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Pathogenic challenge reveals immune trade-off in mussels exposed to reduced seawater pH and increased temperature

Robert P. Ellis [a,c,*], Steve Widdicombe [a], Helen Parry [a], Thomas H. Hutchinson [b], John I. Spicer [c]

[a] Plymouth Marine Laboratory, Prospect Place, Plymouth PL1 3DH, UK
[b] School of Biological Sciences, University of Plymouth, Plymouth PL4 8AA, UK
[c] Marine Biology and Ecology Research Centre, School of Marine Science & Engineering, University of Plymouth, Plymouth PL4 8AA, UK

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ABSTRACT

Ocean acidification (OA) and warming pose a considerable threat to marine ecosystems. Previous studies show that these environmental co-stressors significantly impact upon a number of key physiological functions, including calcification, metabolism and growth, in many marine organisms. Yet despite the importance of the immune system, to date only a handful of studies have investigated the impact of reduced seawater pH on an organism's immune response. Furthermore, whilst temperature has received far greater attention with respect to host defence, there is a dearth of information concerning the possible synergism of these two stressors on immune defence. Here we show that a 90 day exposure to reduced seawater pH led to a reduction in the antibacterial activity of cell-free haemolymph in the blue mussel *Mytilus edulis*, whilst temperature led to an increase in this immune parameter. However in contrast to previous research, following this initial 90 day exposure, mussels in the current study were then exposed to the pathogenic bacterium, *Vibrio tubiashii*. Crucially, whilst reduced seawater pH initially appeared to impair immunological functioning, as has been interpreted previously, mussels demonstrated the ability to restore haemolymph bactericidal activity when required. This indicated that the initial reduction in antibacterial activity was in fact a reversible physiological trade-off, rather than an irreversible impairment of immune function. By demonstrating this plasticity, the current study illustrates the need to measure organism responses within a realistic natural context (i.e. measuring the immune response of an organism in the presence of a pathogen). Failure to do so may result in a misleading interpretation of the ecological relevance of experimental data, and thus the sensitivity of different species in a rapidly changing environment.

1. Introduction

Ocean acidification (OA) is one of the greatest threats currently faced by marine ecosystems (Halpern et al., 2008; Harley et al., 2006). Caused by anthropogenic CO_2 emissions, OA has already led to measureable changes in ocean carbonate chemistry (Olafsson et al., 2009; Santana-Casiano et al., 2007) and it is projected to reduce surface seawater pH in the open ocean by 0.4 units before the end of this century (Caldeira and Wickett, 2003, 2005). In some coastal habitats, where the seasonal upwelling of hypoxic and hypercapnic seawater naturally produces carbonate chemistry conditions analogous to the current worst case acidification models (Hauri et al., 2013; Hofmann et al., 2011, 2014; Melzner et al., 2012; Thomsen et al., 2010), OA is likely to exacerbate these seasonal events, creating conditions that far exceed any current OA projections for open ocean surface waters (Dorey et al., 2013; Hauri et al., 2013; Hofmann et al., 2014; Melzner et al., 2012). Furthermore, such alterations in seawater carbonate chemistry are occurring against a background of warming (IPCC, 2007), pollution (Rodgers and Laffoley, 2011) and disease outbreak (Elston et al., 2008), posing a significant challenge to the many marine organisms that inhabit these coastal regions. It is therefore vital to characterise species sensitivity to multiple stressors (Dupont and Pörtner, 2013a, 2013b).

Whilst a reduction in seawater pH impacts a number of important physiological processes including calcification (e.g. Gazeau et al., 2007; Wood et al., 2010), photosynthesis (e.g. Langdon and Atkinson, 2005; Schneider and Erez, 2006), acid–base balance (e.g. Miles et al., 2007; Spicer et al., 2007), metabolism (e.g. Michaelidis et al., 2005; Thomsen and Melzner, 2010), growth (Berge et al., 2006; Thomsen et al., 2010) and behaviour (e.g. Bibby et al., 2007; Nilsson et al., 2012) in a wide range of marine organisms, the investigation of host defence and its interaction with climate change stressors is still in its infancy.

The immune response is vital to all animals, controlling or fighting any pathogenic challenge (Ellis et al., 2011). It is therefore a key determinant of host survival under changing environmental conditions. Despite this importance only a handful of studies have, to date, investigated the impact of OA on the immune system of a marine organism. The first study undertaken, carried out by Bibby et al. (2008), exposed the blue mussel, *Mytilus edulis*, to reduced seawater pH for 32 days,

* Corresponding author.
E-mail address: roblis@pml.ac.uk (R.P. Ellis).

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measuring a number of key immune responses over the course of the experiment. Mussels maintained in reduced pH seawater (7.7, 7.5 or 6.7) displayed reduced phagocytic activity, compared with controls maintained at pH 7.8, after the 32 day exposure. Hernroth et al. (2011) also demonstrated a suppression of phagocytic activity, as well as a reduction in coelomocyte counts and an inhibition of p38 MAP-kinase activity, in *Asterias rubens* exposed to pH 7.7. Similarly Matozzo et al. (2012) demonstrated a reduction in host defence in mussels exposed to reduced seawater pH, with the lysozyme-like activity of cell free haemolymph reduced in *Mytilus galloprovincialis* exposed to pH 7.7 and 7.4 for 7 days.

As with reduced seawater pH, only two studies to date have measured the combined effects of reduced seawater pH and temperature on the immune response of a marine organism, despite there being a significant body of literature that has investigated the impact of environmental temperature on host defence in marine invertebrates in isolation (see Ellis et al., 2011). In exposing the Norway lobster, *Nephrops norvegicus*, to a seawater pH of 7.6 for 4 months over a range of temperatures from 5 °C to 18 °C, Hernroth et al. (2012) found that a combined exposure to these two stressors led to a reduction in total haemocyte counts in this crustacean. Yet, in this species phagocytic capacity was only impacted by reduced seawater pH. Matozzo et al. (2012), in exposing *M. galloprovincialis* and *Chamelea gallina* to reduced seawater pH, also maintained these bivalves at two different temperatures, 22 °C and 28 °C. However, despite demonstrating a significant impact of a combined temperature and reduced pH exposure on a number of immune parameters (including total haemocyte counts, lysozyme like activity of cell-free haemolymph and neutral red retention) these authors highlight the lack of clear response of bivalve host defence. Thus encouraging the authors to call for further investigation to elucidate any possible mechanisms of temperature/reduced pH immune modulation in a range of marine species. The dearth of studies undertaken to date that have investigated a combined temperature and OA exposure on organism host defence highlights the pressing need to characterise the synergistic effects of these stressors on the immune response in a range of marine organisms.

Measuring an organism's immune response in the presence of a realised pathogenic threat arguably offers the best assessment of any altered immune functionality or immune suppression (Ellis et al., 2011; Viney et al., 2005), yet to date only one study has investigated the impact of reduced seawater pH in a marine organism in the presence of a pathogen. Asplund et al. (2013) investigated the impact of a reduced seawater pH on the host pathogen interaction between the blue mussel, *M. edulis*, and a common bivalve bacterial pathogen, *Vibrio tubiashii*. This study exposed both the bivalve host and bacterial pathogen to reduced seawater pH (pH 7.74), demonstrating no adverse effects of OA on either the host immune response (total haemocyte count, phagocytic capacity) or the growth or viability of the bacterium. Conversely, when investigating the interaction of these two organisms, *V. tubiashii* were clearly able to infect OA exposed mussels easier than their counterparts maintained under ambient conditions. This study therefore highlights the pressing need to undertake studies that investigate the combined effects of climate change stressors on host defence, in the context of a pathogenic challenge.

Being sessile filter feeders, marine bivalves are perhaps more predisposed to encountering marine microbes (Hernroth et al., 2002), and an elevated threat of a pathogenic insult. Yet, effective defence mechanisms ensure that mussels may tolerate this pathogen exposure without getting infected (Asplund et al., 2013; Venier et al., 2011). Furthermore, bivalves may also be particularly vulnerable to reduced seawater pH (Hendriks et al., 2010; Kroeker et al., 2010, 2013), which impacts on acid–base balance, metabolism and calcification in this group (e.g. Gazeau et al., 2013; Hüning et al., 2013; Melzner et al., 2011), whilst being ectothermic, means environmental temperature also plays an important role in determining body temperature, directly impacting all physiological processes in this group (Pörtner et al., 2006; Young et al., 2011). The need to balance the allocation of energy between competing physiological processes under conditions of environmental stress, and under the combined challenge of reduced seawater pH, increased temperature and pathogen exposures in particular, means marine bivalves are thus an ideal model system for investigating the impact of climate change stressors on the invertebrate immune response.

With energy being a key limiting resource, it has been hypothesised that the increased energetic burden of an exposure to reduced seawater pH, increased temperature or the possible synergistic effects of these stressors will divert resources from processes such as host defence or growth, in favour of higher priority processes such as maintaining physiological homeostasis, metabolism or calcification (Bibby et al., 2008; Sokolva et al., 2012; Wood et al., 2008). If this is the case then an OA/temperature induced trade-off in host defence should reduce immunocompetence, and increase pathogen susceptibility, as was hypothesised by both Bibby et al. (2008) and Matozzo et al. (2012). However, it is important to note that these two studies were undertaken in the absence of a pathogen. Therefore it is possible that the reduction in host defence measured by these authors may be a result of mussels trading-off the cost of maintaining a maximal immune response under OA conditions, with no permanent impairment of immune functionality or increased disease susceptibility.

To test this hypothesis we investigated how a combined exposure to reduced seawater pH (ranging from 8.05 to 6.50) and increased temperature impacted the antibacterial activity of cell-free haemolymph in the blue mussel, *M. edulis* and how a subsequent challenge with the pathogenic bacterium, *V. tubiashii* (Elston et al., 2008), altered the initial response of this immune parameter to environmental stress.

2. Materials and methods

2.1. Organism collection, exposure and sampling protocol

The mussels used in this study were collected and maintained as described by Ellis et al. (2014). Briefly, adult *M. edulis* (shell length = 50–70 mm) were collected from Exmouth, UK, and transported to Plymouth Marine Laboratory, in December 2009. Mussels were divided between 60 experimental chambers (Vol. = 250 ml; 4 mussels per chamber), continuously supplied with seawater from 1 of 10 header tanks. Header tank pH_{NBS} was adjusted using the computerised feedback mechanism described by Widdicombe and Needham (2007), maintaining seawater at one of five nominal pH levels: 8.05 (present day ambient seawater pH), 7.80 (2100, IS92 emissions scenario; IPCC, 2007), 7.60 (2100, A2 scenario; Caldeira and Wickett, 2005), 7.35 (2300, IS92 emissions scenario; IPCC, 2007) and 6.50 (CCS leak; Blackford et al., 2009). Chambers were distributed between 10 water baths, set to either 12.5 °C or 17.0 °C (representing natural seasonal temperature measured in the English Channel in December 2009, or a 4.5 °C warming event above this seasonal temperature), creating a fully crossed experimental design. Mussels were fed *Isochrysis galbana* (algal concentration maintained between 100,000 and 150,000 cells ml^{-1}). pH_{NBS}, temperature and salinity were measured 3 times per week. Total alkalinity (A_T) was measured weekly using an open-cell potentiometric titration technique. All other carbonate system variables were calculated using CO_2SYS (Pierrot et al., 2006), according to Findlay et al. (2013). Carbonate chemistry data for both header tanks and experimental chambers for the experimental exposure are presented in Table 1.

Mussels were maintained as above for 90 days, following which antibacterial activity of cell-free haemolymph was measured in one mussel per chamber. After 91 days the remaining mussels were challenged with *V. tubiashii* (2×10^6 bacterial cells, injected into the posterior adductor muscle), before being returned to the system. The immune response was then measured again in one mussel per chamber after 92 days (1 day post-exposure) and again after 98 days (7 days post-exposure). Mortality was assessed daily throughout the duration

Table 1
Carbonate chemistry of sea water in **a)** header tanks and experimental chambers at **b)** 12.5 °C and **c)** 17.0 °C for each pH exposure level for each pH exposure level. Data are expressed as mean (±S.E.). Significant differences ($p \leq 0.05$) between treatment levels are indicated by different letters based on pair-wise tests.

Parameter	8.05	7.80	7.60	7.35	6.50
a)					
pH_{NBS}	8.09 ± 0.01[a]	7.77 ± 0.01[b]	7.60 ± 0.01[c]	7.33 ± 0.01[d]	6.46 ± 0.02[e]
Temperature (°C)	13.83 ± 0.11	13.94 ± 0.10	13.85 ± 0.10	14.01 ± 0.10	13.87 ± 0.10
Salinity	34.17 ± 0.07	34.15 ± 0.07	34.17 ± 0.07	34.16 ± 0.07	34.15 ± 0.07
A_T (μmol kg^{-1} SW)	2402.32 ± 30.27	2396.70 ± 27.89	2412.67 ± 30.79	2392.42 ± 31.71	2420.65 ± 30.62
pCO_2 (μatm)*	518.02 ± 22.25[a]	1177.85 ± 64.12[b]	1940.40 ± 240.51[c]	5075.37 ± 1402.88[d]	25,242.14 ± 1938.82[e]
b)					
pH_{NBS}	8.02 ± 0.01[a]	7.68 ± 0.01[b]	7.51 ± 0.01[c]	7.30 ± 0.01[d]	6.55 ± 0.01[e]
Temperature (°C)	12.36 ± 0.09	12.27 ± 0.10	12.30 ± 0.10	12.32 ± 0.09	12.46 ± 0.10
Salinity	34.00 ± 0.06	34.01 ± 0.06	34.04 ± 0.06	34.00 ± 0.06	33.98 ± 0.06
A_T (μmol kg^{-1} SW)	2395.15 ± 20.60[a]	2394.45 ± 20.01[a]	2436.43 ± 19.58[a]	2434.78 ± 17.50[a]	2584.84 ± 44.15[b]
pCO_2 (μatm)*	787.87 ± 30.60[a]	1704.95 ± 46.67[b]	2413.11 ± 92.87[c]	3786.12 ± 174.12[d]	24,751.24 ± 1109.09[e]
c)					
pH_{NBS}	7.95 ± 0.01[a]	7.63 ± 0.01[b]	7.51 ± 0.01[c]	7.29 ± 0.01[d]	6.51 ± 0.01[e]
Temperature (°C)	17.12 ± 0.07	17.04 ± 0.07	17.11 ± 0.07	17.16 ± 0.06	17.07 ± 0.08
Salinity	34.07 ± 0.06	34.03 ± 0.06	34.06 ± 0.06	34.03 ± 0.06	34.03 ± 0.06
A_T (μmol kg^{-1} SW)	2407.38 ± 17.57[a]	2408.32 ± 18.32[a]	2424.32 ± 12.09[a]	2440.25 ± 17.63[a]	2630.13 ± 36.18[b]
pCO_2 (μatm)*	624.60 ± 21.85[a]	1535.21 ± 77.30[b]	2341.68 ± 104.76[c]	3494.13 ± 148.63[d]	21,846.26 ± 1055.31[e]

* Calculated using CO_2SYS software.

of the experiment, with any dead mussels removed from the set-up upon discovery.

V. tubiashii NCIMB 1337 (ATCC19106) was chosen as it is pathogenic to bivalves and due to its recent re-emergence having been linked to a reduction in hatchery bivalve populations (Elston et al., 2008). *V. tubiashii* was grown under thermo-stable conditions (24 °C) in marine broth (sterile marine saline [salinity = 35, pH_{NBS} 8.05] + 1 g l^{-1} yeast extract and 0.5 g l^{-1} tryptone). Log phase broth culture was harvested into a sterile centrifuge tube (vol = 15 ml; Sarstedt®), centrifuged (10 min, 800 ×g; 15 °C; Centrifuge 5810R, Eppendorf) and re-suspended in sterile marine saline. Bacteria were rinsed a further two times before being re-suspended in fresh marine saline at a concentration of ca. 2×10^8 ml^{-1} ($OD_{600\ nm}$ ca. 2). Bacteria were then further diluted by two serial tenfold dilutions in marine saline to give a final working concentration of 2×10^6 ml^{-1} (Parry and Pipe, 2004).

2.2. Antibacterial activity of cell-free haemolymph

To quantify the immune response haemolymph (vol. = 500 μl) was extracted using a syringe (vol. = 1.0 ml), the needle (21G) of which was inserted into the large sinus within the posterior adductor muscle. Antibacterial activity of cell-free haemolymph was estimated by measuring the growth inhibition of a log phase bacterial culture by turbidimetry, according to Wootton and Pipe (2003). Briefly, an aliquot of haemolymph (250 μl) was added to an equal volume of sterile 3% NaCl solution and centrifuged (400 ×g, 150 s). The supernatant was carefully removed and transferred to a microcentrifuge tube (Eppendorf® vol. = 1.5 ml) and stored at T = 20 °C until analysis could be carried out.

Antibacterial activity of the cell-free haemolymph was measured by pipetting a 100 μl aliquot of cell-free haemolymph into 4 replicate wells of a 96-well microplate (Nunc Microwell) with an equal volume of *V. tubiashii* suspension (2×10^6 bacterial cells per ml^{-1} suspended in sterile 3% NaCl solution). 50 μl of sterile 3% NaCl solution and 50 μl of marine broth (sterile 3% NaCl solution, 5 g l^{-1} peptone, 1 g l^{-1} yeast extract) with 100 μl of *V. tubiashii* suspension were added to 4 replicate wells as controls. 150 μl of sterile 3% NaCl solution added to 50 μl of marine broth was used as the blank. Plates were read using a microplate reader (Molecular Devices VersaMax Microplate Reader) at λ = 340 nm over 22 h. Activities are expressed as percentage bacterial growth inhibition in haemolymph exposed samples compared to control bacterial growth.

2.3. Data analysis

Carbonate chemistry and haemolymph antibacterial activity data were analysed using the PERMANOVA+ add-on (Anderson et al., 2008), in PRIMER 6.1 (Clarke and Gorley, 2006). Data were first tested for homogeneity of variance using PERMDISP, a dissimilarity-based multivariate extension of Levene's test (Anderson et al., 2008). An appropriate transformation of the data (Log(1.03-V)) was performed when required, prior to the construction of similarity matrices. Euclidean distance similarity matrices were constructed, and p-values were calculated using 9999 permutations of the residuals under a reduced model. Pair-wise comparisons were then made where significant p-values were present for a factor with more than two levels, or where two factors were shown to significantly interact. To test survival during the experimental exposure, the impact of treatment and replicate on overall mortality was first tested using PERMANOVA+. This showed a significant treatment effect (Pseudo-F_9 = 11.17, $p \leq 0.01$), however no effect of replicate (Pseudo-F_5 = 1.07, p = 0.39). As there was no effect of replicate on the overall mortality, replicate data were then pooled within treatment to test mussel survival using a Log-rank survival analysis of Kaplan–Meier curves in Sigmaplot 12.0 (Systat Software, California, US). This enabled a more in depth assessment of mussel mortality over the experimental duration. Pair-wise comparisons were then made using the Holm–Sidak multiple comparison test when Log-rank survival analysis indicated a significant difference in the survival between different treatment groups.

3. Results

3.1. Antibacterial activity of cell-free haemolymph

Reduced seawater pH and increased temperature were both shown to significantly affect the antibacterial activity of cell-free haemolymph in mussels (Table 2). Pair-wise analyses indicated that mussels maintained at pH 6.50 had a significantly lower antibacterial activity compared to mussels maintained at all other pH levels, whilst mussels maintained at pH 7.80 were also significantly different to those maintained at pH 7.35 (Fig. 1a). Additionally, organisms maintained at 17.0 °C had a higher antibacterial activity compared to those maintained at 12.5 °C (Fig. 1b).

As well as showing separate effects of both reduced seawater pH and increased temperature on the bactericidal activity of mussel

Table 2
Three-way PERMANOVA for the impact of pH, temperature and bacterial exposure on antibacterial activity of cell-free haemolymph in *Mytilus edulis*. Degrees of freedom (DF), sum of squares (SS), mean squares (MS), Pseudo-F (pF) and permutation probability value (p). (Bold p values indicate significance at $p < 0.05$.).

Source	DF	SS	MS	pF	p	Perms
pH	4	12.784	3.196	7.7949	**0.0001**	9947
Temp	1	5.534	5.534	13.4960	**0.0006**	9841
Bact	2	1.009	0.505	1.2309	0.2939	9946
pH × Temp	4	5.053	1.263	3.0812	**0.0180**	9952
pH × Bact	8	10.808	1.351	3.2950	**0.0017**	9941
Temp × Bact	2	1.708	0.854	2.0831	0.1267	9941
pH × Temp × Bact	8	3.052	0.381	0.9304	0.4966	9936
Residual	110	45.102	0.410			
Total	139	94.507				

haemolymph, these two factors were shown to interact (Table 2). As with the main pH effect, pair-wise analyses showed that at 12.5 °C organisms maintained at pH 6.50 were significantly different to those exposed to all other pH treatments, whilst mussels maintained at pH 7.80 were also different from those maintained at pH 7.60 and 7.35 (Fig. 2). Conversely, at 17.0 °C there was no significant reduction in the antibacterial activity of mussels maintained at pH 6.50, whilst those maintained at pH 7.35 were significantly different to those at pH 7.80 and 7.60 (Fig. 2).

As with temperature, seawater pH was also shown to interact with pathogen exposure (Table 2) in the current study. Pairwise analysis demonstrated that the antibacterial activity of cell-free haemolymph was significantly lower in mussels exposed to pH 6.50, compared to all other pH treatments, prior to a bacterial exposure (Fig. 3a). Furthermore, the bactericidal activity of mussel haemolymph was significantly lower in mussels maintained at pH 7.80 compared to pH 7.35 pre-challenge (Fig. 3a). Conversely, whilst mussels maintained at pH 7.35 were significantly different to those maintained at pH 7.80, 7.60 and 6.50 when measured 1 day post-exposure, there was no difference in

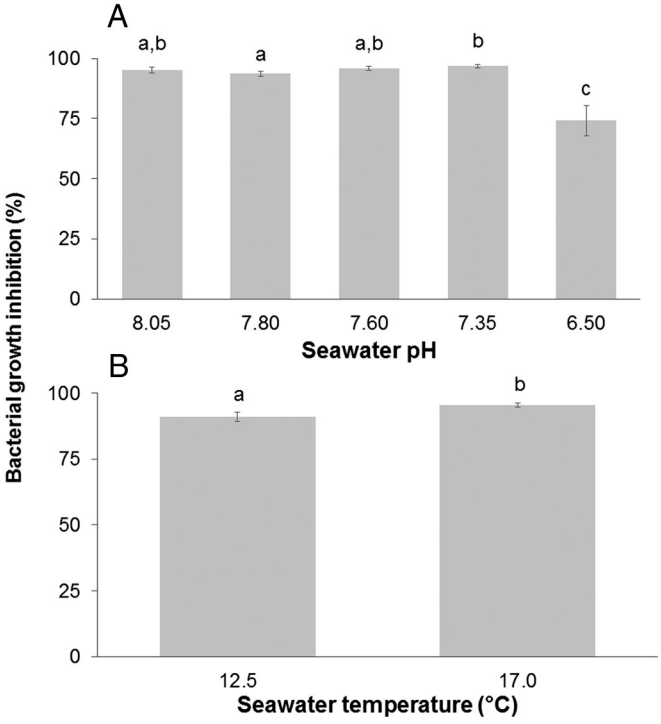

Fig. 1. A) The effect of seawater pH on the antibacterial activity of mussel cell-free haemolymph. B) The effect of water temperature on the antibacterial activity of mussel cell-free haemolymph. Values are expressed as means (±SEM). Data are pooled across temperature and bacterial exposure (A) or bacterial exposure and seawater pH (B). Significant differences ($p \leq 0.05$) are indicated by different letters and based on pair-wise tests.

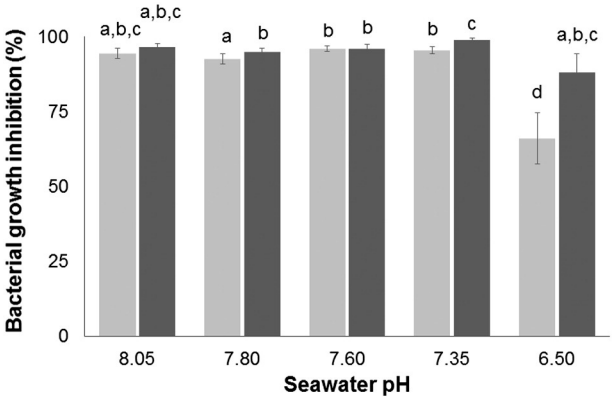

Fig. 2. The effect of temperature and seawater pH on the antibacterial activity of cell-free haemolymph in mussels maintained at 12.5 °C (light grey bars) or 17.0 °C (dark grey bars). Values are means (±SEM) for data pooled across bacterial exposure. Significant differences ($p \leq 0.05$) are indicated by different letters and based on pair-wise tests.

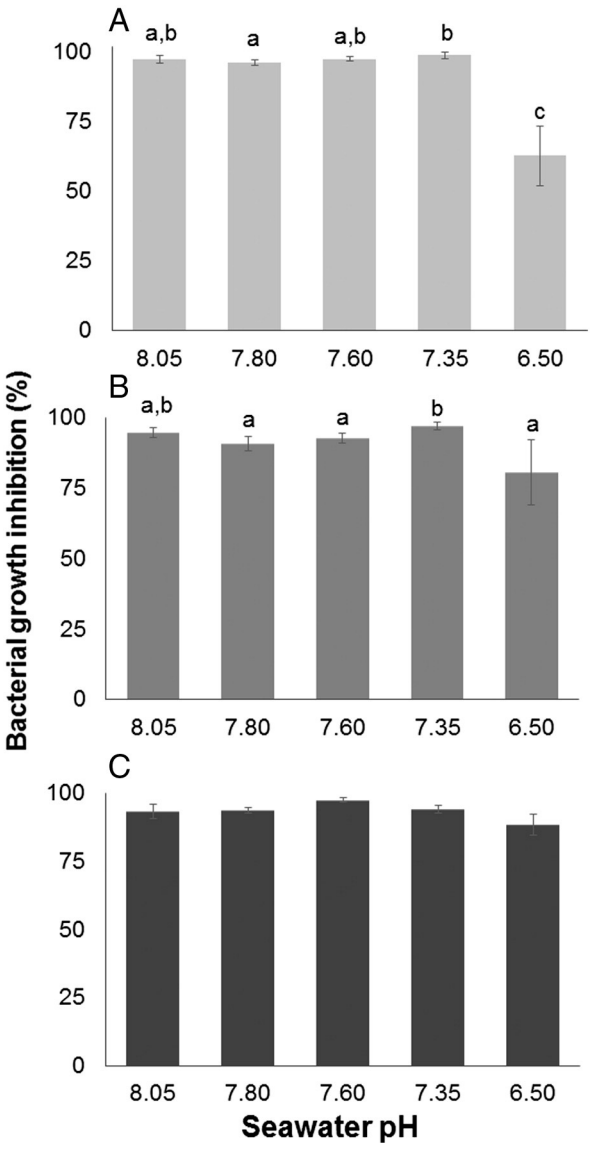

Fig. 3. The effect of seawater pH and a bacterial exposure on the antibacterial activity of cell-free haemolymph in mussels measured A) prior to a bacterial exposure, B) 1 day post-inoculation or C) 7 days post-inoculation. Values are means (±SEM) for data pooled across temperature. Significant differences ($p \leq 0.05$) are indicated by different letters and based on pair-wise tests.

the antibacterial activity in any other treatment group (Fig. 3b). By 7 days post-bacterial exposure there was no significant difference in the bactericidal activity of organisms maintained at any pH exposure level (Fig. 3c). There was no significant three-way interaction detected between reduced seawater pH, increased temperature and bacterial exposure in the current study.

3.2. Mortality

Mortalities occurred in all treatments during the experiment; however as reported by Ellis et al. (2014), cumulative mortality was higher at low pH. Log-rank survival analysis of Kaplan Meier curves highlighted that there was a significant effect of reduced seawater pH on survival in organisms maintained at both 12.5 °C ($Z_4 = 44.70$, $p \leq 0.001$) and 17.0 °C ($Z_4 = 54.40$, $p \leq 0.001$). Pairwise comparisons revealed that at 12.5 °C the probability of survival was significantly lower in organisms maintained at pH 6.50 compared to all other treatments. Cumulative survival did not fall below 90% at pH 8.05, pH 7.80, pH 7.60 and pH 7.35 (Fig. 4a) and was not significantly different between these groups, however at pH 6.50 survival fell below 50%, with the mean survival time of organisms in this group being 67 days.

As at 12.5 °C, pairwise analyses revealed that the probability of survival was significantly lower in organisms maintained at pH 6.50 and 17.0 °C, compared to all other pH treatments at this temperature. Whilst mussel survival was maintained above 90% at pH 8.05, at pH 7.80 and pH 7.60 survival was 87%. At pH 7.35 survival fell to 67%. At pH 6.50, in mussels maintained at 17.0 °C, survival dropped to just 21% during the experimental exposure (Fig. 4b), with the mean survival time of organisms in this group being 59 days. No additional mortality was noted following *V. tubiashii* exposure.

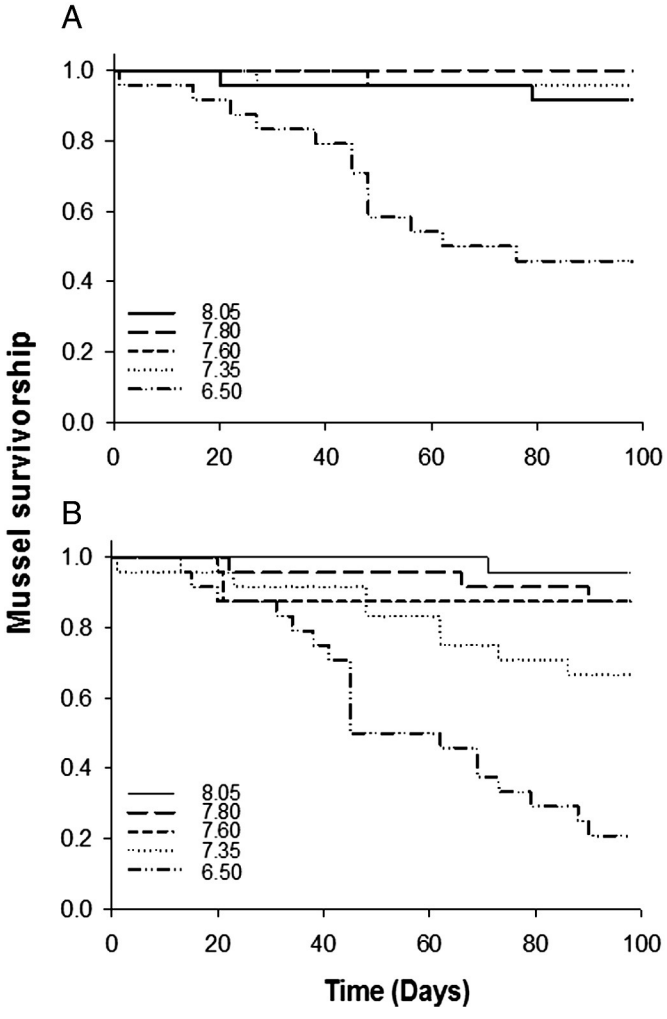

Fig. 4. *Mytilus edulis* survivorship in organisms maintained under control and acidified seawater conditions at A) 12.5 °C and B) 17.0 °C, over the duration of the experimental exposure. Step-wise curves were calculated and generated using Kaplan Meier Log-rank survival analysis.

4. Discussion

In agreement with previous research (Beesley et al., 2008; Ellis et al., 2014; Ries et al., 2009; Thomsen and Melzner, 2010; Thomsen et al., 2010), mussels in the present study were tolerant of a level of seawater acidification projected to occur in the open ocean over the next 300 years (pH 7.35; Caldeira and Wickett, 2005). Under these experimental conditions the antibacterial activity of cell-free haemolymph remained unaffected and survival was maintained above 90% at 12.5 °C. Conversely, when exposed to an extreme level of CO_2 enrichment (pH 6.50), the bactericidal activity of mussel haemolymph was significantly reduced. Furthermore, the survival at this pH fell below 50%.

In showing immune system maintenance to be compromised by exposure to a reduced seawater pH of 6.50, this study would appear to support previous research on the impact of elevated seawater pCO_2 on the invertebrate immune response (Bibby et al., 2008; Matozzo et al., 2012). Bibby et al. (2008) found that a 32 day exposure to OA reduced the ability of impacted mussels to increase phagocytic activity when compared to controls, suggesting that an increased haemolymph calcium concentration interfered with cellular signalling pathways, and thus immunocompetence. Similarly, Matozzo et al. (2012) demonstrated that a 7 day exposure to pH 7.7 or 7.4 reduced lysozyme-like activity of cell-free haemolymph in *M. galloprovincialis*. These authors, and subsequent reviewers, have therefore interpreted this reduced immune activity as a reduction in both organismal health and disease resistance. However, Bibby et al. (2008) and Matozzo et al. (2012) undertook their studies in the absence of a pathogenic challenge, and therefore interpreting a reduction in immune defence as reduced immunocompetence may not be correct as it assumes that a maximal immune response always maximises fitness, irrespective of the cost of immune system maintenance (Viney et al., 2005).

Our understanding of the marine invertebrate immune response has significantly advanced over the past decade following the development and employment of novel genetic techniques (Philipp et al., 2012). However, the observation that immune defences are often induced by infection rather than being constitutively active suggests that immune activity is costly (Lazzaro and Little, 2009). These costs are central to our understanding of ecological immunology (Rolff and Siva-Jothy, 2003; Sheldon and Verhulst, 1996). Measuring an organism's susceptibility to a realised pathogenic threat therefore offers a better assessment of impaired immune function (Ellis et al., 2011; Viney et al., 2005). Indeed, by showing that an exposure to reduced seawater pH failed to impact host defence of mussels in isolation, yet haemocytes of OA exposed mussels were more susceptible to a bacterial exposure than their control counterparts, Asplund et al. (2013) demonstrate the importance of considering the immune response in the context of a realised pathogen exposure.

Whilst reduced seawater pH significantly reduced the antibacterial activity of cell-free haemolymph in the present study, to fully understand the response of the immune system in mussels exposed to elevated seawater pCO_2 pathogen exposure is shown to be vital. Despite the antibacterial activity of mussels maintained at pH 6.50 being 40% lower than the control group prior to a bacterial exposure, when this immune parameter was measured again following a bacterial challenge there was no significant difference in the bactericidal activity of mussels maintained at this pH compared to the control group. From this it might be suggested that the initial reduction in host defence noted in these mussels was in fact a reversible, physiological trade-off, rather than an

irreversible impairment of immunological functioning, as concluded previously (Bibby et al., 2008; Matozzo et al., 2012). Furthermore, by maintaining immune plasticity, and an ability to up-regulate the immune response when required, mussels in the current study were able to respond to a bacterial infection when the need arose.

Whilst reducing the energetic investment in immune system maintenance is a strategy that may improve the survival of mussels exposed to stressful conditions in the short term, conserving energy for higher priority functions such as calcification or maintaining homeostasis (Sokolva et al., 2012), the fact that organisms maintain immune system maintenance under favourable conditions would suggest that evolutionarily, reducing immune maintenance is a more risk prone strategy. This may be due to the enhanced prevalence of disease in bivalves, due to their filter feeding lifestyle (Hernroth et al., 2002), with the benefits of maintaining a constitutive immune response outweighing the costs incurred by the host, when resources allow.

In the current study elevated temperature was also shown to significantly impact the mussel immune response, significantly increasing the antibacterial activity of cell-free haemolymph. This is consistent with Monari et al.'s (2007) finding of a temperature-related increase in antibacterial activity in the striped venus clam, *C. gallina*. It is widely accepted that temperature affects enzymatic activity and metabolism in ectotherms (Somero, 2002). Therefore, the increase in antibacterial activity measured in the current study, and the increase in lysozyme-like activity measured by Monari et al. (2007), could represent an increased activity of hydrolytic enzymes at elevated temperature. Such an increase in antimicrobial activity with increased temperature has been demonstrated in the shore crab, *Carcinus maenas*, where the activity of antimicrobial proteins was higher at elevated temperatures (Chrisholm and Smith, 1994). The sensitivity of antimicrobial activity to temperature means it is possible that the rise in seawater temperature predicted to occur within the next 100–300 years (IPCC, 2007) may counteract any reduction in immune system maintenance caused by a concomitant reduction in seawater pH. This is further supported by the significant interaction between temperature and seawater pH, with the increased antibacterial activity of cell-free haemolymph at 17.0 °C counteracting the impact of reduced seawater pH on this immune parameter, even under extreme enrichment of seawater pCO_2 (pH 6.50).

Whilst seawater temperature increased the bactericidal activity of mussel haemolymph, it also led to an apparent increase in the sensitivity of mussels to OA, as measured by the higher cumulative mortality in mussels maintained at both pH 7.35 and 6.50 at 17.0 °C. This study is therefore consistent with previous research that highlighted the importance of measuring the impact of environmental stressors in combination (Harvey et al., 2013; Kroeker et al., 2013; Wood et al., 2010), as doing so indicates that organisms may be more vulnerable to climate change than suggested by single stressor studies.

5. Conclusions

From this study it appears that mussels can trade-off immune system maintenance, whilst maintaining an ability to up-regulate their immune response when a pathogen is encountered, even under extreme environmental conditions. We conclude that by overlooking, or not accounting for, physiological trade-offs, investigators are liable to misinterpret experimental results. By comparing our results with previous research (Bibby et al., 2008; Matozzo et al., 2012), we illustrate that it is possible that a natural mechanism for managing environmental stress may be interpreted and reported as a negative result, possibly incorrectly. This study therefore cautions any assumption that physiological responses encountered in laboratory experiments are solely driven by direct effects of environmental stress. As environmental stress increases so too will the energy demands on marine organisms. Individuals will increasingly be required to adjust and balance their energy budget to meet the costs of a variety of physiological challenges as and when they arrive. Understanding and measuring physiological parameters functionally (i.e. measuring the immune response in the presence of a pathogen), will therefore allow the successful differentiation between pathological responses and physiological trade-offs. Ultimately this should allow the OA and climate change research community to better predict population and ecosystem level impacts with greater confidence.

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