ICES Journal of Marine Science

ICES Journal of Marine Science (2017), 74(4), 1159–1169. doi:10.1093/icesjms/fsw192

Contribution to Special Issue: 'Towards a Broader Perspective on Ocean Acidification Research Part 2'

Original Article

Effects of elevated CO_2 and temperature on an intertidal harpacticoid copepod community

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Sarmento, V. C., Parreira Santos, P. J., Hale, R., Ingels, J. and Widdicombe, S. Effects of elevated CO_2 and temperature on an intertidal harpacticoid copepod community. – ICES Journal of Marine Science, 74: 1159–1169.

Received 6 June 2016; revised 30 September 2016; accepted 10 October 2016; advance access publication 10 December 2016.

Warming and ocean acidification have been shown to have significant impacts on marine organisms. However, none studies have addressed the impact of these two stressors on harpacticoid copepod community structure. A mesocosm experiment was conducted to assess the potential interactive impact of different levels of elevated CO_2 and temperature on an intertidal harpacticoid copepod community. Artificial substrate units (ASUs) colonized by meiofauna from the extreme low intertidal zone were exposed to eight experimental treatments (four pH levels: 8.0, 7.7, 7.3 and 6.7, crossed with two temperature levels: 12 and 16 °C). After 60 days exposure communities were significantly affected by both stressors. The dominant harpacticoid species were mainly affected at treatments held at pH 6.7, but with divergent biological response patterns. At pH 6.7 *Tisbe* sp and *Ectinosoma* sp2 exhibited important density reductions, while considerable density increases were observed for *Amphiascus longarticulatus* and *Amphiascoides golikovi*. This study has demonstrated that elevated levels of CO_2 and ocean warming may have substantial effects on the structure of harpacticoid communities. Importantly, the increase in malformations observed at pH 6.7 indicated that we need to consider sub-lethal effects that could have consequences for populations after long periods of exposure.

Keywords: benthos, climate change, meiofauna, ocean acidification, warming.

Introduction

The increasing concentration of atmospheric carbon dioxide (CO_2) is altering the levels of co-occurring stressors, resulting in increasing sea surface temperatures and seawater pCO_2, as well as decreasing the oceans' pH and its level of saturation of carbonate minerals (Feely *et al.*, 2009). Since the beginning of the industrial revolution in the mid-eighteenth century, the release of CO_2 from human activities has resulted in an increase in atmospheric CO_2 concentrations by nearly 40% (Feely *et al.*, 2009). The present Earth's atmospheric CO_2 levels are higher than at anytime in at least the last 800 000 years (Lüthi *et al.*, 2008), and is expected to continue to rise at an accelerating rate (Feely *et al.*, 2009). Globally averaged combined land and ocean surface temperature data show a warming of 0.85 [0.65–1.06]°C over the period 1880–2012 (IPCC, 2014).

Owing to its large volume and the ability of seawater to buffer CO_2, the ocean has absorbed nearly one-third of all the anthropogenic carbon added to the atmosphere, attenuating the overall effects (Sabine *et al.*, 2004). However, oceanic uptake of CO_2 has resulted in changes in seawater carbonate chemistry, a process known as "ocean acidification" and the pH of ocean

surface water has decreased by 0.1 units since the beginning of the industrial era, corresponding to a 26% increase in acidity (IPCC, 2014).

The rise in greenhouse gas (primarily CO_2) atmospheric concentration is predicted to continue, with estimates for the year 2100 ranging from 475 to 1313 ppm (IPCC, 2013). Best estimates of ocean warming in the top 100 m of the water column are about 0.6–2.0 °C by the end of the 21st century (IPCC, 2013). However, an additional warming of global mean surface temperatures is forecasted to reach 2.6–4.8°C by the end of 21st century (IPCC, 2014). As a consequence of ocean CO_2 uptake, an additional drop in ocean pH of 0.3 units by 2100 and 0.7 units by 2250 is predicted (Caldeira and Wickett, 2003).

Climate change is causing alterations to marine ecosystems with impacts that are evident from polar to tropical regions (Harley *et al.*, 2006; IPCC, 2014). Temperature and pH are among the most important environmental factors controlling the distribution, physiological performance, morphology, and behaviour of marine invertebrates (e.g. Pörtner, 2008; Widdicombe and Spicer, 2008; Doney *et al.*, 2009). However, global warming and ocean acidification are the two major consequences of climate change that are being observed.

Environmental stressors can have simple additive effects (both significant, but no significant interaction) or have complex interactive effects where they have synergistic (increased stress) or antagonistic (decreased stress) effects on biological processes (Folt *et al.*, 1999). Despite the well-known controlling influence of temperature on metabolism and development, the interactive effects of ocean warming and CO_2-driven acidification on organisms at community level have been poorly studied and require use of factorial experimental designs.

Harpacticoid copepods are usually the most important meiofaunal group in terms of abundance in phytal areas with high diversity (Giere, 2009). Owing to their high-nutritional value, they are a predominant element in the diet of many fishes of both ecological and economic importance (Huys and Boxshall, 1991). Furthermore, copepods have been extensively used to show natural environmental changes (e.g. Sarmento *et al.*, 2012; Kitahashi *et al.*, 2014) as well as to evaluate different types of human impacts (e.g. Sarmento and Santos, 2012; Costa *et al.*, 2016). Owing to the greater sensitivity that harpacticoid copepods can exhibit in comparison to other dominant meiofaunal groups such as nematodes (Hale *et al.*, 2011; Sarmento *et al.*, 2015), they have been recently suggested as a valuable group for predicting climate changes (Zeppilli *et al.*, 2015). However, no studies on how the combination of elevated seawater CO_2 and temperature will impact intertidal harpacticoid multi-species assemblages are available as yet.

The present study used a mesocosm experiment to assess the potential interactive impacts of different levels of elevated CO_2 and temperature on the fauna from an intertidal zone using the harpacticoid copepod community as a model system to evaluate changes in community structure and species responses.

Material and methods

The meiofauna samples used in this study are from a mesocosm experiment carried out at Plymouth Marine Laboratory (PML) in 2009 (Hale *et al.*, 2011), where intertidal benthic communities were exposed to elevated temperature crossed with different levels of reduction in the pH of seawater. Sample collection and mesocosm experimental set-up were described in detail by Hale *et al.* (2011) and are summarized here.

Material collection

Fifty artificial substrate units (ASU, each one made from four nylon mesh pan scourers tied together, 9 cm ø, 2.5 cm thick) were deployed in a sheltered area of rocky intertidal at Mount Batten, Plymouth, UK (50.3567N, 4.1277W). The area is characterized as a kelp habitat dominated by brown and red algae. The ASUs were attached to the rock between 0.6 and 1 m above lowest chart datum, during the spring low tide on 14 January 2009. They were left for a period of 12 weeks to allow colonization and collected on 8 April 2009. The ASUs were retrieved and transported in plastic bags to the mesocosm facility at the PML 1 h after collection. Once at PML, five ASUs were randomly selected and preserved in 10% formaldehyde solution, to represent the standard invertebrate communities at the start of the exposure period.

Mesocosm experiment

Forty of the remaining ASUs were each placed individually in separate food grade plastic buckets (vol. 6 l) containing seawater at ambient pH and temperature. Each bucket was randomly allocated to one of the eight treatments (four pH levels crossed with two temperature levels), with five replicates for each combination. Control pH was 8.0 (the ambient seawater pH measured at the fauna collection site), and the decreased pH levels used were 0.3 units below ambient (the predicted drop in ocean pH by 2100), 0.7 units below ambient (the predicted drop in pH by 2250, Caldeira and Wickett, 2003) and a pH of 6.7 (to simulate CO_2 storage site continuous point source leakage under already acidified conditions, Blackford *et al.*, 2009). The mesocosm was held at a control temperature of 12 °C (the ambient temperature measured at the fauna collection site) and the elevated temperature treatment was 4 °C above the control (simulating a rise in temperature midway within the range predicted, 2–6.4 °C, as a result of increased atmospheric CO_2 by 2099 (Sokolov *et al.*, 2009). Buckets containing the ASUs were maintained in water baths (five buckets per water bath) and the artificial manipulation of temperature was achieved and regulated by heaters (Hale *et al.*, 2011).

Seawater was bubbled with CO_2 into the header tanks. Each 6-l bucket was continuously supplied with high pCO_2 seawater (8–10 ml min^{-1} using peristaltic pumps) and oxygen was bubbled through the water held within the buckets to assist with maintenance of the correct pH and to increase water mixing and oxygen levels. The monitoring system as described in Hale *et al.* (2011) maintained the nominated pH and temperature treatments throughout the experimental period with little variation (Table 1) and was therefore considered a suitable method for the artificial manipulation of seawater pH and temperature. The natural light regime was approximated using daylight simulation lights within the mesocosm with an average 8-h photoperiod per day. The experiment ran for 60 days, with little variation in the treatment levels (Table 1). During that time, each bucket received 1.68 ml of shellfish feed one time a week to simulate the food availability at the Mount Batten collection site. No tidal simulation was applied during the experiment (Hale *et al.*, 2011).

Table 1. Seawater chemistry within a) buckets and b) reservoir tanks during the experimental exposure period (Hale *et al.*, 2011).

	Nominal pH	Temperature (°C)	pH	Sal	TCO_2	TA	pCO_2 (μatm)	Ω_{Ca}	Ω_{Ar}	HCO_3-	CO_3^{2-}
Buckets											
12 °C	8	11.78	7.86	34.88	1858.80	1956.61	729.23	1.59	1.01	1784.99	66.59
		0.35	0.09	0.19	314.04	293.36	160.4	0.3	0.19	275.4	12.5
		0.07	0.02	0.05	72.54	74.23	40.59	0.08	0.05	69.68	3.16
	7.7	11.93	7.66	34.89	2084.49	2155.52	1295.53	1.17	0.75	2031.97	49.03
		0.35	0.08	0.24	331.50	303.58	244.51	0.24	0.15	286.88	10.09
		0.07	0.01	0.06	76.57	76.81	61.87	0.06	0.04	72.59	2.55
	7.3	11.66	7.35	34.94	2181.94	2098.44	2729.23	0.55	0.35	2039.57	23.22
		0.41	0.07	0.13	227.68	228.21	499.36	0.11	0.07	222.75	4.5
		0.09	0.01	0.03	52.59	57.74	126.35	0.03	0.02	56.36	1.14
	6.7	11.53	6.81	34.82	2409.95	1942.01	2268.73	0.16	0.1	1925.14	6.6
		0.38	0.23	0.14	313.33	221.04	3127.42	0.11	0.07	220.99	4.56
		0.08	0.04	0.04	72.37	55.93	791.33	0.03	0.02	55.92	1.15
16 °C	8	16.04	7.85	35.31	1915.97	1984.25	822.18	1.91	1.23	1779.26	80.3
		0.40	0.13	0.26	216.12	226.77	743.4	0.38	0.27	204.34	15.83
		0.08	0.02	0.07	49.92	57.38	188.1	0.1	0.06	51.7	4.01
	7.7	16.01	7.61	35.13	2046.30	2072.27	1422.98	1.21	0.78	1943.53	50.94
		0.63	0.15	0.21	241.83	246.16	388.9	0.29	0.19	233.63	12.18
		0.13	0.03	0.05	55.86	62.29	98.4	0.07	0.05	59.12	3.08
	7.3	15.76	7.37	35.06	2105.32	2051.74	2611.66	0.67	0.43	1980.85	28.01
		0.31	0.1	0.15	254.15	232.74	547.02	0.15	0.1	225.48	6.43
		0.07	0.02	0.04	58.70	58.89	138.41	0.04	0.02	57.05	1.63
	6.7	15.48	6.66	34.99	2423.61	1957.02	3010.36	0.15	0.1	1940.63	6.5
		1.52	0.19	0.18	284.04	25.08	4141.2	0.08	0.05	212.54	3.41
		0.32	0.04	0.04	65.60	54.42	1047.85	0.02	0.01	53.78	0.86
Reservoir tanks											
	8	14.08	7.89	34.95	1930	2018.97	680.76	1.93	1.24	1811.93	81.15
		0.44	0.14	0.10	273.13	307.01	156.68	0.31	0.2	287.81	12.89
		0.19	0.06	0.05	138.22	166.89	85.17	0.17	0.11	156.45	7.01
		15.56	7.98	34.84	1860	1970.58	527.15	2.38	1.53	1715.41	99.82
		0.41	0.14	0.13	257.68	290.3	96.68	0.39	0.25	231.67	16.49
		0.18	0.06	0.07	130.40	157.81	52.56	0.21	0.14	142.08	8.97
	7.7	15.46	7.68	34.82	2086.67	2116.32	1211.65	1.38	0.89	1970.45	57.92
		0.43	0.19	0.12	206.13	225.18	243.27	0.23	0.15	216.55	9.6
		0.19	0.08	0.06	104.31	122.41	132.24	0.12	0.08	117.71	5.22
		14.33	7.63	34.75	2033.33	2084.55	1308.57	1.21	0.77	1957.12	50.53
		0.56	0.18	0.15	287.90	323.71	230.09	0.37	0.23	297.01	15.36
		0.24	0.08	0.08	145.70	175.97	125.07	0.2	0.13	161.46	8.35
	7.3	15.39	7.26	34.86	2156.67	2066.85	3279.5	0.54	0.35	2010.12	22.48
		0.55	0.19	0.13	130.21	141.82	584.35	0.12	0.08	135.28	5.21
		0.24	0.08	0.07	65.90	77.09	317.65	0.07	0.04	73.54	2.83
		14.26	7.35	34.82	2106.67	2043.55	2905.94	0.55	0.35	1984.88	23.14
		0.43	0.2	0.14	264.49	286.94	484.55	0.12	0.08	278.31	5.16
		0.19	0.08	0.07	133.85	155.98	263.4	0.07	0.04	151.29	2.8
	6.7	13.86	6.33	34.76	2686.67	1770.66	243.69	0.05	0.03	1765.47	2.16
		0.44	0.14	0.14	311.7	247	42.29	0.02	0.01	245.84	0.64
		0.19	0.06	0.08	157.74	134.27	22.99	0.01	0.01	133.64	0.35
		15.3	6.34	34.76	2693.33	1763.27	238.83	0.06	0.04	1757.66	2.31
		0.7	0.2	0.16	358.17	220.64	41.23	0.02	0.01	219.47	0.63
		0.3	0.08	0.09	181.26	119.94	22.41	0.01	0.01	119.31	0.34

Values: mean, ±SD, 95% CI.

Sal, salinity; TCO_2, total water carbon dioxide concentration; TA, total alkalinity; $_pCO_2$, partial pressure of carbon dioxide; Ω_{Ca}, calcite saturation state; Ω_{Ar}, argonite saturation state; HCO_3-, bicarbonate concentration; CO_3^{2-}, carbonate concentration.

At the end of the exposure period, the ASUs were removed from the buckets and the resident fauna were extracted. The collected material was passed through two sieves (0.5 mm and 63 μm) to separate the macrofauna fraction from the meiofauna fraction (Somerfield *et al.*, 2007). Results from the macrofauna fraction are published in Hale *et al.* (2011) and meiofauna (major taxonomic groups) and Nematoda community results have been published in Meadows *et al.* (2015). Owing to the high number of meiofauna organisms in each sample, 36 samples (the 4 first replicates per treatment + 4 initial samples; instead of 5) were selected for copepod species identification. Under a stereo microscope, the first 60 copepod individuals were selected from each replicate, placed in Eppendorf tubes and preserved in 75% Industrial Methylated Spirit (IMS). The identification of

Copepoda Harpacticoida (copepodite V and adult stages) was done under a compound microscope (1000× magnification) and identified to species by the analyses of the entire animal following the taxonomic keys of Lang (1948, 1965), Huys *et al.* (1996), and Wells (2007) as well as publications with specific descriptions.

Statistical analysis

Permutational multivariate analysis of variance (PERMANOVA) (McArdle and Anderson, 2001), based on Bray–Curtis dissimilarities of copepod abundance $\log_{(x+1)}$ transformed data, was used to evaluate the impact of different temperatures (factor Temperature) and pH levels (factor pH) on the community structure. For all analyses, 9999 random permutations were used. Pairwise *a posteriori* comparisons (the multivariate version of the *t* statistic) were made for the calculation of significant differences. A similarity percentage (SIMPER) analysis was applied to determine which species were responsible for the dissimilarities among pH and temperatures. The Shannon–Wiener (H', using $\log_2$), Pielou's evenness (J'), and the number of species in each sample (S) were calculated. The population parameters malformed animal ratio, copepodite ratio, ovigerous female, and female/male ratios were also calculated. In the present study, malformations were considered as external morphological abnormalities in important taxonomic characteristics, i.e., when parts of the body do not have the normal or expected shape for a given species (e.g. the number and shape of limbs or thorns). Minor variations in ornamentation were not considered malformations.

Two-way analysis of variance (ANOVA) was used to examine the effects of the different pH and temperatures on the densities of harpacticoid's more abundant species (>2% of total), on the ecological descriptors (S, J', and H') and on population parameters (ratios of copepodites, female/male, ovigerous females, and malformed animals).

PERMANOVA, SIMPER, and Non-metric Multi-Dimensional Scaling (MDS) were applied using the software Primer® 6 with add-on PERMANOVA+ (Plymouth Routines in Multivariate Ecological Researches). The two-way ANOVAs were calculated using the software STATISTICA 12. Distribution normality and homogeneity of variance were checked. The level of significance was set at $p < 0.05$ for all analyses. Confidence intervals of 95% (CI) were used to express the variation of the calculated means. Parametric statistical analysis followed Zar (1996).

Results

A total of 2160 copepod individuals were analysed, 60.09% of which were identified as harpacticoids at the species level, 0.79% were adult cyclopoids, 38.7% were copepodites (total), and 0.42% were broken animals that could not be identified to species level. Among the harpacticoids, 12 families, 33 genera, and 51 species were recorded (Table 2).

Tisbe sp. (37.42%), *Harpacticus obscurus* (11.91%), *Ectinosoma* sp2 (5.45%), *Ectinosoma* sp1 (4.8%), *Amphiascoides* sp1 (4.55%), *Paradactylopodia* sp. (4.49%), *Dactylopusia vulgaris dissimilis* (4.26%), *Ameiropsis mixta* (3.94%), *Amphiascus longarticulatus* (3.25%), *Amphiascoides golikovi* (3.06%), and *Ameira* sp. (1.67%), accounted for ∼85% of total.

MDS ordination analyses indicated marked differences in the structure of copepod community among field and treatment samples. Among treatments, the most important difference was observed between samples maintained at pH 6.7 and those from the other pH treatments (Figure 1).

The pattern illustrated in the MDS ordination (Figure 1) was confirmed by PERMANOVA. Significant differences in the copepod community structures were detected for the factor pH and Temperature, but not for the interaction between the two factors (Table 3).

The pattern of response of copepod community structure to the different pH levels was mainly caused by differences among samples kept at pH 6.7, whereas no differences were detected among control and the other treatments (Table 4).

SIMPER analyses showed that decreases in the density of *Ectinosoma* sp2 and *Tisbe* sp. in samples at pH 6.7 were important to dissimilarities among treatments (Table 5).

SIMPER analyses showed that many species were important contributors to the dissimilarity between communities kept at the two different temperature levels (Table 6).

ANOVA results for species richness, evenness, and diversity showed no significant differences for the factors pH and temperature nor for interaction between the two factors ($P > 0.1$ for all) (Figure 2).

ANOVA results for copepod population parameters showed no differences for female/male ratio ($P > 0.6$ for all comparisons) nor for the percentage of ovigerous females ($P > 0.15$ for all comparisons) for both pH and temperature or interaction between factors. The copepodite ratio showed significant differences for the factor pH [$F_{(3,24)} = 3.12$; $p = 0.045$] and for the factor Temperature [$F_{(1,24)} = 5.41$; $p = 0.029$], but not for their interaction ($p > 0.18$). The *a posteriori* Fisher test indicated that copepodite ratio at pH 6.7 was lower than at pH 7.7 ($p = 0.007$) and higher at 16°C compared with 12°C. Malformed animals ratio showed significant differences for factor pH [$F_{(3,24)} = 3.24$; $p = 0.039$]. The Fisher test indicated that the ratio of malformed animals at pH 6.7 was significantly higher when compared with pH 8.0 ($p = 0.014$), 7.7 ($p = 0.013$) and 7.3 ($p = 0.041$) (Figure 3).

Only few harpacticoid species showed significant differences among treatments, and the majority were sensitive only to pH 6.7 (Figure 4). The two-way ANOVA indicated that *Tisbe* sp. showed significant interaction between the factors pH and Temperature [$F_{(3,24)} = 5.22$, $p < 0.01$]. At pH 6.7, *Tisbe* sp. showed higher densities at 12°C than at 16°C ($p < 0.001$). Moreover, the density of *Tisbe* sp. in samples held at pH 6.7 and 16°C was lower than in the all other treatments ($p < 0.001$). *Ectinosoma* sp2 was sensitive to the pH [$F_{(3,24)} = 6.99$, $p < 0.01$] and Temperature [$F_{(1,24)} = 5.33$, $p = 0.03$] changes, but the interaction was not significant [$F_{(3,24)} = 0.44$, $p = 0.72$]. Results of the *a posteriori* test showed that the density of *Ectinosoma* sp2 was lower at pH 6.7 when compared with all other pH levels ($p < 0.01$ for all comparisons). Considering the factor temperature, *Ectinosoma* sp2 density was higher at 16°C than at 12°C ($p = 0.03$). The species *Amphiascoides* sp1 showed significant differences only for the factor Temperature [$F_{(1,24)} = 5.11$, $p = 0.033$], with higher densities found at 16°C.

Discussion

The results presented here indicate the potential impacts to intertidal copepods that are likely to occur across a range of predicted pH (in the context of global change at highly variable coastal areas and CO_2 storage leakage) and temperature levels (Caldeira and Wickett, 2003; IPCC, 2014). As ocean acidification and warming are the two major consequences that have already been

Table 2. List of Copepoda *Harpacticoida* species from collected at the rocky shore at Mount Batten, Plymouth, UK.

Order Harpacticoida Sars, 1903	
Suborder Oligoarthra Lang, 1944	
Family Laophontidae T. Scott, 1905	**Family Canthocamptidae Brady, 1880**
Laophonte cornuta Philippi, 1840	*Mesochra pygmaea* (Claus, 1863)
Laophonte setosa Boeck, 1865	*Nannomesochra arupinensis* (Brian, 1925)
Laophonte sima Gurney, 1927	**Family Dactylopusiidae Lang, 1936**
Laophonte sp	*Dactylopusia vulgaris dissimilis* Brian, 1921
Laophontinae sp1	*Diarthrodes* sp
Laophontinae sp2	*Paradactylopodia* sp
Paralaophonte brevirostris (Claus, 1863)	**Family Ectinosomatidae Sars, 1903**
Family Miraciidae Dana, 1846	Ectinosomatidae sp1.
Amonardia normani (Brady, 1872)	*Ectinosoma* sp1.
Amphiascoides golikovi Chislenko, 1977	*Ectinosoma* sp2.
Amphiascoides sp1	*Halectinosoma* sp1.
Amphiascopsis sp	*Halectinosoma* sp2.
Amphiascus minutus (Claus, 1863)	*Halectinosoma* sp3.
Amphiascus longarticulatus Marcus, 1974	*Pseudobradya* sp1.
Amphiascus (varians) sp	*Pseudobradya* sp2.
Amphiascus parvus Sars, 1906	*Pseudobradya* sp3.
Amphiascus angustipes Gurney, 1927	*Sigmatidium* sp.
Bulbamphiascus sp	**Family Longipediidae Boeck, 1865**
Delavalia sp	*Longipedia* sp.
Haloshizopera lima Becker, 1974	**Family Normanellidae Lang, 1944**
Paramphiascella sp	*Normanella* sp.
Robertgurneya sp	**Family Harpacticidae Dana, 1846**
Robersonia sp	*Harpacticus obscurus* T. Scott, 1895
Family Ameiridae Boeck, 1865	**Family Pseudotachidiidae Lang, 1936**
Ameira sp	*Idomene purpurocincta* (Norman & T. Scott, 1905)
Ameiropsis mixta Sars, 1907	**Family Peltidiidae Claus, 1860**
Nitocra sp	*Alteutha depressa* (Baird, 1837)
Proameira hiddensoensis (Schäfer, 1936)	*Eupelte* sp.
Proameira thetiensis Pallares, 1982	**Family Tisbidae Stebbing, 1910**
Psyllocamptus (L) *triarticulatus* Lang, 1965	*Tisbe* sp.
	Harpacticoida sp.

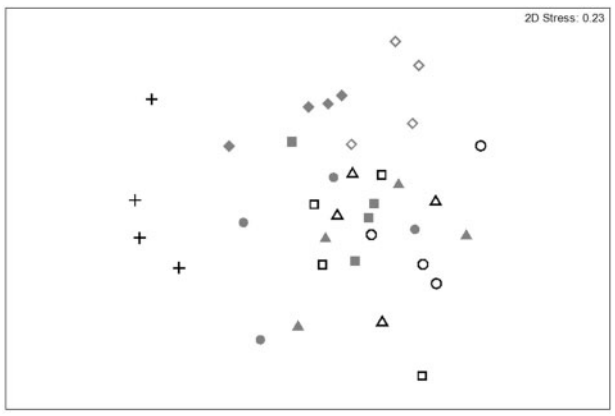

Figure 1. MDS ordination plots for the Bray–Curtis similarity for Copepod community structure. + (cross) Field samples, • (circle) 8.0, ▲ (triangle) 7.7, ■ (square) 7.3, ◆ (diamond) 6.7 (12 °C closed symbols, 16 °C open symbols).

Table 3. PERMANOVA results for the Copepod community exposed to different pH and temperatures.

Source	*Df*	MS	*F*	*P*
Temperature (*T*)	1	3584.5	**2.52**	0.0044
pH	3	3147.1	**2.22**	0.0004
pH × *T*	3	1467.1	1.03	0.422
Residual	24	1420.3		

Significant values are highlighted in bold.

Table 4. Pair-wise *a posteriori* comparisons for pH.

pH comparisons	*t*	*P*
8.0 x 7.7	0.821	0.759
7.7 x 7.3	1.000	0.466
7.7 x 6.7	**1.951**	0.0001
8.0 x 7.3	0.911	0.6551
8.0 x 6.7	**2.066**	0.0001
7.3 x 6.7	**1.839**	0.0002

Significant values are highlighted in bold.

observed in response to increased atmospheric CO_2, organisms are being exposed to these stressors simultaneously in natural ecosystems (Byrne, 2011; Hale *et al.*, 2011; Melatunan *et al.*, 2013). In the present study, harpacticoid community structure was affected by pH and temperature separately, with samples exposed to pH 6.7 showing the strongest effect. Using the same experimental procedures, Meadows *et al.* (2015) found that the total density of copepods was significantly affected by pH and temperature separately, and that copepod abundance at pH 6.7

Table 5. Percent contribution (Contrib. %) of Cyclopoida and species of *Harpacticoida* to average dissimilarity (Diss.) among different pH (Cut off for low contributions: 70%).

8.0 *vs* 6.7		**7.7 *vs* 6.7**		**7.3 *vs* 6.7**	
Diss.= 63.88	**Contrib.%**	**Diss.= 60.36**	**Contrib.%**	**Diss.= 58.61**	**Contrib.%**
Amphiascus longarticulatus	5.84	*Ectinosoma* sp2	6.63	*Ectinosoma* sp2	6.18
Tisbe sp.	5.80	*Tisbe* sp	6.45	*Tisbe* sp	6.02
Ectinosoma sp2.	5.62	*Amphiascoides golikovi*	5.40	*Paradactylopodia* sp	5.51
Dactylopusia vulgaris dissimilis	5.04	*Amphiascoides* sp1	4.89	*Amphiascus longarticulatus*	5.31
Amphiascoides golikovi	4.81	*Delavalia* sp	4.85	*Amphiascoides* sp1	4.66
Laophonte cornuta	4.65	*Amphiascus longarticulatus*	4.82	*Laophonte cornuta*	4.44
Amphiascoides sp1.	4.65	*Laophonte cornuta*	4.70	*Robertgurneya* sp	4.01
Paradactylopodia sp.	4.54	*Ameiropsis mixta*	4.44	*Ameiropsis mixta*	3.68
Normanella sp.	4.09	*Paradactylopodia* sp	4.32	*Pseudobradya* sp2	3.67
Ectinosoma sp1.	4.07	Cyclopoida	4.23	*Ectinosoma* sp1	3.58
Ameira sp.	4.01	*Dactylopusia vulgaris dissimilis*	4.14	*Dactylopusia vulgaris dissimilis*	3.54
Cyclopoida	3.76	*Ectinosoma* sp1	4.01	*Amphiascoides golikovi*	3.52
Ameiropsis mixta	3.22	*Normanella* sp	3.69	*Pseudobradya* sp1	3.25
Pseudobradya sp1.	2.88	*Idomene purpurocincta*	3.39	*Idomene purpurocincta*	3.18
Pseudobradya sp2.	2.86	*Pseudobradya* sp1	3.35	*Ameira* sp	3.17
Laophonte sima	2.84			*Normanella* sp	2.82
				Laophontinae sp2	2.53

Table 6. Percent contribution (Contrib. %) of Cyclopoida and species of Harpacticoida to average dissimilarity (Diss.) between temperatures (Cut off for low contributions: 70%).

12 *vs* 16°C	
Diss.= 57.39	**Contrib.%**
Amphiascoides sp1.	5.18
Paradactylopodia sp.	4.98
Amphiascus longarticulatus	4.81
Ectinosoma sp.	4.68
Amphiascoides golikovi	4.38
Dactylopusia vulgaris dissimilis	4.25
Cyclopoida	4.11
Tisbe sp	4.11
Ameiropsis mixta	4.06
Ectinosoma sp1.	3.83
Laophonte cornuta	3.81
Normanella sp.	3.72
Ameira sp.	3.68
Pseudobradya sp1.	3.42
Delavalia sp.	3.38
Robertgurneya sp.	3.15
Idomene purpurocincta	2.93

was significantly lower compared with other pH levels (Meadows *et al.*, 2015). However, the here presented detailed analysis at lower taxonomic level showed that different harpacticoid species exhibited divergent response patterns highlighting the species-specific nature of responding to stressors.

The direct effects on taxa and the indirect effects through ecological interactions are both important factors influencing the response of a multispecies intertidal community to increasing pCO_2 and warming (Hale *et al.*, 2011; Melatunan *et al.*, 2013). Species interactions may attenuate or amplify the direct effects on individual species (Kroeker *et al.*, 2012). In the present study, harpacticoid species responded mainly to treatments held at pH 6.7. Differences between pH 6.7 and the other pH treatments were caused by important reductions in the densities of *Tisbe* sp. and *Ectinosoma* sp2, followed by a considerable increase in the densities of *A. longarticulatus* and *A. golikovi* at pH 6.7. These changes were also accompanied by differential species response to temperature at this pH, where the densities of *A. longarticulatus* and of *Amphiascoides* sp1 were higher at 16°C and the density of *Tisbe* sp. was higher at 12°C. These results show that in multispecies communities it is likely that certain trade-offs between species will occur under environmental change, with both species that suffer and benefit from the changing conditions altering the resulting communities.

Most of the studies investigating the effects of increased pCO_2 on copepods have found that acidification associated with a wide range of CO_2 concentrations predicted for this century cause no significant effects on mortality, development, metabolism, or reproductive parameters (Mayor *et al.*, 2012; McConville *et al.*, 2013; Vehmaa *et al.*, 2013; Hildebrandt *et al.*, 2014; Li *et al.*, 2015). However, when copepods are exposed to a range of pCO_2 predicted for a future ocean in combination with increasing temperatures they become more sensitive to high pCO_2 with impacts on offspring viability, hatching success, egg production, and metabolic rates (Mayor *et al.*, 2012; Vehmaa *et al.*, 2013; Hildebrandt *et al.*, 2014; Zervoudaki *et al.*, 2014).

Some studies have applied CO_2 concentrations far beyond those expected for the next 100 years as reported by the IPCC reports. However, such predictions are for open ocean conditions and coastal environments already experiencing pCO_2 levels much higher than those predicted under future open ocean scenarios (Blackford *et al.*, 2009; Hofmann *et al.*, 2011). Consequently, these higher treatment levels can still be considered as relevant to future ocean acidification impacts in highly variable inshore systems. At these levels (5000–10,000 ppm CO_2, pH 7.02–6.7), copepods were negatively affected in terms of reproduction but not in terms of mortality, with reductions in hatching success and egg production and with increases in hatching and nauplius mortality (Kurihara *et al.*, 2004a, b; Mayor *et al.*, 2007; McConville *et al.*, 2013).

Contrarily to what was observed for single-species experiments, in the present study, the copepod communities were negatively affected in terms of mortality at these high pCO_2 levels. Under these conditions, the response patterns were complex.

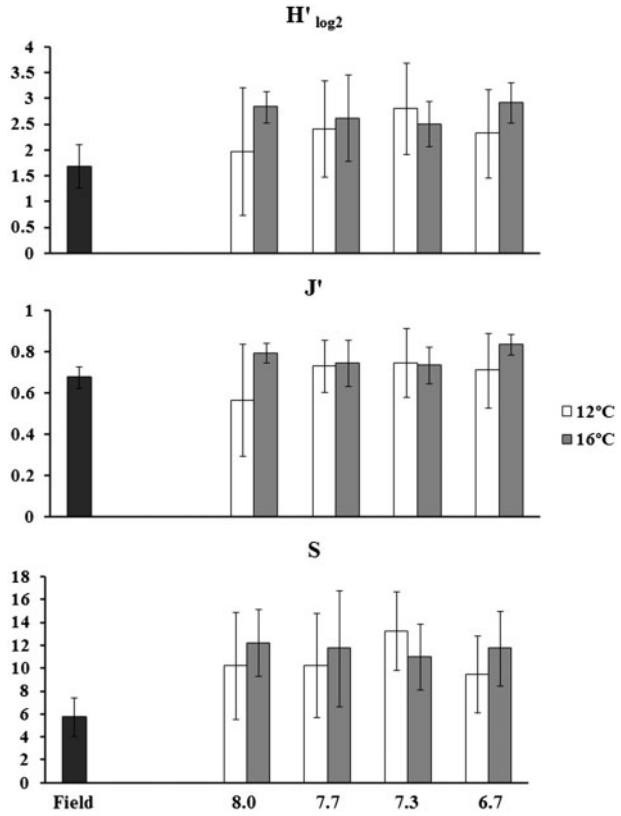

Figure 2. Shannon diversity (H' $\log_2$), Pielou's evenness (J') and species richness (S) for copepod community at different pH and temperatures. Values: mean ± 95% confidence intervals.

Despite the sensitivity of the dominant species *Tisbe* sp, the densities of other species increased, suggesting that the impact of increased pCO_2 in combination with warming can cause responses that are not predictably unidirectional. Our results confirm the increasing consensus in literature that experiments on whole assemblages should be prioritized performed alongside species and population specific studies. The former can reveal complex changes in ecological and biological interactions and present an ecosystem-level view of changes, whilst the latter may provide the necessary information on physiology and ecology of species and population to interpret a more complex system with many ecological interactions. This way, results and predictions from single species studies can be interpreted in the light of multi-specific assemblages (e.g. Kroeker *et al.*, 2013; Gaylord *et al.*, 2015).

In the present study, strong impacts were observed only at the most severe pH treatment, and copepods were not affected at less severe pH reductions. This apparent high tolerance is to be expected considering that communities from temperate intertidal environments experience high variability of abiotic factors in their natural environment and thus, most intertidal animals would have developed effective physiological adaptations for surviving such a highly variable and testing environment. In fact, there have been studies that found no effects of high pCO_2 (pH between 7.78 and 7.2) on copepods from the Arctic or temperate environments and/or from laboratory cultures (Mayor *et al.*, 2012; McConville *et al.*, 2013; Vehmaa *et al.*, 2013; Hildebrandt *et al.*, 2014; Li *et al.*, 2015). It is expected that fauna from habitats characterized by strong abiotic variability (e.g. areas with volcanic emissions in the sea, areas with excessive respiration in confined areas filled with plant and animal life, like rockpools of the intertidal zone, marine sediments, or hypoxic bottom waters) would exhibit greater tolerance to climate change predictions expected for this century (Pörtner *et al.*, 2004). Pascal *et al.* (2010) suggested that among two harpacticoid species, the species (*Shizopera knabeni)* that came from environments that are more

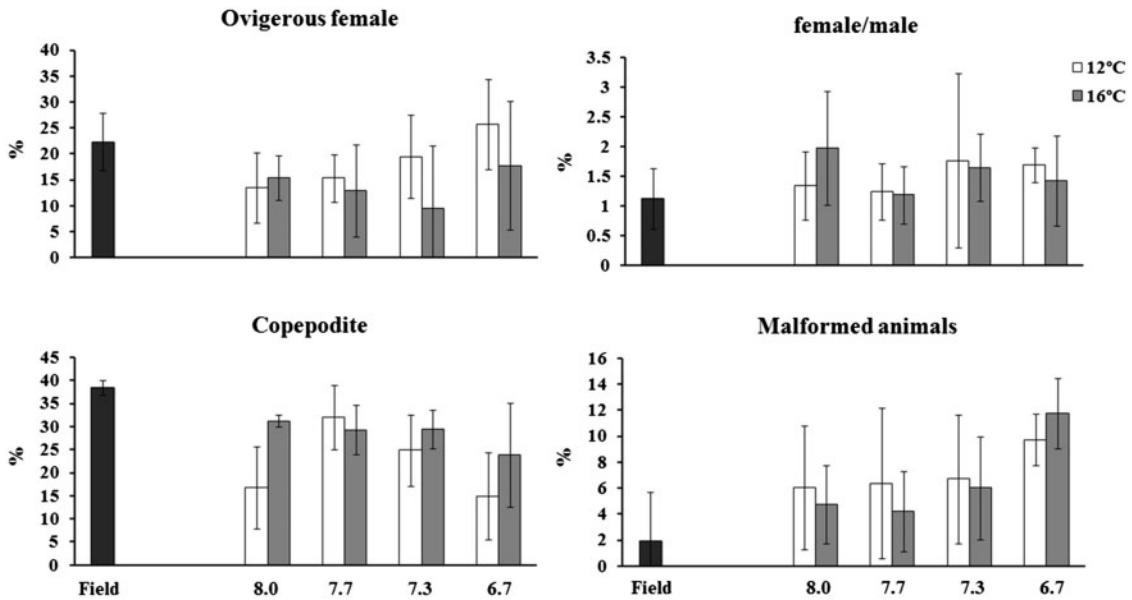

Figure 3. Mean (±95% confidence intervals) of ovigerous female, female/male ratios, copepodite, and malformed animals' ratios at different pH and temperatures.

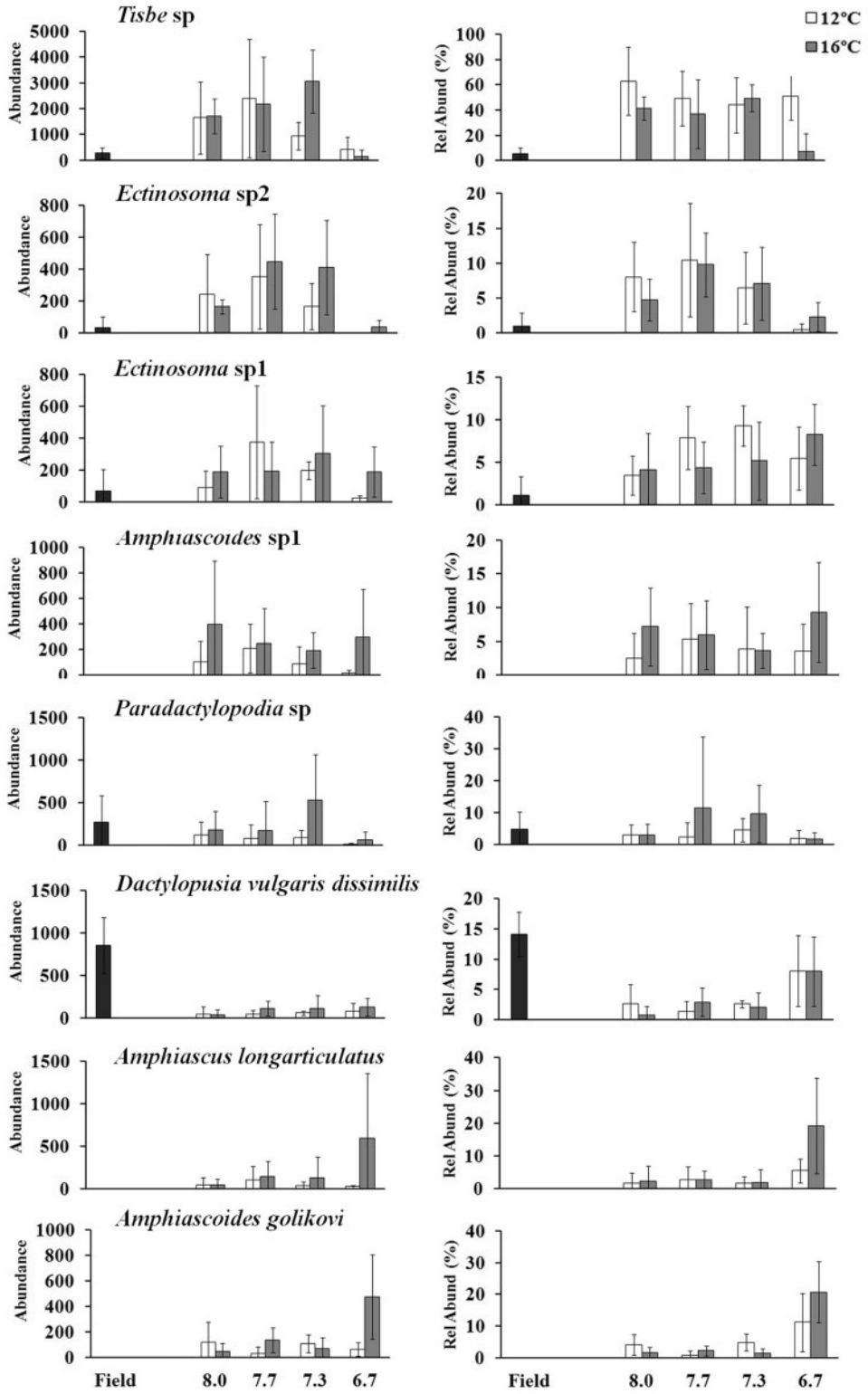

Figure 4. The effects of pH and temperature on the mean abundance and relative abundance (±95% confidence intervals) of the main harpacticoids species.

prone to hypercapnia (e.g. mudflats) was less sensitive to future acidification than *Amphiascoides stopus*, which is found on large grained beaches. Li *et al.* (2015) found that the combination of heat shock and high pCO_2 did not affect the mortality of *Tigriopus japonicus*, a harpacticoid copepod which inhabits a highly variable intertidal environment. However, when exposed to very high CO_2 levels, reduction in egg production rate and hatching success was observed for *Centropages typicus*, but not for *Temora longicornis* (both calanoid copepods). As both species were collected from the western English Channel the results suggest that even species from the same locality and with similar life histories could present different tolerances to ocean acidification (McConville *et al.*, 2013). Additionally, alternative theories suggest that for some of the organisms, particularly if they are from populations at the edges of their geographical distribution, individuals may already be close to their upper tolerance capacity and even a slight increase in pCO_2 could have significant impacts on community structure (Findlay *et al.*, 2010).

Tisbe sp. was the dominant species and the general community response to increased pCO_2 and warming observed was influenced by this species. Species of this genera are characterized by having high fecundity and a short generation time (7–16 days, Williams and Jones, 1994; Pinto *et al.*, 2001), a wide range of body sizes, tolerance to a wide range of environmental changes and by having the ability to grow on different food sources and attaining high-population densities (e.g. 205 ind. mL^{-1}) (Souza-Santos *et al.*, 2006), characteristics that allows *Tisbe* species to be easily reared in the laboratory (e.g. Williams and Jones, 1999; Souza-Santos *et al.*, 2015). However, despite the absence of significant effects on *Tisbe* sp. density at pH 7.7 and 7.3, it is possible—if not likely—that sub-lethal impacts could occur (Fitzer *et al.*, 2012).

In the present study, the positive increase of malformed adult animals with the increased level of warming and pCO_2 is presented for the first time for copepods. This kind of approach has been conducted only for large representatives of macrobenthic species at early development stages. For those animals, an increase in abnormal development in larval and juveniles stages of some coral, molluscs and echinoderms has been correlated to increases in pCO_2 and warming (Byrne, 2011). Since the time difference between the nauplii stage and the last copepodite stage is very short (10 and 18 days) for most of harpacticoid species (Giere, 2009), the evaluation of abnormality at these stages would be a very difficult task, and for studies at community level almost impossible. On the other hand, assessing the presence of malformed appendices in adults during microscope identification does not increase time significantly. The analysis of this parameter indicates that, species that do not suffer mortality in response to high pCO_2 and warming entering the adult stage (like *Tisbe* sp) are not free from sub-lethal symptoms that could have negative consequences for populations after long periods of exposure.

The exposure period of 60 days could be a brief time scale to detect subtle effects for other communities such as macrofauna (Hale *et al.*, 2011). However, owing to the rapid life cycles of harpacticoid copepods, the present results were most probably the response of a natural community exposed to elevated pCO_2 and warming over 6–8 generations (Giere, 2009). A limitation of the present study is the exposure of intertidal communities to artificially constant low pH. Consequently, high priorities for future research should consider the natural variability of pH and temperature that organisms are subjected in field in order to investigate climate change effects on species and community responses through natural and manipulative experiments.

The results presented in this study demonstrated that the combination of elevated levels of CO_2 and ocean warming may have substantial effects on harpacticoid communities from intertidal environments. Moreover, they showed that ecological interactions may lead to complex community responses to pH and temperature changes that cannot be encompassed by single species and/or single stressor experiments.

Acknowledgments

V.C.S. gratefully acknowledges a PhD scholarship from the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco (FACEPE), P.J.P.S. akcnowledges research fellowship (CNPq 306444/2014-3) from the Conselho Nacional de Ciência e Tecnologia (CNPq). J.I. was supported by a Plymouth Marine Laboratory Post-doctoral Research Fellowship in collaboration with University of Exeter and a Marie Curie Intra-European Fellowship within the 7th European Commission Framework Programme (Grant Agreement FP7-PEOPLE-2011-IEF no. 00879). The initial study was undertaken while R.H. was in receipt of a NERC Advanced Course Studentship. Thanks are also owing to anonymous referees for providing comments that improved the manuscript.

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Handling editor: Joanna Norkko