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Variable metabolic responses of Skagerrak invertebrates to low O_2 and high CO_2 scenarios

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Abstract. Coastal hypoxia is a problem that is predicted to increase rapidly in the future. At the same time, we are facing rising atmospheric CO_2 concentrations, which are increasing the pCO_2 and acidity of coastal waters. These two drivers are well studied in isolation; however, the coupling of low O_2 and pH is likely to provide a more significant respiratory challenge for slow moving and sessile invertebrates than is currently predicted. The Gullmar Fjord in Sweden is home to a range of habitats, such as sand and mud flats, seagrass beds, exposed and protected shorelines and rocky bottoms. Moreover, it has a history of both natural and anthropogenically enhanced hypoxia as well as North Sea upwelling, where salty water reaches the surface towards the end of summer and early autumn. A total of 11 species (Crustacean, Chordate, Echinoderm and Mollusc) of these ecosystems were exposed to four different treatments (high or low oxygen and low or high CO_2; varying pCO_2 of 450 and 1300 µatm and O_2 concentrations of 2–3.5 and 9–10 mg L^{-1}) and respiration measured after 3 and 6 days, respectively. This allowed us to evaluate respiration responses of species of contrasting habitats to single and multiple stressors. Results show that respiratory responses were highly species specific as we observed both synergetic as well as antagonistic responses, and neither phylum nor habitat explained trends in respiratory responses. Management plans should avoid the generalized assumption that combined stressors will result in multiplicative effects and focus attention on alleviating hypoxia in the region.

1 Introduction

Resolving the responses of marine organisms to the multiple pressures associated with global change is a major challenge for marine scientists (Duarte et al., 2014). This challenge is particularly pressing for coastal ecosystems, where human populations and impacts often concentrate. Among these pressures, decreasing O_2 concentrations (hypoxia) and ocean acidification (OA) are receiving particular attention (Diaz and Rosenberg, 2008; Vaquer-Sunyer and Duarte, 2008; Doney et al., 2009; Kroeker et al., 2013). Whereas uptake of anthropogenic CO_2, leads to a decreased pH in the open ocean (Caldeira and Wickett, 2003; Doney et al., 2009), explaining fluctuations of pH in coastal waters is more complex, often involving metabolic processes (Duarte et al., 2013). The involvement of metabolic processes in the regulation of pH in coastal waters is particularly evident when eutrophication stimulates algal blooms, leading to increased sedimentation of organic matter, subsequently degraded through microbial respiration, thereby consuming O_2 and releasing CO_2 (Conley et al., 2009). Hence, elevated pCO_2 through excess respiration is associated with reduced O_2 in coastal ecosystems, as these two gases are linked through metabolic processes. Indeed, hypoxia is affecting a growing number of coastal ecosystems (Diaz and Rosenberg, 2008; Vaquer-Sunyer and Duarte, 2008), suggesting that eutrophication-driven acidification (Borges and Gypens, 2010; Cai et al., 2011) should be spreading as well.

Whereas scientists have acknowledged this coupling over decades, the impacts of these two pressures have continued to be studied in isolation, although the synergistic effect of O_2 depletion and CO_2 accumulation is likely to provide a significant challenge to coastal invertebrates and mostly to sessile species.

The consequences of elevated pCO_2 for marine organisms reach further than the highly documented impacts on calcification rates (Doney et al., 2009). For example, extracellular acid–base regulation is a challenge as some organisms are unable to compensate for increased environmental acidity (e.g. Pane and Barry, 2007), which may lead to elevated CO_2 and low pH in their blood fluids. Depending on the severity of the pH change, organisms can experience mortality and a range of sub-lethal effects such as increased respiration, reduced growth, behavioural changes such as compromised ability to avoid predators (see summary by Kroeker et al., 2013) and increased susceptibility to parasites (Burgents et al., 2005). Similarly, hypoxia has been shown to cause mortality, reduced growth and reproduction, decrease respiration rates and induce behavioural changes such as forced migrations, which may make organisms more susceptible to predation (Vaquer-Sunyer and Duarte, 2008). In a review of 460 studies, half showed 50 % population mortality in response to low O_2 that occurred within just five days (Vaquer-Sunyer and Duarte, 2008). There are growing concerns that the combined impacts of elevated pCO_2 and hypoxia may prove to be a more significant challenge for marine life than the predictions from isolated effects (Burnett, 1997; Brewer and Peltzer, 2009; Mayol et al., 2012; Melzner et al., 2013). While other studies have considered combined stressors such as low O_2 and high or low temperature (reviewed by Vaquer-Sunyer and Duarte, 2011), low O_2 and increased hydrogen sulphide (reviewed by Vaquer-Sunyer and Duarte, 2010) or increased pCO_2 and temperature (Doney et al., 2009; Lischka et al., 2011), research focusing on how elevated pCO_2 and low O_2 will impact marine organisms has been a recent focus (e.g. Gobler et al., 2014; Steckbauer et al., 2015; Sui et al., 2016a, b). For example, recent reports have shown that low O_2 and elevated pCO_2 can cause additive impacts for the growth and survivorship of bivalve larvae and later stage clams (Gobler et al., 2014); however, similar research indicates some early life stage bivalves are largely tolerant of these combined effects (Frieder et al., 2014). Moreover, invertebrates along the coastline of Chile show rather additive than synergetic affects to the combination of low O_2 and elevated pCO_2 (Steckbauer et al., 2015).

Here we examine the independent and synergistic impacts of short-term elevated pCO_2 and low O_2 scenarios on the respiration of Skagerrak marine invertebrates at projected future levels, as down-regulation of metabolic rates has been proposed as a short-term evolutionary strategy to balance energy supply and demand when physiological processes are impaired by environmental stresses (Calosi et al., 2013). Elevated pCO_2 and low O_2 impose a significant strain on aerobic animals as the available energy acquired from oxic respiration can be reduced in the presence of increased pCO_2 (Brewer and Peltzer, 2009). This energy could otherwise be directed towards growth, reproduction and other biologically and ecologically important activities. Reduced respiration is known to occur during hypoxia and both increases and decreases have been observed when pH is reduced (e.g. Whiteley, 2011; Wei and Gao, 2012). However, responses are often highly species-specific (Fabry, 2008; Malakoff, 2012; Calosi et al., 2013). Reduced metabolism is a beneficial response for organisms in the short-term but could become problematic over extended periods (Melzner et al., 2009; Rosa et al., 2013), as they may be unable to produce the energy required to support key processes.

We evaluated the respiratory responses of 11 species of Skagerrak marine invertebrates representing four phyla and contrasting habitats, such as shallow rocky shores, typically growing in Baltic waters, and deeper (about 30 m), muddy sediments in Atlantic waters, as well as behavioural strategies, including sessile (e.g. blue mussels) and mobile (e.g. hermit crab, sea stars and sea snails; see Table 1). We used a two-way full factorial design enabling us to resolve additive and interactive effects. We hypothesize that responses could be driven by phyla and/or the habitat or niche the species occupy (Table 1). In particular, we expect all species to be able to cope with elevated pCO_2, as they experience broad fluctuations of pCO_2 in their habitat (Table 1), but should be vulnerable to hypoxia, as they experience high O_2 levels in their habitat, except for those with an infaunal growth habitat, which are expected to be resistant to low O_2 and elevated pCO_2, as in their habitat (Table 1). We also expect calcifiers to be particularly vulnerable to elevated pCO_2 as additional energy to support calcification is required to cope with the reduced saturation state of carbonate minerals associated with elevated pCO_2 (Hendriks et al., 2015).

2 Methods and materials

2.1 Site and location

The experiments were conducted during August 2013 at the Sven Lovén Centre for Marine Sciences in Kristineberg, of the University of Gothenburg, Sweden (58°14′58″ N, 11°26′44″ E). The centre provided access to a diversity of marine life as it is located at the mouth of the Gullmar Fjord. This fjord is home to a mix of habitats with varying complexity and a salinity gradient of three distinct water masses: (1) the surface layer from the Kattegat Sea (salinity 24–27); (2) the more saline mid-waters (32–33) from the Skagerrak; and (3) the salty (34.4) North Sea water mass in the deeper sections of the fjord (Polovodova et al., 2011). The fjord is home to a range of habitats such as sand and mud flats, seagrass beds, exposed and protected shorelines and rocky bottoms, which together with the diversity of water masses results in high biodiversity (Sven Loven Centre, 2011). Natural

Table 1. Species used in the experiment, along with the habitat where they were collected and the characteristic pH and O_2 levels in these habitats. Values of pH and O_2 at the habitats are taken from Dorey et al. (2013), Hu et al. (2014) and Grans et al. (2014).

Species	Sampling site	pH	Oxygen ($mg\,L^{-1}$)
Ciona intestinalis	Mooring rope, surface	Highly variable (8.7–7.6)	High (8)
Pagurus bernhardus	Gravels, 30 m	Variable (8.1–7.7)	High (8)
Littorina littorea	Rocky shore, surface	Highly variable (8.7–7.6)	High (8)
Tarebia granifera	Soft sediment, 30 m, infaunal	Low (7.6)	Low (1.6)
Mytilus edulis	Rocky shore, surface	Highly variable (8.7–7.6)	High (8)
Ophiocomina nigra	Gravels, 30 m	Variable (8.1–7.7)	High (8)
Ophiothrix fragilis	Gravels, 30 m	Variable (8.1–7.7)	High (8)
Amphiura filiformis	Soft sediment, 30 m, infaunal	Low (7.6)	Low (1.6)
Asterias rubens	Rocky shore, surface	Highly variable (8.7–7.6)	High (8)
Psammechinus miliaris	Gravels, 30 m	Variable (8.1–7.7)	High (8)
Brissopsis lyrifera	Soft sediment, 30 m, infaunal	Low (7.6)	Low (1.6)

and anthropogenically enhanced hypoxia both occur within the fjord when enrichment is high and seasonal water exchange over the sill is slow (Josefson and Widbom, 1988; Arneborg, 2004).

2.2 Species, collection and maintenance

Specimens from 11 invertebrate species (Table 1) were collected from either surface or deep water within the Gullmar Fjord. *Ciona intestinalis* and *Littorina littorea* were collected by hand from mooring ropes and rocky shores in the Grunsund boat harbour, respectively. *Asterias rubens* was also collected by hand from the rocky shore at the research station. All other specimens were retrieved with an Agassiz trawl aboard the research vessel *Oscar von Sydow* at up to 30 m depth over both rocky bottom and muddy sediment. *Amphuira filiformis* were collected with a 0.5 m sediment grab at 20 m depth. Only the top 10 cm of sediment from each grab was retained, as this was the oxygenated layer where organisms could be found. All organisms were maintained in flow-through tanks water for at least two days before being placed into experimental aquaria. Water conditions followed the natural fluctuations occurring in the fjord (average pH ~ 8.0, salinity $= 32.1 \pm 0.02$ ranging from 31.5 to 32.7, and temperature $= 16\,^{\circ}C \pm 0.06$ ranging from 14.1 to 17.3 °C, data from http://www.weather.loven.gu.se/en/data, last access: 2 October 2013).

Based on earlier experience in holding these species for experimental purposes, *Pagrus bernhardus* was fed, by allowing them to feed *ad libidum* on blue mussel meat, while being held in the tank prior to the experiment. No animals were fed during the experimental period. *C. intestinalis* and *L. littorea* were placed in plastic mesh cages ($\sim 0.5\,cm^2$) so that they were not lost through the outflow or escaped the aquarium. All gastropods, bivalves and hermit crabs were cleaned with a toothbrush prior to use in order to remove any algae that could alter O_2 concentrations during measurements.

Invertebrates were exposed to one of the four treatments for a maximum of six days. Mortality events were rare across species (7 individuals died out of 168 used in the experiments) and insufficient to allow robust calculations of mortality rates. Of these seven, three specimens (one each of *P. bernhardus*, *P. miliaris* and *A. rubens*) died at the same time in the same aquarium indicating that there was likely an anomaly in the tank, although we could not determine its nature. The other four specimens that experienced mortality were *A. rubens* under $L_{O_2}L_{CO_2}$, and *P. bernhardus, M. edulis*, and *A. filiformis* under $L_{O_2}H_{CO_2}$. Survivorship in the control was 100 %, 97.6 % in $L_{O_2}L_{CO_2}$, and 92.9 % in the $H_{O_2}H_{CO_2}$ and $L_{O_2}H_{CO_2}$ treatment.

2.3 Treatment protocol

Four treatments (three replica aquaria each) with two levels of dissolved oxygen (DO) and pCO_2 concentration were used: (a) $H_{O_2}L_{CO_2}$ – ambient pCO_2 (400 µatm) and high O_2 (100 % saturation or 9–10 $mg\,L^{-1}$); (b) $L_{O_2}L_{CO_2}$ – ambient pCO_2 and reduced O_2 (20–35 % saturation or 2–3.5 $mg\,L^{-1}$); (c) $H_{O_2}H_{CO_2}$ – elevated pCO_2 (~ 1300 µatm) and high O_2; and (d) $L_{O_2}H_{CO_2}$ – elevated pCO_2 and reduced O_2.

The high O_2 aquaria were bubbled with ambient air, whereas the low O_2 aquaria were bubbled with a mixture of air and N_2 using an Aalborg GFC17 Mass Flow Controller (MFC) and a jar filled with glass marbles (allowing even mixing of gases) to create a mixture with reduced O_2 content. This was then bubbled through the six low O_2 treatments maintaining the DO between 2.0–3.5 $mg\,L^{-1}$, which was chosen after the meta-analysis of Vaquer-Sunyer and Duarte (2008) to be a bit higher than the traditional definition of hypoxia by Diaz and Rosenberg (1995, 2008). The DO

content of each aquaria was measured daily with PresSens oxygen micro-optodes (OXY 4 v2.11 Micro) that were calibrated in O_2 saturated deep-sea water ($\sim 10\,mg\,DO\,L^{-1}$ for 100 % DO) and a $1\,g\,mL^{-1}$ sodium sulphite solution ($0\,mg\,DO\,L^{-1}$ for 0 % DO).

To increase the pCO_2, pure CO_2 was bubbled through elevated pCO_2 treatment aquaria. The low O_2 treatments also received CO_2 gas to maintain pCO_2 at an ambient level due to the displacement of CO_2 in the presence of N_2. A reduction of 0.4 pH units (equivalent to 1300 µatm for elevated pCO_2) from the ambient waters (at ~ 450 µatm in low CO_2) was chosen. These values correspond to the annual average atmospheric pCO_2 level for the high-end projected level for 2100 (IPCC, 2007) and for 2005, respectively. The pH was controlled with Aqua Medic pH computers and 2.5 W M-ventil valves. Each pH controller had a sensor attached to the aquarium, which opened the valve to release a burst of CO_2 when the pH was increasing beyond the set level (i.e. 7.6 or 8.0). The pH_{NBS} (NBS scale) was measured daily in all aquaria with a Metrohm 827 pH meter, calibrated at 15 °C (with pH solutions of 3.99, 7.04, and 9.08).

Aquaria were continuously replenished by allowing water to flow through the tanks (filtered through a 20 µm mesh) in a flow-through system with aquaria volume maintained at 17 L. Each replica aquaria held one individual from each species with the exception of *C. intestinalis* and *L. littorea*, which had two individuals per replica tank.

2.4 Carbonate chemistry

The Gran titration method was used to measure total alkalinity (TA) every third day. Two 25 mL water samples were collected from each aquarium and filtered through a 45 µm filter. TA was measured at room temperature with a SI Analytics TitroLine alpha plus machine and TitriSoft 2.6 software. Spectrophotometry was used to measure the pH_{TOT} (total scale) at 25 °C of treatments with a Perkin Elmer Lambda 25 UV/VIS spectrometer and Perkin Elmer UV WinLab software to confirm the values of the daily pH_{NBS} measurements (after Dickson, 2009). TA, pH_{NBS}, with temperature and salinity, were used to calculate the pCO_2, and aragonite and calcite saturation states (Ω_{arag} and Ω_{cal} respectively) in CO2SYS (Pierrot and Wallace, 2006) with K_1 and K_2 constants from Mehrbach et al. (1973; refit by Dickson and Millero, 1987) and KSO_4 from Dickson (1990).

Respiration Index (RI) was calculated after Brewer and Peltzer (2009) as follows:

$$RI = \log_{10}(pO_2/pCO_2), \quad (1)$$

where $RI \leq 0$ corresponds to the thermodynamic aerobic limit, a formal dead zone; at $RI = 0$ to 0.4 aerobic respiration does not occur; the range $RI = 0.4$ to 0.7 represents the practical limit for aerobic respiration, and the range $RI = 0.7$ to 1.0 delimits the aerobic stress zone (Brewer and Peltzer, 2009). Therefore, an RI less than 1.0 represents conditions in which organisms experience a physiological constraint on the free energy available to them to do work, with increasing severity of this constraint as the RI declines.

2.5 Metabolic rate

Respiration was measured on day 3 and 6 of exposure (with the exception of *O. nigra* and *C. intestinalis* which were recorded on days 2 and 4, and 3 and 5, respectively) to detect any change in metabolic rate in response to short-term hypoxia, elevated pCO_2 water or both. Invertebrates were placed in pre-treated water for approximately 5 h (depending on their size) in hermetically sealed containers. DO was measured at the beginning and end of the incubation (max. 5.5 h) using the PresSens micro-optodes. A blank sample was measured to see if there was any natural "drift". Initial and final measurements were used to calculate the consumption rate standardized to dry weight (DW) as $DO\,mg\,L^{-1}\,min^{-1}\,g\,DW^{-1}$. DW was measured after placing the individuals in the dry oven at 60 °C for at least 24 h to remove any moisture. All weight measurements were recorded with a Mettler Toledo AT261 Delta Range analytical balance (readability 0.01 mg).

The response ratio of the respiration rate was calculated as the average metabolism in the experimental treatment (X_E), divided by the average metabolism in the control (X_C). The effect size for each treatment was the ln-transformed respiration rate (Kroeker et al., 2010):

$$\text{Ln Effect Size} = \text{LnRR} = \ln(X_E) - \ln(X_C), \quad (2)$$

where X_E and X_C are the mean values of the response variable in the experimental and control treatments, respectively, where the control treatment was represented by the $H_{O_2}L_{CO_2}$ treatment. Bias-corrected bootstrapped 95 % confidence interval was calculated after Hedges et al. (1999) and Gurevitch and Hedges (1999). The zero line indicates no effect, and significance of mean effects is determined when the 95 % confidence interval does not overlap zero.

2.6 Data analyses

One-way ANOVA's were conducted to test for differences in the respiration rate between treatments for each species. As there was no significant difference between time (i.e. difference between day 3 and 6) all data from day 3 and 6 were pooled together. Where the respiration showed significant differences between treatments, a Student's t test and post hoc Tukey HSD test were conducted to resolve which treatments resulted in different respiration rates. A regression comparison was done to test the overall differences between the treatments. Moreover, a general linear model (GLM) was used to quantify species response to changes in pCO_2, oxygen and their interaction. A significant, positive interaction term indicates synergistic effects between the stressors, while a significant, but negative interaction term implies antagonis-

Table 2. Realized carbonate chemistry and oxygen concentrations for the four treatments ($H_{O_2}L_{CO_2}$, $L_{O_2}L_{CO_2}$, $H_{O_2}H_{CO_2}$, and $L_{O_2}H_{CO_2}$). Values are averages ±SE of measurements and calculations (using CO2SYS). Respiration Index (RI) as defined by Brewer and Peltzer (2009) (see Sect. 2.4 for details).

	$H_{O_2}L_{CO_2}$		$L_{O_2}L_{CO_2}$		$H_{O_2}H_{CO_2}$		$L_{O_2}H_{CO_2}$	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Temperature (°C)	15.4	0.2	15.4	0.2	15.4	0.2	15.4	0.2
Oxygen (mg L^{-1})	9.43	0.06	2.91	0.20	9.60	0.06	3.05	0.24
pH_{NBS}	8.02	0.01	8.06	0.02	7.58	0.03	7.61	0.04
Salinity	32.16	0.12	32.16	0.12	32.16	0.12	32.16	0.12
Total alkalinity (µmol kg^{-1})	2245.9	11.2	2249.5	9.1	2249.5	7.2	2258.8	11.5
pCO_2 (µatm)	591.9	22.4	536.7	26.6	1783.5	131.7	1687.7	146.7
HCO_3^- (µmol kg^{-1})	1975.4	15.9	1951.8	16.7	2143.6	9.0	2144.8	16.6
CO_3^{2-} (µmol kg^{-1})	111.3	3.0	120.4	4.1	44.5	2.9	47.6	3.5
Ω Aragonite	1.73	0.05	1.87	0.06	0.69	0.04	0.74	0.05
Ω Calcite	2.71	0.07	2.93	0.10	1.08	0.07	1.16	0.08
RI	1.60	0.02	1.15	0.03	1.14	0.03	0.69	0.04

tic effects, using the statistical software JMP (version 10.0; https://www.jmp.com, last access: 5 November 2012) with the level for significance set at 0.05.

3 Results

3.1 Water conditions

The average measurements and calculated carbonate chemistry data for the experimental period are shown in Table 2. On average (±SE) the targeted pH levels of 8.04 ± 0.07 in low pCO_2 treatments and 7.59 ± 0.02 in the elevated pCO_2 treatments were achieved, respectively, and significantly different from each other ($p < 0.0001$, ANOVA). The corresponding atmospheric CO_2 levels were higher than our expected target of 380 µatm ($H_{O_2}L_{CO_2}$ and $L_{O_2}L_{CO_2}$) and 1300 µatm ($H_{O_2}H_{CO_2}$ and $L_{O_2}H_{CO_2}$).

The desired average (±SE) O_2 content of 9.51 ± 0.05 mg L^{-1} for high O_2 treatments and 2.98 ± 0.15 mg L^{-1} for low oxygen treatments were also attained (Table 1; $p < 0.0001$, ANOVA). O_2 concentrations remained relatively stable for the $H_{O_2}L_{CO_2}$ and $H_{O_2}H_{CO_2}$ treatments (SE = 0.06 for both), where 100 % saturation was targeted. DO concentrations in the $L_{O_2}L_{CO_2}$ and $L_{O_2}H_{CO_2}$ treatments were more variable ranging from 1.81 up to 3.88 mg L^{-1} over the course of the experiment. The pH was also most variable where manipulation was required in the $H_{O_2}H_{CO_2}$ (SD = 0.08 units) and $L_{O_2}H_{CO_2}$ (SD = 0.09 units) treatments; however, there was also natural variation in the seawater as seen in the $H_{O_2}L_{CO_2}$ and $L_{O_2}L_{CO_2}$ treatments (SD = 0.28). Overall pH and O_2 levels were well maintained around the targeted averages.

The RI averaged 1.60 ± 0.02 for the $H_{O_2}L_{CO_2}$, 1.15 ± 0.03 for the $L_{O_2}L_{CO_2}$, 1.14 ± 0.03 for the $H_{O_2}H_{CO_2}$ and 0.69 ± 0.04 for the $L_{O_2}H_{CO_2}$ treatment (Table 2). The RI values for the hypoxic and elevated pCO_2 treatment were similar, as the differences in pO_2 and pCO_2 had a similar effect on RI. All treatments matched the target values and were held to an acceptable level and variability within each treatment (Table 2).

3.2 Respiration

Although the overall response was not significant for any experimental treatment ($p = 0.357$; ANOVA), when plotting the mean respiration rate of each species of the $H_{O_2}L_{CO_2}$ treatment vs. the different experimental treatments (Fig. 1), results of regression analysis show that there is a significant difference between the 1 : 1 line in the $H_{O_2}H_{CO_2}$ treatment ($p < 0.05$; regression comparison), whereas the other two treatments did not differ significantly ($L_{O_2}L_{CO_2}$: $p = 0.701$; $L_{O_2}H_{CO_2}$: $p = 0.070$; regression comparison). When comparing results of the different habitats a significant difference between treatments and habitats was observed ($p < 0.01$; two-way ANOVA), as the result the mooring was different from the other three habitats throughout treatments (Student's t).

The general trend for the $L_{O_2}L_{CO_2}$ and $L_{O_2}H_{CO_2}$ treatment was for organisms to reduce their metabolism, as metabolic rates for most species fell below the 1 : 1 line (Fig. 1). The metabolic rate for *C. intestinalis* under ambient conditions was over 2.5 times greater than that for any other species. Echinoderms generally displayed lower respiration rates, with the exception of *A. filiformis* who had comparatively high metabolism (Fig. 2). The three species of molluscs had similar metabolic rates, which differed amongst treatments.

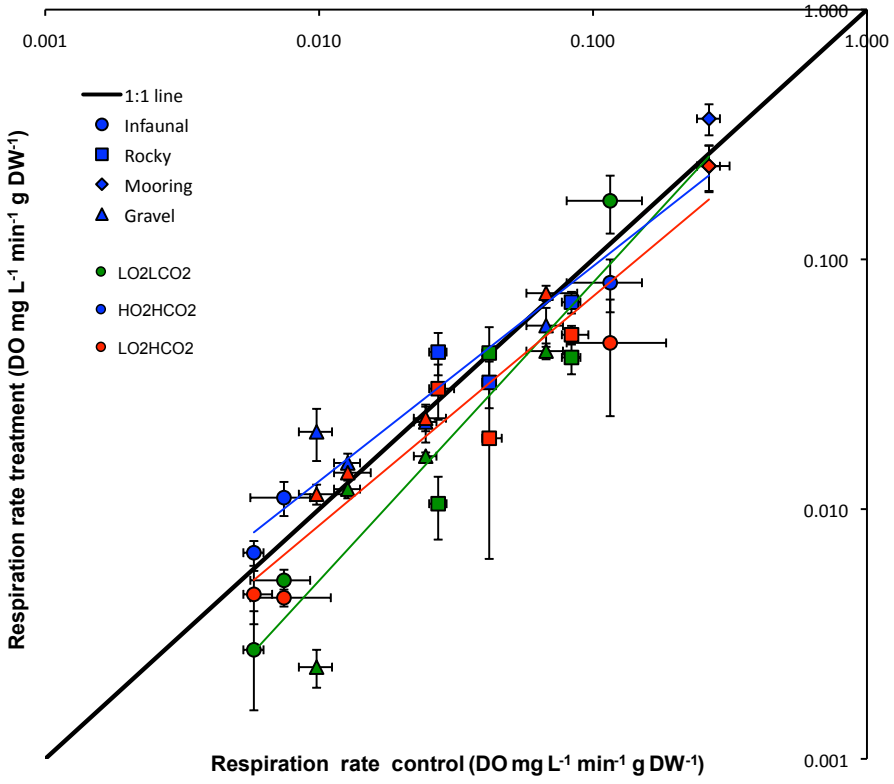

Figure 1. Respiration rate (Average ±SE) control vs. treatments of all tested species: green – $L_{O_2}L_{CO_2}$ ($y = 0.9571x - 0.0046$, $R^2 = 0.90$), blue – $H_{O_2}H_{CO_2}$ ($y = 1.2905x - 0.0122$, $R^2 = 0.93$) and red – $L_{O_2}H_{CO_2}$ ($y = 0.8301x - 0.0032$, $R^2 = 0.92$). The 1 : 1 line represents where treatment metabolism is equal to ambient metabolism.

When looking at the Ln effect size of each species separately, 6 of the 11 species tested experienced reduced respiration in response to the $L_{O_2}L_{CO_2}$ treatment compared to the $H_{O_2}L_{CO_2}$ treatment, with a further three species experiencing a trend towards reduced respiration (Fig. 2; Table 3). The species *A. filiformis* and *A. rubens* responded with increased respiration, although not significantly (Fig. 2). As for the $H_{O_2}H_{CO_2}$, six species increased respiration, with a significant difference in *O. fragilis* and *M. edulis*. The other five species responded with decreased metabolic rates (Fig. 2). The $L_{O_2}H_{CO_2}$ treatment also had quite variable results with seven species experiencing lower respiration rates than the control, with significant differences in the species *A. rubens*, *A. filiformis*, *L. littorea* and *T. granifera* (Fig. 2). The majority of species exposed to $L_{O_2}L_{CO_2}$, $H_{O_2}H_{CO_2}$ and $L_{O_2}H_{CO_2}$ did not experience changes in respiration that differ significantly from those observed under $H_{O_2}L_{CO_2}$ conditions. This is confirmed by the results of the GLM (Table 3), which showed that the responses to oxygen and CO_2 are highly species specific, as we observed synergetic effects in only 4 out of 11 species (*O. fragilis*, *O. nigra*, *A. rubens* and *T. granifera*; Table 3).

4 Discussion

The Baltic species tested were highly resistant to short-term hypoxia and elevated pCO_2, alone or in combination, as they experienced very high survival rates across treatments in the relatively short-duration experiment reported here. Whereas lethal responses to elevated pCO_2 are seldom observed (Kroecker et al., 2013), the level of hypoxia imposed is sufficient to cause mortality of half of the populations of most marine species (Vaquer-Sunyer et al., 2008), with elevated pCO_2 expected to enhance respiratory stresses (Brewer and Peltzer, 2009). This suggests that the species tested have adapted to hypoxia and elevated pCO_2, which are experienced regularly in the ecosystem (Table 1), as more vulnerable species would already have been removed from the community.

The resistance of all species tested to short-term (up to 6 day exposure) hypoxia, elevated pCO_2 and their combined effects, reflected in negligible mortality rates and modest metabolic responses, suggest that the community in the Gullmar Fjord have already been sieved to contain species and lineages resistant to these stressors, to which they have been exposed, at least for short periods of time, for generations (Josefson and Widbom, 1988; Arneborg, 2004). Whereas

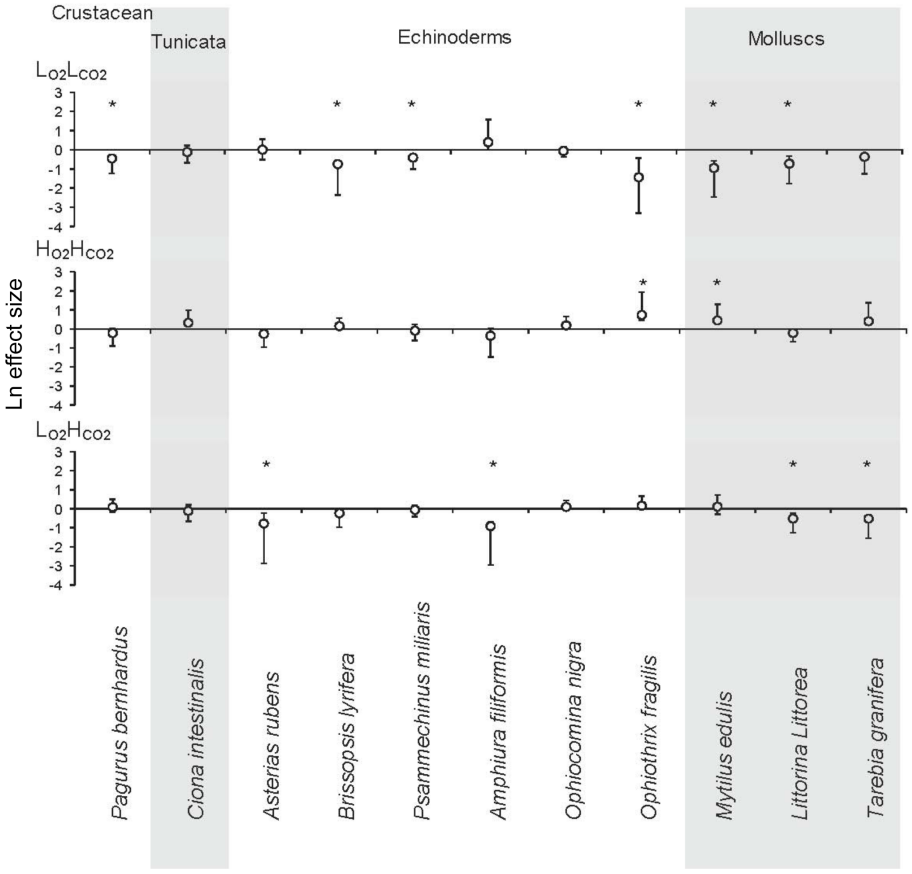

Figure 2. The Ln effect size of the response ratios for invertebrate species and phyla in response to three treatments: low O_2 ($L_{O_2}L_{CO_2}$), low pH ($H_{O_2}H_{CO_2}$) and coupled low O_2 and low pH ($L_{O_2}H_{CO_2}$) compared to control levels ($H_{O_2}L_{CO_2}$). LnRR = ln(treatment)–ln(control) ± Bias-corrected bootstrapped 95 % confidence interval (after Gurevitch and Hedges, 1999; Hedges et al., 1999; Kroeker et al., 2010). The zero line indicates no effect, and significance of mean effects is determined when the 95 % confidence interval does not overlap zero (significant results marked with "*"). Grey background was added to summarize the species by phyla.

physiological limits to low O_2 concentrations seem unavoidable (Brewer and Peltzer, 2009), the existence of thresholds for elevated pCO_2 are less evident. Moreover, the fact that no or negligible mortality was experienced in our experiments at RI's of 0.69, where Brewer and Peltzer (2009) predict the organisms to be severely compromised, in the thermodynamic limit of aerobic respiration, supports the idea that organisms have acclimatized to reoccurring events of low O_2 (and low pH), which are well documented within the Gullmar Fjord (Rosenberg, 1985; Johannessen and Einar, 1996; Nordberg et al., 2000; Polovodova and Nordberg, 2013). While there is a relatively long history of monitoring in the Gullmar Fjord, one of the longest-studied ecosystems in the world (seawater temperature records exist since the 1700s), pH data collection has been erratic and often only recorded at the surface (SMHI, 2011). However, available data shows pH has fluctuated between 7.6 and 8.7 over the last six decades (Dorey et al., 2013).

Our experimental treatments explored a more limited range of O_2 and CO_2 than present across Gullmar Fjord. The community in this area has already been sieved of species vulnerable to low O_2 concentrations due to a history of hypoxia and even complete anoxia within the last four decades (Nordberg et al., 2000; Polovodova et al., 2011). Indeed, our $H_{O_2}L_{CO_2}$ values, involving saturating O_2 concentrations, are unlikely to be experienced at the fjords depths (Fig. 3). It is, therefore, possible that the low O_2 conditions better represented the environment in which the organisms were growing prior to the experiments. The experimental CO_2 values tested need also be compared with ambient levels. Dorey et al. (2013) found that pH in the Gullmar Fjord fluctuated as much as 8.7 to 7.6 over the last 66 years (average monthly fluctuation was 0.34 to 0.89 units). Therefore, the minimum pH level conducive to a rise in pCO_2 to 1,000 µatm would be closer to 7.2. Dorey et al. (2013) conducted lab experiments with pH values as low as 6.5 for urchin larvae, which are generally more sensitive to pH change than adults (Dupont and Thorndyke, 2009). Exposed *A. filiformis* live in sediment burrows that experience much lower oxygen and higher pCO_2 than surrounding water which intensifies with depth (Hu et

Table 3. Respiration Rate in DO mg L^{-1} min^{-1} g DW^{-1} (±SE) and results of the general linear model (GLM) of all tested species (pooled data where we had data from different days). Levels not connected by the same letter are significantly different (after Student's *t* and Tukey HSD tests). Numbers written in bold colour highlight significant differences.

Species	Taxa	Day	Prob. > *F*		Average respiration rate (±SE) DO mg L^{-1} min^{-1} g DW^{-1}				General linear model (GLM)
					$H_{O_2}L_{CO_2}$	$L_{O_2}L_{CO_2}$	$H_{O_2}H_{CO_2}$	$L_{O_2}H_{CO_2}$	$CO_2{*}O_2$
Pagurus bernhardus	Crustacean	3,6	**0.0417**	Average	0.067	0.043	0.054	0.073	**−0.0434**
				(±SE)	0.010	0.003	0.010	0.005	
$n = 12$				Students' *t*	A	B	AB	A	
				Tukey HSD	AB	B	AB	A	
Brissopsis lyrifera	Echinoidea	3	0.0715	Average	0.0058	0.0027	0.0067	0.0046	**−0.0009**
				(±SE)	0.0005	0.0012	0.0008	0.0011	
$n = 12$				Student's *t*	A	B	A	AB	
				Tukey HSD	A	A	A	A	
Psammechinus miliaris	Echinoidea	3,6	0.1202	Average	0.024	0.016	0.022	0.023	**−0.0090**
				(±SE)	0.002	0.001	0.004	0.003	
$n = 12$				Student's *t*	A	B	AB	AB	
				Tukey HSD	A	A	A	A	
Asterias rubens	Echinoidea	3	0.3302	Average	0.042	0.042	0.032	0.019	**0.0133**
				(±SE)	0.002	0.011	0.007	0.013	
$n = 12$				Student's *t*	A	A	A	A	
				Tukey HSD	A	A	A	A	
Amphiura filiformis	Echinoidea	3	0.1678	Average	0.115	0.172	0.081	0.046	0.0904
				(±SE)	0.035	0.045	0.019	0.023	
$n = 12$				Student's *t*	AB	A	AB	B	
				Tukey HSD	A	A	A	A	
Ophiothrix fragilis	Echinoidea	3	**0.0023**	Average	0.0098	0.0023	0.0204	0.0115	**0.0015**
				(±SE)	0.0014	0.0004	0.0048	0.0011	
$n = 12$				Student's *t*	B	C	A	B	
				Tukey HSD	BC	C	A	AB	
Ophiocomina nigra	Echinoidea	3	0.2054	Average	0.013	0.012	0.015	0.014	**0.0006**
		2,4,6		(±SE)	0.001	0.001	0.001	0.001	
$n = 24$				Student's *t*	AB	B	A	AB	
				Tukey HSD	A	A	A	A	
Mytilus edulis	Bivalve	3,6	**0.0063**	Average	0.027	0.011	0.043	0.030	**−0.0045**
				(±SE)	0.002	0.003	0.008	0.008	
$n = 12$				Student's *t*	A	B	A	A	
				Tukey HSD	AB	B	A	AB	
Littorina littorea	Gastropoda	3,6	**< 0.0001**	Average	0.083	0.040	0.067	0.050	**−0.0254**
				(±SE)	0.006	0.006	0.007	0.004	
$n = 24$				Student's *t*	A	B	A	B	
				Tukey HSD	A	C	AB	BC	
Tarebia granifera	Gastropoda	3,6	**0.0073**	Average	0.007	0.005	0.011	0.004	**0.0044**
				(±SE)	0.002	0.001	0.002	0.000	
$n = 12$				Student's *t*	AB	B	A	B	
				Tukey HSD	AB	B	A	B	
Ciona intestinalis	Tunicata	3,5	0.1578	Average	0.265	0.236	0.366	0.236	0.1001
				(±SE)	0.025	0.050	0.052	0.049	
$n = 24$				Student's *t*	A	A	A	A	
				Tukey HSD	A	A	A	A	

al., 2014). *A. filiformis* have been shown to withstand a pH of 7.0 and O_2 levels below 2.0 mg L^{-1} and experience no mortality (Hu et al., 2014). Hence, the species tested here already has O_2 and pH values comparable to those used as treatments here, particularly for infauna, such as *A. filiformis* and *B. lyrifera* which appear to be exposed to low O_2 and pH conditions on a regular basis.

Sublethal responses, in terms of metabolic depression or enhancement, were observed in response to hypoxia and elevated pCO_2, alone or in combination. We expected that $L_{O_2}H_{CO_2}$ would be the most significant respiratory stress for organisms, as it would affect all species except those with an infaunal growth habit, and thus would result in a reduced metabolism. However, only one species (*A. filiformis*) with an infaunal growth habit (Table 1) experienced a significantly

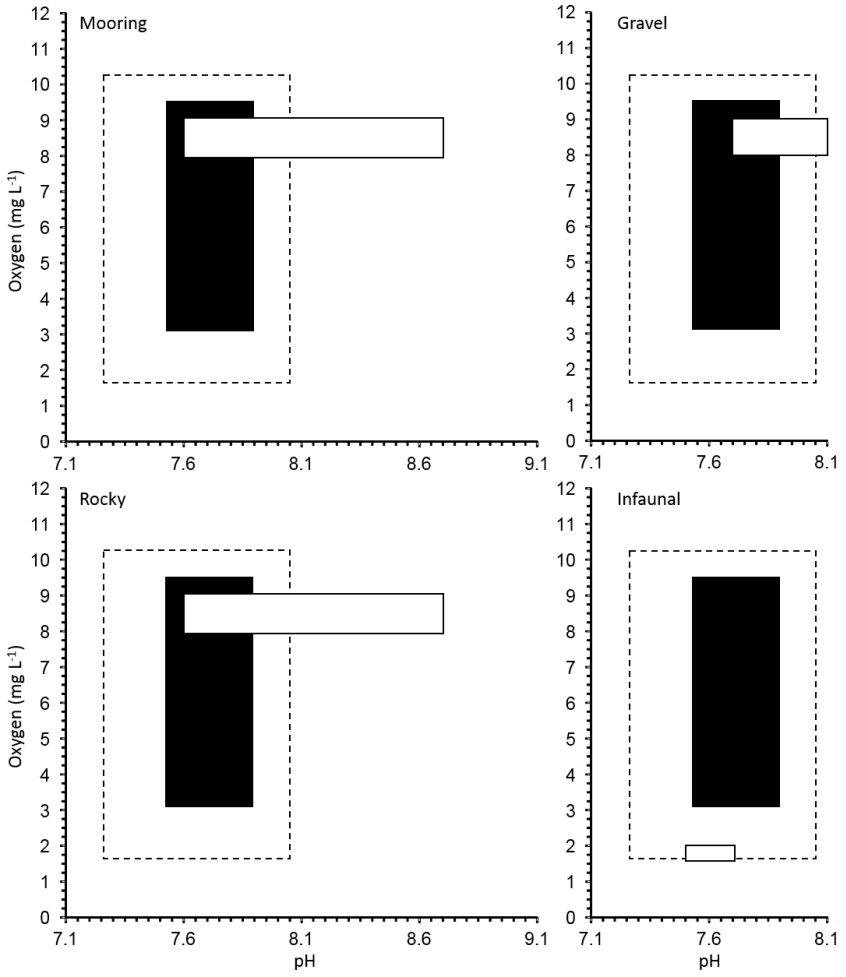

Figure 3. Realized oxygen concentration and pH conditions for a manipulation experiment for 11 invertebrate species from four different natural habitats in the Gullmar Fjord: gravel, infaunal, mooring and rocky. Average and extreme (maximum and minimum) O_2 and pH conditions during experimental exposure are shown in black and with dotted line, respectively. The natural O_2 and pH conditions expected for each habitat are shown in the white boxes.

reduced metabolism due the coupled impacts of H_{CO_2} and L_{O_2}. Two of the three species with an infaunal growth habit showed no metabolic response to hypoxia, whereas all except two of the species growing in other habitats, generally experiencing high O_2 levels, experienced a metabolic depression under hypoxia. Whereas there were no consistent patterns in the responses across phyla, they showed consistency among habitats, reflecting the conditions the species were adapted to in their natural habitat.

There is growing interest in understanding the response of marine organisms to multiple stressors, such as rising temperature, OA, increased UVB radiation and reduced O_2 (e.g. Pörtner et al., 2005; Fredersdorf et al., 2009; Vaquer-Sunyer and Duarte, 2010, 2011; Duarte, 2014). For example, Mayol et al. (2012) examined the co-occurrence of low O_2 with elevated pCO_2 in the Pacific Ocean off the Chilean coast, identifying layers where both stressors co-occur. Yet, most experimental evidence of the response of marine invertebrates to stressors focuses on individual effects, where their combined effects may differ from those derived (or calculated) from combinations of individual effects (cf. in Kroeker et al., 2013). Indeed, multiplicative, rather than additive, effects of the impacts of the individual stresses are expected (Pörtner et al., 2005; Clapham and Payne, 2011; Ateweberhan et al., 2013). However, our results demonstrate that there is a broad range of possible impacts within species from the Gullmar Fjord ecosystem, including species that show an amplification of the responses beyond that expected under an additive model and those that show a buffering or compensation of responses when multiple stressors co-occur. The *A. rubens* exhibited a synergistic response to hypoxia and elevated pCO_2 as it showed a significant metabolic depression under both stressors, but no significant response to either one alone. The echinoderm *O. fragilis* experienced enhanced metabolic rates when exposed to elevated pCO_2, consistent with the sensitivity to high CO_2 reported for their larvae, which experi-

enced 100 % mortality when pH was reduced by just 0.2 units (Dupont and Thorndykee, 2008). In contrast, *M. edulis* experienced depressed metabolism when exposed to hypoxia. As a result, these effects operated into an antagonistic mode, resulting in no significant change in metabolic rates when the organisms were exposed to both hypoxia and elevated pCO_2. However, there was no general trend for responses to be either additive or synergistic across species. Indeed, our result suggests that responses are mostly dependent on the fluctuations in the stressors in their habitats, so that the prior selective and adaptive history of the species plays an important role in determining their vulnerability to different stressors.

Whereas a theoretical framework to predict the response of marine organisms to multiple stressors is generally lacking, Brewer and Pelzer (2009) derived a theoretical expectation of the expected responses in the particular case of combined hypoxia and high CO_2, the organisms tested show a RI decrease with intensity of alterations in our treatments as expected. Although we reached the 0.7 threshold value (0.69 under $L_{O_2}H_{CO_2}$), which represents the thermodynamic limit for aerobic respiration, the organisms are expected to be severely compromised. Yet, we observed little or no mortality and the organisms exposed to $L_{O_2}H_{CO_2}$ should have experienced aerobic stress, yet our results showed that they were more likely to reduce respiration under hypoxia. Hence, the RI does not appear to hold predictive power as to the response of marine invertebrates to the interactions between O_2 and CO_2. All but one of the tested species were calcifiers, and were expected to be impacted by elevated pCO_2. Indeed, the treatments with elevated pCO_2 reached an undersaturated concentration of aragonite ($\Omega_{arag} < 1$), where calcifiers are expected to be stressed (Doney et al., 2009). Molluscs rely chiefly on aragonite to construct their shells (Porter, 2007), while echinoderms and crustaceans use calcite (Raup, 1959; Raabe et al., 2005). Yet, the impacts of elevated pCO_2 were not greater in molluscs than for echinoderms and crustaceans in our experiments. Hence, the RI does not hold predictive power on the effects of hypoxia and/or pCO_2 on the species tested here, which seemed best predicted from consideration of the ranges of O_2 and CO_2 they experience in their habitat.

Responses to low O_2 and elevated pCO_2 were variable amongst phyla and species in the community tested here, ranging from antagonistic to synergistic responses. The very limited impacts of low O_2 and elevated pCO_2 of the invertebrates from this ecosystem, which showed little or no mortality in the presence of both stressors, reflects the range of conditions in the habitats these organisms occupy. This ecosystem has been reported to experience recurrent seasonal hypoxic events characterized by low pH values and elevated pCO_2 (Nordberg et al., 2000; Dorey et al., 2013). Hence, the organisms tested were resistant to both stressors within the levels used in this experiment, which, while ranging within values reported to negatively impact on marine invertebrates for both O_2 (Vaquer-Sunyer and Duarte, 2008) and CO_2 (Kroeker et al., 2013), were within the range present in their ecosystem. Eutrophication-driven hypoxia, such as that experienced in Baltic fjords, derives from excess metabolic O_2 consumption and is, therefore, coupled with elevated pCO_2 (e.g. Duarte et al., 2013; Melzner et al., 2013; Wallace et al., 2014). Hence, low O_2 and elevated pCO_2 often co-occur in areas affected by hypoxic events, such as Gullmar Fjord. Haselmair et al. (2010) observed that pH declined by up to 0.7 units during an induced anoxic event in the Adriatic Sea and Melzner et al. (2013) predict that pCO_2 can reach up to 3200 µatm during anoxic events in brackish waters (salinity of 20), with those values decreasing as salinity increases. Hence, adaptive responses of organisms in the Gullmar Fjord should be coupled for low O_2 and elevated pCO_2, thereby accounting for the limited effects to the experimentally imposed stressors used here.

5 Conclusions

Respiratory responses to low O_2 and elevated pCO_2 were variable amongst phyla and species in the community tested here, ranging from buffered to amplified metabolic responses. The very limited impact of low O_2 and elevated pCO_2 of the invertebrates from this ecosystem, which showed little or no mortality in the presence of both stressors, reflects the past history of this ecosystem, which has been reported to experience recurrent hypoxic events characterized by low pH values and elevated pCO_2 (Nordberg et al., 2000; Dorey et al., 2013). Hence, the organisms trialled were resistant to both stresses within the levels used in this experiment, which were within values reported to negatively impact on marine invertebrates for both O_2 (Vaquer-Sunyer and Duarte, 2008) and CO_2 (Kroeker et al., 2013). Therefore, hypoxia impacted the greatest number of organisms and represents the most concerning stress in the region. Management plans addressing hypoxia should also avoid the generalized assumption that synergistic stressors will result in multiplicative effects, and focus research into understanding the mechanisms calcifiers and other invertebrates employ to cope with these changes. Our results also highlight the idiosyncratic nature of responses, which were strongly species-specific, suggesting that extrapolations from experiments conducted on a few species to the phylum level may be strongly misleading. This adds complexity to the challenge of predicting how global stressors will affect marine ecosystems in the future.

Data availability. Data can be accessed at https://doi.org/10.1594/PANGAEA.890918 (Fontanini et al., 2018).

Author contributions. The experiment was designed by AF, AS and CMD. The experiment was conducted by AF, AS and SD. The analysis was conducted by AF, AS, SD and CMD. The writing was performed by AF, AS, SD and CMD.

Competing interests. The authors declare that they have no conflict of interest.

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