, 1973) and associated
238 significance levels (p values) to compare geometric mean abundance anomalies for
239 *Pseudocalanus* spp., *P. moultoni*, and *P. newmani* versus depth-averaged temperature and
240 salinity anomalies calculated from temperature and salinity CTD profiles at each station using
241 data from the NEFSC database (see ftp://ftp.nefsc.noaa.gov/pub/hydro/matlab_files/yearly;
242 Fratantoni et al., 2017). These temperature and salinity values were averaged across all stations
243 sampled each year; anomalies were computed by subtracting the 14-year average from each
244 year's average value. Regression analyses were also done using the depth-averaged temperature
245 and salinity values (not anomalies) for the geometric mean abundance anomalies for the
246 combined species, as well as for *P. moultoni* and *P. newmani*.

247 Non-metric multidimensional scaling (NMDS) analysis (Clarke, 1993) was done using
248 the FATHOM Toolbox for MatLab (Jones, 2017) to examine trends in the patterns of variation
249 of the geometric mean abundance of the species in relation to temperature and salinity. The
250 importance of the different variables in driving the observed patterns of variation were visualized
251 by plotting the abundances of the two species (numbers per 10 m^2) in scaled bubbles in the two

MDA dimensions. Relationships between species-specific abundances and temperature and salinity were further examined using regression analysis.

3. Results

3.1. Calculation of species-specific abundances using qPCR

The standard curve calculated from quantitative PCR (qPCR) detection of cryptic species based on analysis of mixtures of DNA from previously-identified *P. newmani* and *P. moultoni* specimens, with all values within the 95% confidence interval (Figure 2), allowed reliable estimation of individual species abundances from the morphological taxonomic count data for *Pseudocalanus* spp. available from the U.S. GLOBEC and NOAA NEFSC EcoMon cruise reports and databases.

3.2. Time-series analysis of *Pseudocalanus* spp. and species-specific abundances

Geometric mean abundances during May-June of *Pseudocalanus* spp., as well as *P. moultoni* and *P. newmani*, varied interannually by an order of magnitude or more on Georges Bank between 1995 and 2012 Table 1; Figure 3A). The geometric mean abundance anomalies differed between *P. moultoni* and *P. newmani* for most years, and showed clear patterns of interannual variation over the sampled period of 1995 – 2012; functional regression analysis revealed the lack of significant relationship between these abundance measures for the two species, although values for each species were significantly correlated to that of the combined species, *Pseudocalanus* spp. (*P. moultoni* vs. *P.* spp; R^2=0.53, P=0.003; *P newmani* vs *P.* spp; R^2=0.52; P=0.004; Figure 3B). There was no significant relationship between interannual mean abundances of *P. moultoni* and *P. newmani* (R^2=0.08, P=0.34; Figure 3C).

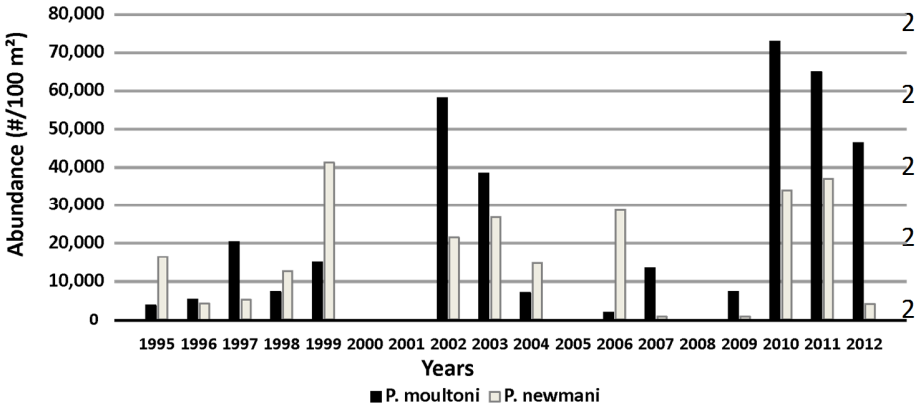


Figure 3A. Geometric mean abundances (numbers per 100 m^2) for *Pseudocalanus moultoni* and *P. newmani*.

Thi s study demonstrated that *P. moultoni* and *P. newmani* exhibited species-specific responses to environmental variables during 1995 – 2012. Regression analysis of the species' geometric mean abundance anomalies and depth-averaged temperature anomalies at each collection location revealed a significant positive relationship between *P. moultoni* and temperature (R^2=0.35, P=0.03), but neither *Pseudocalanus* spp. (R^2=0.14, P=0.19) nor *P. newmani* (R^2=0.01, P=0.79) abundances were significantly correlated with temperature (Figure 4A). Regression analysis of the species' geometric mean abundance anomalies and depth-averaged salinity at each collection location revealed no significant relationship with salinity for *Pseudocalanus* spp. (R^2=0.19, P=0.12), *P. moultoni* (R^2=0.07, P=0.35), or *P. newmani* (R^2=0.18, P=0.13; Figure 4B). Depth averaged temperature and salinity measured at each station were not significantly related based on regression analysis (R^2=0.00, P=0.95; Figure 4C). The same set of regression analyses of species' geometric mean abundance anomalies versus depth-averaged temperature and salinity (actual measurements, not anomalies) at each collection location revealed the same patterns, with the only significant relationship between *P. moultoni* and temperature (R^2=0.35, P=0.03; not shown).

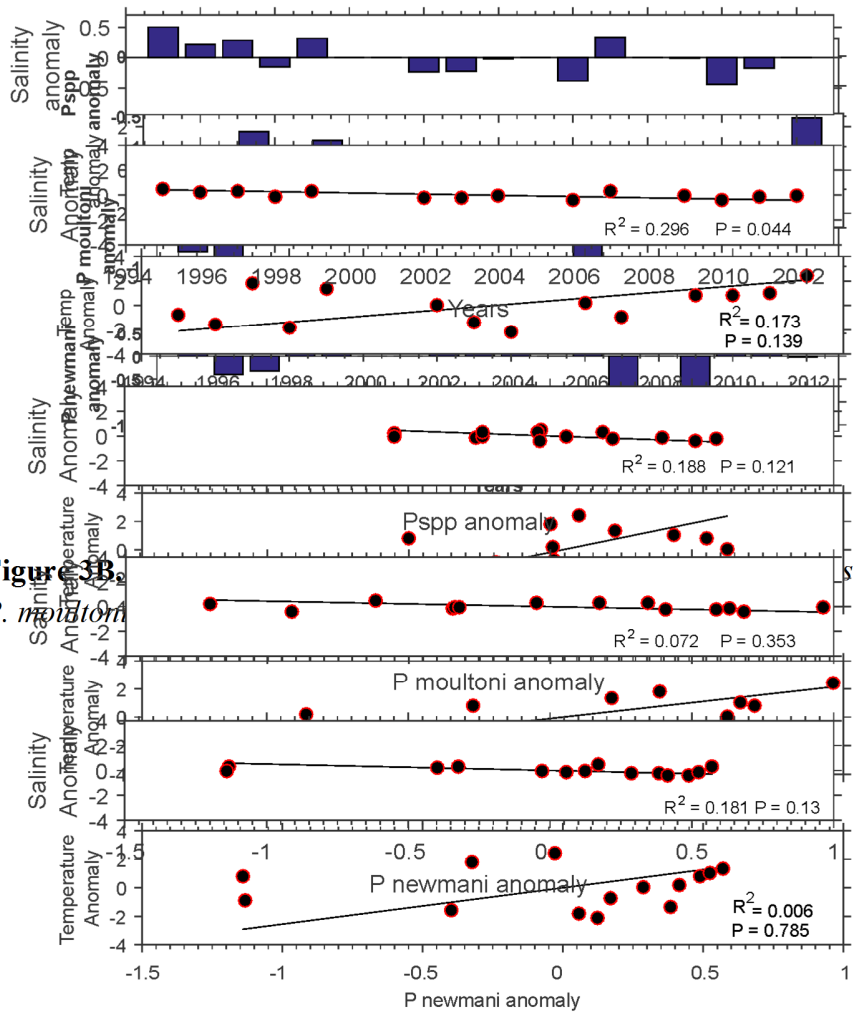


Figure 3B. spp., *P. moultoni*

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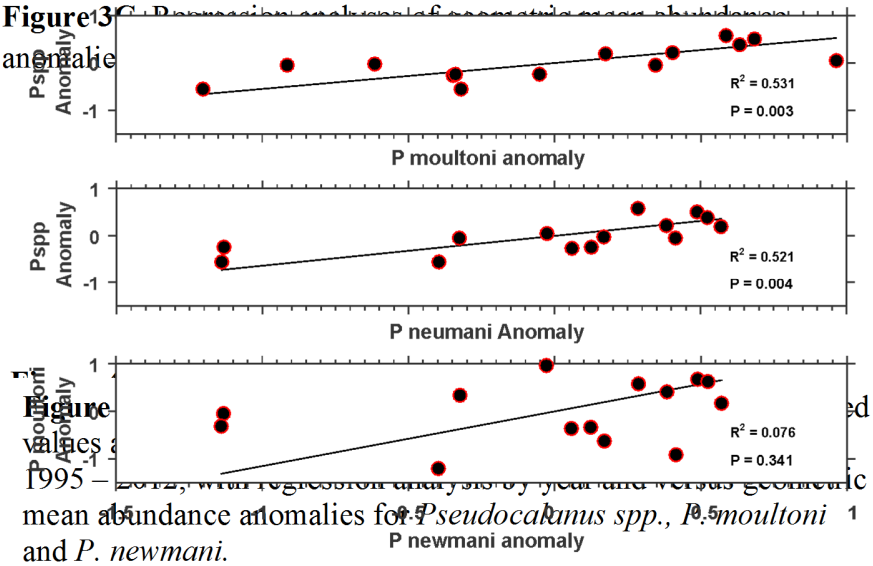

Figure ed values a 1995 – 2012, with regression analysis by year and versus geometric mean abundance anomalies for *Pseudocalanus spp., P. moultoni* and *P. newmani.*

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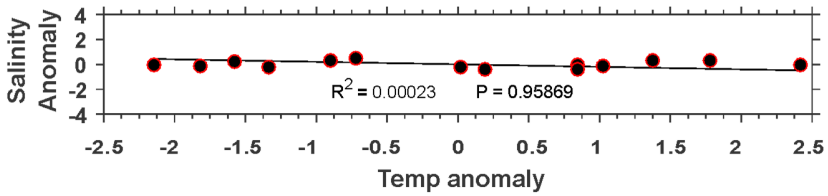

Figure 4C. Regression analysis of interannual variation in temperature versus salinity anomalies, based mean depth-averaged values at all stations each year where samples were collected during 1995 – 2012.

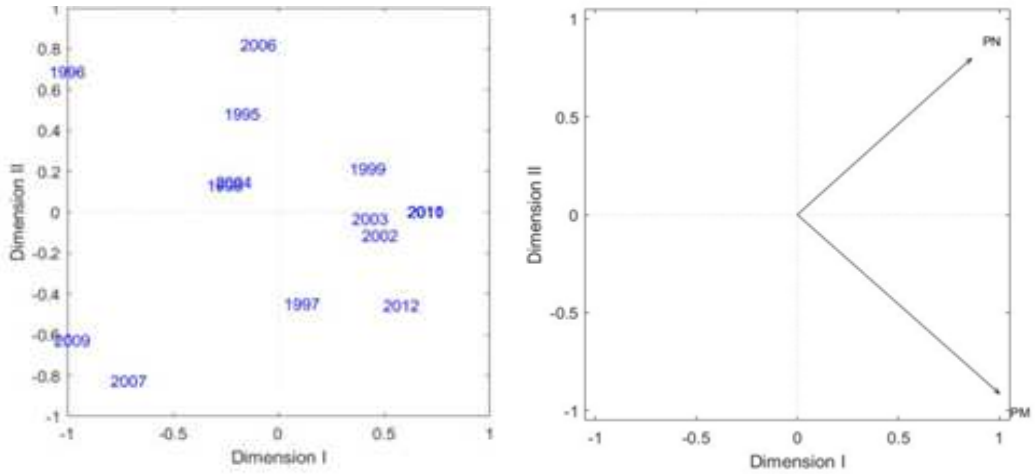

Figure 5. A) Vector analysis showing patterns and drivers of interannual variation in the two NMDS dimensions (Axis I and II) for the geometric mean abundance anomalies of *Pseudocalanus moultoni* and *P. newmani*. NMDS stress criterion = 0.0430. **B)** Vectors indicate trends based on regression analysis of the geometric mean abundance anomalies for each species versus the two dimensions (Axis I and II).

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299 **5A)**

300 **5B)**

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302 **6A)**

303 **6B)**

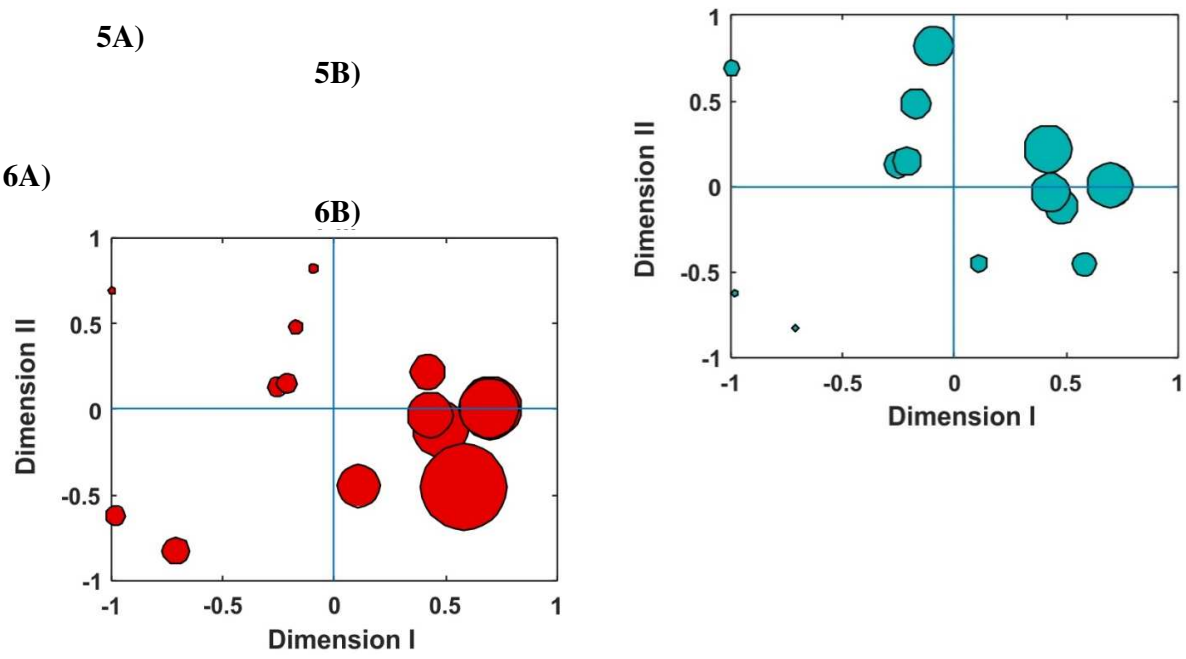

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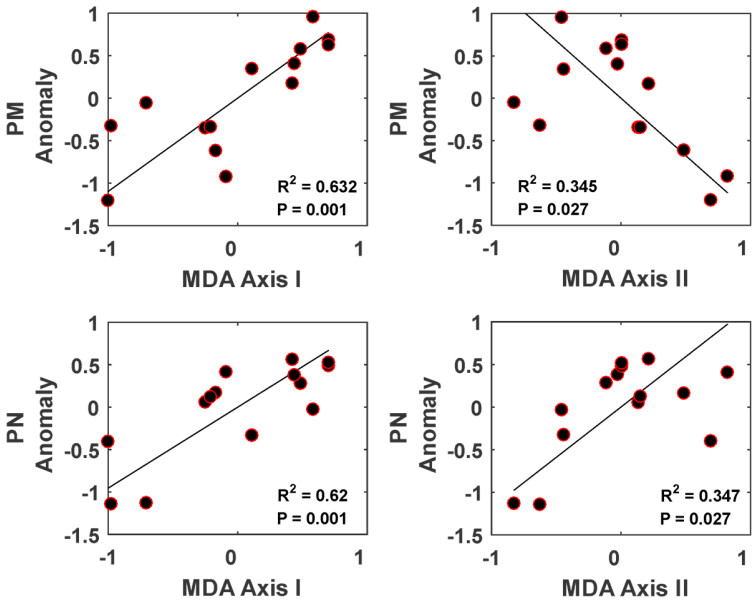
Figure 6. NMDS plot (see Figure 5A) with bubbles proportionally scaled to mean annual abundances (number per 100 m^2) for each species: **A)** *Pseudocalanus moultoni* and **B)** *Pseudocalanus newmani*. Diagrams are designed to allow visualization of species-specific differences in interannual patterns of variation during 1995 - 2012.

306 **7A)**



307 7B)

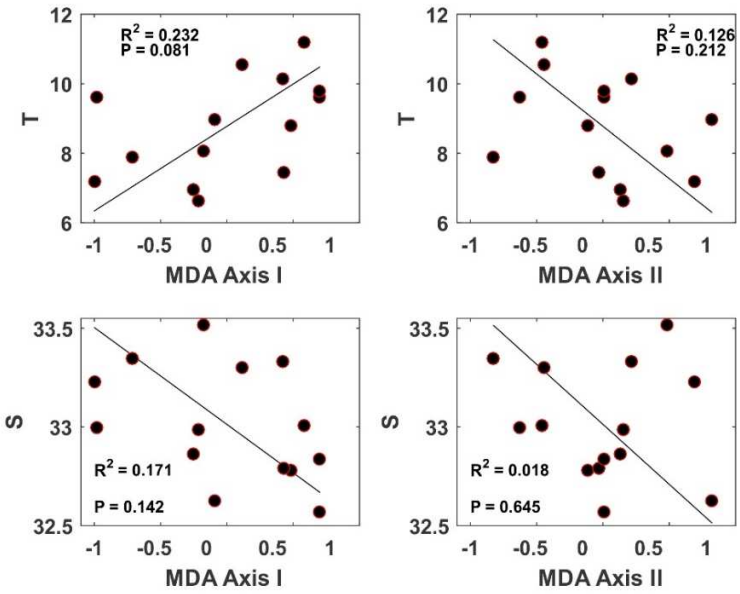


Figure 7. Regression analyses of geometric mean abundance anomalies by year for *Pseudocalanus moultoni* and *Pseudocalanus newmani* versus vectors for the two dimensions from NDMS analysis. **A)** Both species show significant positive trends on Axis I. For Axis II, the *P. newmani* trend is positive; the *P. moultoni* trend is negative. **B)** Annual mean depth averaged temperature and salinity anomalies show different trends on Axis I: positive for temperature and negative for salinity; both variables have negative trends on Axis II.

4. Discussion

4.1. Species-specific abundances and relationships to environmental variation

Comparative analysis of species abundances and environmental variables has been used to gain insight into the underlying causes and drivers of marine zooplankton community composition and change. Environmental drivers, including temperature and salinity, can cause increases or decreases in marine populations and may lead to shifts in biogeographic ranges of species and assemblages; predicted rates of such range shifts in the Georges Bank area are expected to reach 4-6 km/year in future decades (Pereira et al., 2010). However, there are differences among species in their responses to temperature change that impact how taxa and associated assemblages respond; not all species' ranges move poleward under warming (Burrows et al., 2011). Range shifts can be species-specific, but they can also occur across an entire assemblage within a given area or water column depth layer in response to temperature (O'Brien et al. 2013; Pinsky et al., 2013).

Temperature is a primary environmental factor impacting zooplankton species abundance, and may play an especially important role at the biogeographic boundaries and physiological tolerance limits of the species. Due to their sensitivity to environmental variables, including temperature, and short life spans, copepods can act as indicator species for climate variability (Richardson, 2008). Recent studies have reported a variety of relationships among abundances of *Pseudocalanus* species and environmental variables, including temperature, salinity and chlorophyll (Kitamura et al., 2018; Musialik-Kosarowska et al., 2018).

On the NW Atlantic continental shelf, *Pseudocalanus* spp. are exposed to temperatures ranging seasonally from 3˚ to 22˚ C, with an average increase of 1^{o} C over the last 100 years (Friedland et al., 2013). Significant negative correlations between annually averaged abundance

of *Pseudocalanus* spp. and temperature on GB during 1977 - 2012 were described by Kane (2014). Prior to the 1990s, peak copepod abundance was found during March-April; since the 1990s there has been a sharp decline in copepod abundance during spring, with highest numbers found in May-June (Kane, 2014). Our study found no significant correlation between *Pseudocalanus* spp. abundances (geometric mean anomalies) during May-June and averaged temperature anomalies (Figure 4A), although the species-specific abundance of *P. moultoni* did show a significant positive relationship, indicating the necessity of discriminating the cryptic species to understand and predict their responses to variations in temperature.

Stress analysis experiments involving temperature conducted by Stegert et al. (2010) showed that *Pseudocalanus* spp. exhibited positive population growth at temperatures less than 18˚C, but higher temperatures resulted in increased mortality and negative population growth. Model predictions of *Pseudocalanus* spp. populations indicate decreased growth, especially in southern regions, with warming of NW Atlantic waters through 2050 (Stegert et al., 2010). The genus-level responses of *Pseudocalanus* to environmental forcing described by Stegert et al. (2010, 2012) are important, but responses to temperature and other environmental variables are likely to be species-specific (Smith et al., 2019) and studies of the combined species may fail to detect population changes and responses to environmental conditions. Species-level variation and oscillations in relative abundance may impact the entire pelagic community and trophic web (Pershing et al., 2005; Richardson, 2008).

Questions of the relationship between salinity and the abundances of either the combined or individual species of *Pseudocalanus* remain. In this study, *Pseudocalanus* spp. abundance anomalies for May – June of 1995 – 2012 were not significantly correlated with salinity, despite the correlation between temperature and salinity (Figure 4C). Annually-averaged *Pseudocalanus*

354 spp. abundance anomalies showed negative correlations with temperature and salinity at the
355 southern limits of the combined species range (Kane, 2014). This salinity correlation with
356 *Pseudocalanus* spp. abundance disappeared when analyzed with different statistical techniques,
357 over a longer time scale, and with chlorophyll as a proxy (Hare and Kane, 2012; Ji, 2013).
358 Salinity variation on Georges Bank may reflect complex water column dynamics: Pershing et al.
359 (2005) proposed links between decreased salinity and increased stratification, driving increased
360 primary production and zooplankton species abundance in the GOM.
361 *Pseudocalanus* spp. are herbivorous and various phytoplankton taxa (including diatoms
362 and dinoflagellates) have been found to be positively correlated with *Pseudocalanus* spp.
363 abundance in some regions of the NW Atlantic, which has been considered to indicate potential
364 control caused by climate forcing (Davis, 1987; Ji et al., 2013; Kane, 2014). Ji et al. (2013)
365 showed through a modeling study that, when phytoplankton concentration was increased and
366 decreased by 20 percent, a relatively uniform spatial increase and decrease in *Pseudocalanus*
367 spp. abundance occurred across the Gulf of Maine and Georges Bank.
368 Differences in temporal and spatial patterns of distribution and abundance between the
369 two species have been hypothesized to result from species-specific patterns of population
370 dynamics (including growth and mortality), variable responses to environmental parameters, and
371 differences in micro-habitat preferences, among others (Bucklin et al., 2015; McGillicuddy and
372 Bucklin, 2002). Clearly, understanding interactions of *Pseudocalanus* spp. with environmental
373 variation over extended time periods requires discrimination of the congeneric and co-occurring
374 species, which are known to have different habitat preferences (Bucklin et al., 2001, 2015).
375 4.2. Further considerations of time-series analysis
376 There are several important factors to consider regarding best practices for time-series

377 analysis (Chatfield, 2009), including both the length of time examined and the frequency of
378 observations. The relationships between the two *Pseudocalanus* species abundances and
379 environmental variables could possibly result from the selection of years for which samples were
380 analyzed. Time-series analysis of the geometric mean abundance anomalies of the combined
381 *Pseudocalanus* spp. on Georges Bank for 1995-2012 revealed significant interannual variation,
382 although little evidence of multi-year trends (Figure 3B). The years examined in this study were
383 mostly within an extended period of low abundance of *Pseudocalanus* spp. on GB during 1999-
384 2012, based on analysis of the combined species for 1977 – 2012 by Kane (2014). The
385 differences between the results of this study of interannual variation during 1995 – 2012 and the
386 patterns reported by Kane (2014) for 1977 – 2012 may be a consequence of the differing periods
387 and durations of observations analyzed in each case.

388 Analysis of patterns of variation in species abundances and environmental variables are
389 also strongly influenced by the temporal and spatial resolution of sampling. This study sought to
390 characterize interannual variation based on collections at 4 – 5 stations during the May – June
391 peak abundance reported for the target species on GB. The stations were selected to represent
392 possible variation in environmental conditions among GB regions, based on regions used to
393 guide sampling during the US GLOBEC study. This sampling design was intended to improve
394 accuracy and reliability of the data analysis focused on interannual variation. Analysis of
395 additional samples from each cruise and additional cruises each year would allow more detailed
396 estimation of variation on shorter temporal and smaller spatial scales.

397 4.3. Implications for ecosystem responses to climate change

398 Species-level responses to environmental variation and climate change in ocean
399 ecosystems are difficult to predict, but we can hypothesize that the positive relationship between

400 *P. moultoni* abundances and temperature in the time-series records analyzed will prove
401 advantageous for the species if significant warming of the NW Atlantic Ocean occurs. Additional
402 analysis is required to understand the complex interactions and impacts of environmental
403 variables (biological and physical parameters), behavioral patterns, and consequences for
404 retention and advective transport (Bucklin et al., 2015; Ji et al., 2013). In the simplest terms, we
405 may expect that *P. moultoni* will increasingly dominate in the Georges Bank zooplankton
406 assemblage in a warming ocean.

407 **5. Conclusions**

408 Quantitative real time PCR (qPCR) is an accurate and cost-effective tool for
409 discriminating cryptic species and estimating their relative abundances. The species-specific
410 primer design, protocol optimization, and determination of the standard curve for calculation of
411 relative abundances yielded a reliable, accurate, and rapid technique that allowed
412 characterization of *Pseudocalanus* species-specific abundances in the context of a time-series
413 ecosystem monitoring program. During 1995 – 2012, *P. moultoni* and *P. newmani* exhibited
414 significantly different patterns of interannual variation in abundances; the species-specific
415 patterns observed also differed from those of the combined species. During 1995 – 2012,
416 interannual patterns of variation in abundance (geometric mean anomalies) of *P. moultoni* were
417 significantly positively correlated with depth-averaged temperatures at the collection locations
418 each year, while *P. newmani* abundances were not. These results strongly support the conclusion
419 that interannual variation in the abundances of the two cryptic species of *Pseudocalanus* reflect
420 different responses by the individual species to varying environmental conditions on the NW
421 Atlantic continental shelf. Discrimination of closely-related and cryptic species of zooplankton is
422 essential for time-series monitoring, on order to allow accurate assessment and prediction of

responses of pelagic ecosystems to environmental variability and climate change.

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7. Author Contributions

Designed research: AB, LBB, DER, NJC, PHW; Performed research: KEC, LLB; Analyzed data: KEC, LBB, PHW; Wrote the paper: KEC, AB

8. Declarations of interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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