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Effects of ocean acidification on the immune response of the blue mussel *Mytilus edulis*

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ABSTRACT: The effects of medium term (32 d) hypercapnia on the immune response of *Mytilus edulis* were investigated in mussels exposed to acidified (using CO_2) sea water (pH 7.7, 7.5 or 6.7; control: pH 7.8). Levels of phagocytosis increased significantly during the exposure period, suggesting an immune response induced by the experimental set-up. However, this induced stress response was suppressed when mussels were exposed to acidified sea water. Acidified sea water did not have any significant effects on other immuno-surveillance parameters measured (superoxide anion production, total and differential cell counts). These results suggest that ocean acidification may impact the physiological condition and functionality of the haemocytes and could have a significant effect on cellular signalling pathways, particularly those pathways that rely on specific concentrations of calcium, and so may be disrupted by calcium carbonate shell dissolution.

KEY WORDS: *Mytilus edulis* · Ocean acidification · Carbon capture and storage · Seawater pH · Immune response · Phagocytosis

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INTRODUCTION

Pre-industrial (ca. 1750) atmospheric CO_2 levels were approximately 280 ppm, whilst present day atmospheric CO_2 levels have increased to over 380 ppm (Feely et al. 2004), primarily as a result of anthropogenic activities. This increase would have been greater, were it not for the fact that almost one half of the anthropogenically produced CO_2 has been taken up by the oceans. Whilst this has slowed the rate of global warming (Sabine et al. 2004), a negative consequence is ocean acidification. Ocean pH has declined by 0.1 unit, compared with pre-industrial values (Orr et al. 2005), and is predicted to decrease a further 0.4 units by the end of the 21st century, and possibly by 0.7 pH units by 2250 (Caldeira & Wickett 2003). The net effect of the dissolution of CO_2 in sea water is to increase the concentration of hydrogen ions (lowering pH), and of carbonic acid and bicarbonate ions, while decreasing the concentration of carbonate ions (Raven et al. 2005). The reduction in available carbonate ions is likely to have biological implications for marine biota that require carbonate minerals to form their shells or skeletons (Caldeira & Wickett 2003, Feely et al. 2004). However, the concurrent decrease in the pH of sea water may also have biological, physiological and evolutionary consequences for all forms of marine biota at many different organisational levels.

Mitigation strategies to reduce atmospheric CO_2 levels are well developed and their potential is globally recognised (Widdicombe & Needham 2007). One such method involves injecting CO_2 into underground porous reservoir rocks (Holloway 2005). However, sub-seabed storage leaks are possible over time (Hawkins 2004). These leaks could cause severe local reductions in pH, affecting offshore organisms, habitats and marine processes. Impacts could also occur inshore, as CO_2 would be transported from land-based capture plants to offshore storage sites via sub-seabed pipes. Again, leakage from these pipes is possible. Consequently, research which investigates major pH decreases is required to understand the effects that CO_2 leakage may have on the marine environment.

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Exposure to acidified sea water disrupts growth and development in a number of pelagic and intertidal organisms (Riebesell et al. 2000, Ishimatsu et al. 2004, Kikkawa et al. 2004, Pörtner et al. 2004, Bibby et al. 2007). One group which has received particular attention with respect to acidification and climate change is marine mussels (Michaelidis et al. 2005, Berge et al. 2006, Gazeau et al. 2007, Petes et al. 2007). This is because they are important ecosystem engineers, and, like all calcifying organisms, they are vulnerable to ocean acidification. Their byssus threads enhance sediment stability and provide a habitat for smaller invertebrates or 'cryptic fauna'. Mussels are also an important food source for other organisms, including birds and humans (Nagarajan et al. 2006).

Michaelidis et al. (2005) found that seawater pH <7.5 was harmful to shelled molluscs, causing permanent reductions in haemolymph pH, growth and metabolism, and increased haemolymph bicarbonate levels derived from shell dissolution. Berge et al. (2006) also found that acidification negatively affected mussel growth and suggested that this is related to metabolic depression. Gazeau et al. (2007) showed that calcification rates in *Mytilus edulis* declined linearly with increasing CO_2 levels, juveniles being particularly sensitive to acidification. Even with a recent increase in CO_2 research, the overall effects of elevated oceanic CO_2 on organisms and ecosystems are still poorly known and unpredictable, especially regarding the sensitivity of CO_2 on defence functions.

The internal defence system of invertebrates is based on an innate, non-lymphoid immune system (Wootton & Pipe 2003) involving effective methods for cellular recognition and for discriminating foreign cells and harmful micro-organisms (Renwrantz 1990). The predominant mechanism of internal defence in bivalves involves phagocytosis by circulating haemocytes, followed by the release of reactive oxygen metabolites and degradative enzymes, and secretion of cytotoxic molecules (Pipe & Coles 1995). Bivalve haemocytes are also involved in nutrient digestion and transport, wound and shell repair, and excretion (Cheng 1981). Thus, any dysfunction in the immune system may have consequences for the nervous and endocrine systems (Koller 1990). Despite the importance of this form of defence, nothing is known of the effect of acidification on immune function.

Consequently, we conducted a medium-term, i.e. 32 d, mesocosm experiment to investigate the effect of exposure to acidified sea water on key aspects of the immune response of *Mytilus edulis*. Mussels were exposed to pH levels mimicking ocean acidification (pH 7.7 or 7.5) or a leakage from a sub-seabed CO_2 storage site (pH 6.7). A multi-assay approach was used to obtain an overall view of immune competence in mussels under acidification stress.

MATERIALS AND METHODS

Study organisms. On June 17, 2007, *Mytilus edulis* (40 to 50 mm shell length) were collected by hand from Trebarwith Strand, North Cornwall, England (50° 38′ 40.97″ N, 4° 45′ 41.78″ W) and transported to a mesocosm at Plymouth Marine Laboratory (PML) within 3 h of sampling. All individuals were cleaned of epibionts, such as barnacles and algae, using a metal scraper. Mussels were then placed in a large (50 l) aerated seawater storage tank (18.6°C, pH 8.01, salinity 33.3). The mussels were kept in this tank overnight (12 h), during which time they were not fed.

Experimental set-up. Experimentation commenced the next day and lasted for 32 d; 4 covered header tanks (450 l) were each filled with sea water (15°C, salinity 34) taken from the PML storage tank (16 m^3), which is itself supplied periodically with deep water collected approximately 14 km offshore. Using the control system described in Widdicombe & Needham (2007), each of the header tanks was set to maintain seawater through the addition of CO_2 gas at 1 of 4 pH levels: 7.8 (ambient seawater), 7.7, 7.5 and 6.7. Each header tank supplied 2 duplicate exposure tanks (50 l) containing mussels (n = 50 in each tank). Header tank pH was monitored using flat surface, combination pH electrodes (Walchem S650CD), calibrated using NIST standardised buffers prior to experimentation, and CO_2 addition was regulated by a computerised feedback relay system (Walchem WebMaster-GI controller USA). Acidified seawater from the header tanks was continuously supplied by gravity feed (60 ± 0.5 ml min^{-1}) into the mussel holding tanks. Natural sea water (pH 7.92) replaced the water within the header tanks, thus increasing seawater pH and triggering the addition of CO_2 until the pH had reached its pre-set level (Table 1) (Widdicombe & Needham 2007).

Overflow water from each exposure tank was allowed to run off, creating a flow-through system preventing build up of metabolic wastes. pH and pCO_2 within all tanks were monitored every 24 h using a calibrated pH meter (Mettler Toledo InLab 413 SG) and pCO_2 probe (Jenco mV meter 6230). pH in the exposure tanks was maintained around the target pH (Table 1); wide pH ranges occurred, because on rare occasions the holding tanks experienced short-term deviations from the target pH when a CO_2 cylinder became empty (resulting in higher pH) or due to leaks in the CO_2 supply system (resulting in a lower pH). These were quickly remedied, and deviations only lasted for a few hours; we think that they had little or no effect on our findings. Temperature and salinity were measured every 24 h using a Tetra Con 325 salinity and temperature probe. During the experiment, mussels were fed with a

Table 1. Seawater pH, pCO_2 and temperature (mean ± 95% CI, pH range in parentheses) in mussel holding tanks

Treat-ment	pH Measured	pCO_2 Concentration (ppm)	pCO_2 Partial pressure (Pa)	Temperature (°C)
7.9	7.83 ± 0.03 (7.6 to 8.0)	665.7 ± 1.5	65.3 ± 1.4	17.24 ± 0.77
7.7	7.65 ± 0.04 (7.2 to 7.9)	1160.7 ± 2.1	114.1 ±2.1	16.25 ± 0.52
7.4	7.49 ± 0.04 (7.1 to 7.7)	1435.2 ± 1.4	140.8 ± 1.3	16.61 ± 0.48
6.5	6.70 ± 0.09 (6.2 to 7.0)	3316.2 ± 2.5	325.1 ± 2.4	16.95 ± 0.46

solution of live *Pavlova* spp. (30 mg dry mass mussel^{-1} day^{-1}), diluted in sea water and supplied to each mussel holding tank by a peristaltic pump (1 ml min^{-1}; Watson Marlow 2058).

Sampling regime. Sampling took place on Days 0, 2, 4, 8, 16 and 32. On each sampling occasion, 16 mussels were randomly selected from each treatment, and haemolymph was taken from each individual as described below. Samples from 10 mussels were used for the analysis of immune function and the remaining 6 samples were analysed for cell counts. During the experiment, each mussel was only sampled once.

Immunological assays. Haemolymph (0.5 ml) was extracted using a 2.5 ml syringe , the needle (21 gauge) of which was carefully inserted into the large sinus within the posterior adductor muscle. Samples for immune assays were withdrawn into an equal volume of 0.1 M Tris-HCl buffer (TBS), pH 7.6, containing 2% NaCl. Samples for total and differential cell counts were fixed with an equal volume of Baker's formol-calcium fixative (4% formaldehyde, 2% NaCl, 1% calcium acetate) (Wootton & Pipe 2003).

Phagocytosis assay: Aliquots (50 µl) of each sample were pipetted into 4 replicate microplate wells with an equal volume of neutral red stained, heat stabilised zymosan (Sigma) (Parry & Pipe 2004). Zymosan and TBS buffer were used as controls. Haemocytes fixed with zymosan were used as blanks. After incubation and a series of cleaning steps (details in Pipe et al. 1995), haemocytes were solubilised in 0.1 ml of a mixture of acetic acid (1%) and ethanol (50%), and incubated for 30 min at 30°C to solubilise the neutral red from the phagocytosed zymosan particles. Plates were then read using a SpectraMax microplate reader (Molecular Devices) at λ = 550 nm.

Reduction of nitroblue tetrazolium (NBT assay): The NBT assay estimates the intracellular production of superoxide dismutase (SOD) inhibitable reactive oxygen metabolites produced by mussel haemocytes (Parry & Pipe 2004). Aliquots of haemolymph in TBS were pipetted into 6 microplate wells. NBT (2 mg ml^{-1}) in TBS (0.1 ml) was added to 3 of the replicate wells per sample, and NBT containing SOD from bovine erythrocytes (activity = 300 units ml^{-1}) was added to the remaining 3 wells; 3 further replicate wells containing 0.1 ml NBT solution and 0.1 ml TBS acted as blank controls for each plate. The foil-covered plates were incubated at 10°C for 30 min after which they were centrifuged at 120 × *g* for 10 min. After a series of washing steps (described in Pipe et al. 1995) all samples were dissolved in dimethyl sulphoxide (DMSO) (0.14 ml) (Sigma) and KOH (0.12 ml, 2 mol l^{-1}). The plates were read on the microplate reader at λ = 620 nm.

Protein content analysis: To express results of the immune assays as optical density per unit of haemocyte protein, the protein content of the haemolymph was measured using a protein assay kit (bicinchoninic acid, BCA; Pierce). Haemolymph samples (50 µl) from each mussel used in the previous assays were digested in an equal volume of 1%, 3-([3-cholamidopropyl]-dimethylammonio)-1-proanesulfonate (CHAPS) solution (Pierce) for 30 min at 30°C. Sub-samples of this mixture (10 µl) were placed into 3 replicate wells, and a standard calibration curve was constructed using bovine serum albumin standards. BCA (0.2 ml) reagent was added to each well and blanks with TBS were created. Plates were incubated for 30 min at 30°C and read at λ = 550 nm.

Total and differential cell counts: Total and differential blood cell counts were carried out, because not all haemocyte sub-populations are phagocytic, nor do all release reactive O_2 metabolites (Pipe et al. 1995). The number of haemocytes per ml of undiluted haemolymph was counted on the fixed samples using an improved Neubauer haemocytometer (Wootton & Pipe 2003). To determine the proportion of eosinophil and basophil cell types, haemolymph cells were stained on prepared slides (Wootton & Pipe 2003) for determination of eosinophils; the remaining haemocyte population was assumed to be basophilic. A fixed volume of haemolymph (0.25 ml) was centrifuged (150 × *g*, 5 min; Shandon UK Cytospin 3) onto a glass slide and the resultant cell monolayer post-fixed in 100% methanol for 160 s, stained with Wright's stain (diluted 1:7 with 0.05 M TBS, adjusted to pH 7.6) for 90 s, rinsed in tap water, air dried and mounted in DPX mountant. Relative numbers of eosinophils and basophils were calculated after counting 200 haemolymph cells from each mussel (Parry & Pipe 2004).

Statistical analyses. All statistical analyses were carried out using MINITAB 15. Data were tested for heterogeneity of variance using Levene's test. Normality was checked by plotting sample means against sample variances. When significant heterogeneity was

detected, data were appropriately transformed, after which a general linear model (GLM) 2-way ANOVA was used to investigate the effects of pH and Time on each parameter. Where the GLM analysis identified a significant effect of Time, the effect of pH at each sampling interval was examined separately. To test for significant relationships between pH and the selected variable at each time interval, 3 different relationship models (H_0) were compared to a model of no relationship (H_1). These models were:

H_1: $y = \mu$ (no relationship, i.e. one single mean, fits 1 parameter)

H_0: $y = \alpha_1 + \beta x$ (linear relationship, fits 2 parameters)

H_0: $y = \alpha_1 + \beta x + \gamma x^2$ (quadratic relationship, fits 3 parameters)

H_0: $y_1 = \mu_1$; $y_2 = \mu_2$; $y_3 = \mu_3$; $y_4 = \mu_4$ (4 independent means, i.e. ANOVA relationship, fits 4 parameters)

The F-ratio was calculated using:

$$F = \frac{(\mathrm{RSS}_{\mathrm{H}_0} - \mathrm{RSS}_{\mathrm{H}_1})/(p-q)}{\mathrm{RSS}_{\mathrm{H}_1}/(n-p)} \quad (1)$$

where RSS is the residual sum of squares from fitting the model, p is the number of parameters fitted to H_1, q is the number of parameters fitted to H_0 and n is the number of sample points.

The F-ratio was compared to the distribution of F. If $F > F$ (5%), H_1 was rejected. If more than one significant model was identified, the best model was determined by comparing decreasingly unconstrained models (H_0) against a fully unconstrained model (H_1). These models were:

H_1: $y_1 = \mu_1$; $y_2 = \mu_2$; $y_3 = \mu_3$; $y_4 = \mu_4$ (4 independent means, fits 4 parameters)

H_0: $y = \mu$ (no relationship, i.e. one single mean, fits 1 parameter)

H_0: $y = \alpha_1 + \beta x$ (linear relationship, fits 2 parameters)

H_0: $y = \alpha_1 + \beta x + \gamma x^2$ (quadratic relationship, fits 3 parameters)

Again, the F-ratio was calculated using Eq. (1) and compared to the distribution of F and, if $F > F$ (5%), H_1 was rejected, i.e. the constrained model (no relationship, linear or quadratic) did not fit the data as well as the fully unconstrained model (ANOVA). The simplest constrained model that fitted the data, as well as the unconstrained model, were considered to be the most appropriate models.

Where GLM analysis identified a significant effect of pH, the effect of Time was also examined separately, following the methods above. Where GLM analysis identified a significant effect of Time but not pH, the data from the different pH treatments were pooled before a 1-way ANOVA analysis of Time vs. the variable was carried out. The models described above were used to determine the best-fit relationship between Time and the variable.

RESULTS

Data had sufficient homogeneity of variance for ANOVA, with exception of the NBT assay. NBT assay data had sufficient homogeneity of variance after transformation (+ 100; square root). There were no significant differences between mussel holding tanks in any treatment.

Phagocytosis assay

There were significant effects of both pH and Time on phagocytosis (Table 2, Fig. 1). Phagocytosis levels declined with decreasing pH on Days 16 and 32 (Table 3). Compared to Day 0 mussels, controls displayed an increase in phagocytosis of >600% on Day 16 and >800% on Day 32. (Fig. 2) Mussels in reduced pH treatments also increased their phagocytosis levels on Days 16 and 32, compared to previous days, but phagocytosis decreased after Day 16. No significant effect of pH was detected on Days 4 and 8, but a significant quadratic relationship was seen for Day 2 (Table 3); the effect of Time on phagocytosis within each pH treatment was significant (Table 3). Consequently, the duration of exposure to reduced pH contributed to the phagocytosis levels observed.

Table 2. General linear model ANOVA for phagocytosis (number of zymosan particles phagocytosed per mg of haemolymph protein), superoxide dismutase (SOD) inhibitable superoxide produced per mg of haemolymph protein (NBT assay), total cell count per ml of haemolymph, and percentage of eosinophilic haemocytes within the haemolymph, against pH and Time. Adj MS = adjusted mean square; ns: not significant

Source and Factor	df	Adj MS	F	p
Phagocytosis assay				
pH	1	1 675 037	99.58	<0.001
Time	3	103 931	6.18	<0.001
Error	199	16 821		
NBT assay				
pH	1	0.011966	1.20	ns
Time	3	0.080530	8.09	0.005
Error	199	0.009953		
Total cell count				
pH	1	5.760×10^{13}	0.21	ns
Time	3	3.761×10^{12}	3.28	ns
Error	123	1.756×10^{13}		
Percentage of eosinophils				
pH	1	142.99	1.826	ns
Time	3	1709.67	22.21	<0.001
Error	123	76.99		

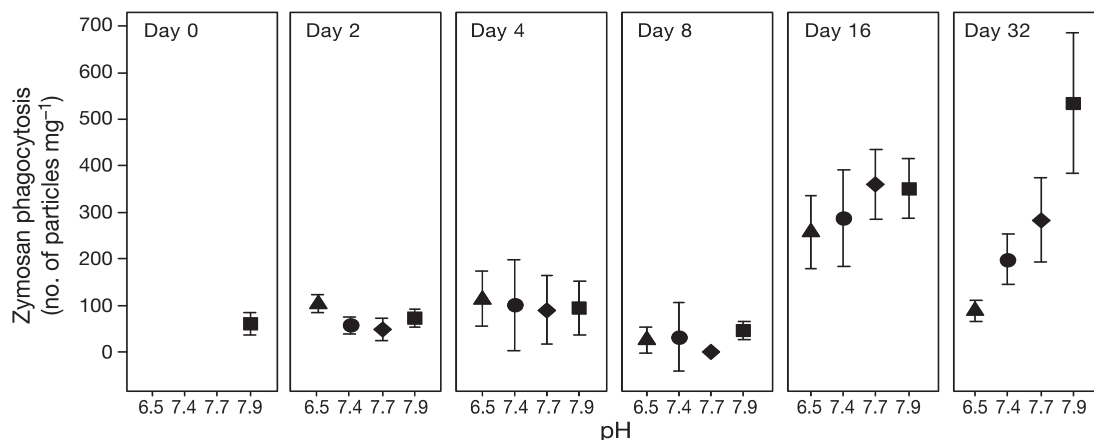

Fig. 1. *Mytilus edulis.* Number of phagocytosed zymosan particles (mean ± 95% CI; n = 10) per mg of haemolymph protein

NBT assay

Time, but not pH, affected the levels of intracellular superoxide anions (O_2^-) produced in the haemocytes (Table 2). Therefore, pH data were pooled and the effect of Time was investigated. Overall, there was an increase in superoxide production with Time (Fig. 3), but this increase did not fit a linear or quadratic relationship (Table 4).

Total and differential cell counts

There was no significant effect of pH or Time on the number of circulating haemocytes (Table 2). The overall mean cell count ±95% CI was $6.44 \times 10^6 \pm 0.74 \times 10^6$ haemocytes per ml of undiluted haemolymph. Time, but not pH, significantly affected the differential cell counts (Table 2). Therefore, pH data were pooled and the effect of Time investigated. An increase in the percentage of circulating eosinophilic cells occurred on Days 16 and 32 (Fig. 4). The fraction of eosinophilic cells increased from 40 to 50% of the haemocyte population over the 32 d period. An ANOVA model, rather than a linear one was applicable (Table 4), due to a reduction on Day 8.

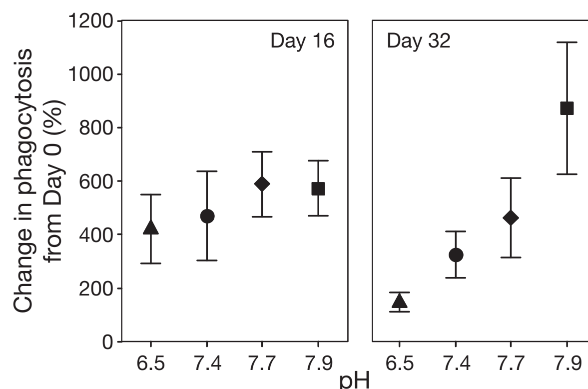

Fig. 2. Change (%) in number of zymosan particles phagocytosed per mg of haemolymph protein for Days 16 and 32, compared to Day 0 (mean ± 95% CI; n = 10)

Table 3. Best fit relationship for effect of pH at each time period and effect of Time for each pH treatment for phagocytosis; values are r^2 adjusted to account for number of predictors in the model. **Bold:** H_0 rejected ($p < 0.05$), i.e. constrained model did not fit the data as well as the fully unconstrained ANOVA model. Best model identified is the least constrained model not significantly different from the full ANOVA model

	Model				Best relationship	
	None	Linear	Quadratic	ANOVA	Model	Equation
pH effect on Day						
2	0	18.5	**32.3**	**32.35**	Quadratic	$y = 3310 - 875.1$ pH $+ 58.77$ pH^2
4	0	0	0	0	None	$y = 100.545$
8	0	0	0	2.96	None	$y = 74.59$
16	0	**8.7**	**6.5**	**6.48**	Linear	$y = -194.2 + 68.74$ pH
32	0	**25.9**	**27.1**	**26.91**	Linear	$y = -1890 + 298$ pH
Time effect at pH						
6.7	0	0	14.7	53.93	ANOVA	
7.5	0	17.9	26.5	42.14	ANOVA	
7.7	0	19.3	20	16.37	Linear	$y = 61.21 + 13.55$ Day
7.8	0	72.7	72.3	78.71	ANOVA	

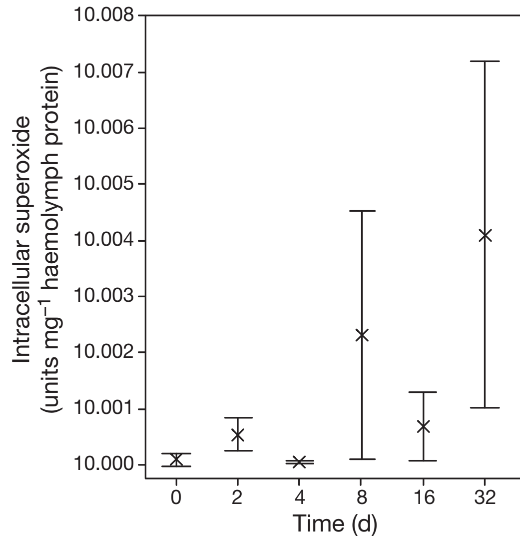

Fig. 3. *Mytilus edulis*. Effect of Time on levels of SOD inhibitable superoxide produced by the haemocytes (transformed data; mean ± 95% CI based on pooled data for pH treatments; n = 40)

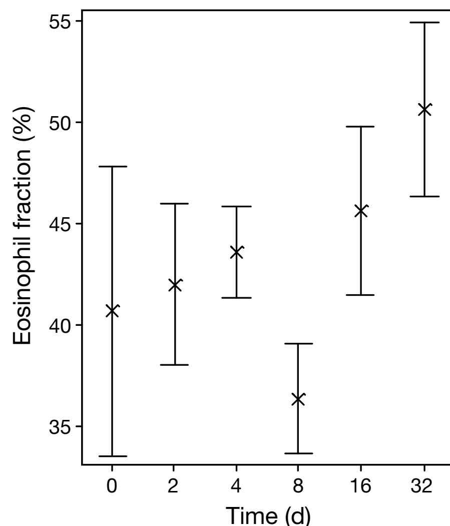

Fig. 4. *Mytilus edulis*. Effect of Time on the percentage of circulating eosinophilic haemocytes (mean ± 95% CI based on pooled pH treatments; n = 6)

Table 4. Best fit relationship for intracellular superoxide anion production, and for percentage of eosinophilic haemocytes, vs. Time; values are r^2 adjusted to account for number of predictors in the model. **Bold:** H_0 rejected ($p < 0.05$), i.e. constrained model did not fit the data as well as the fully unconstrained ANOVA model. Best model identified is the least constrained model not significantly different from the full ANOVA model

	Model				Best
	None	Linear	Quadratic	ANOVA	relationship
NBT assay	0	4.0	3.6	**4.69**	ANOVA
% eosinophils	0	13.0	13.8	**20.22**	ANOVA

DISCUSSION

This is first evidence that medium term (32 d) exposure to elevated CO_2 levels disrupts the ability of *Mytilus edulis* to express an immune response by suppressing levels of phagocytosis. Phagocytosis exhibited a strong decline as a function of decreasing pH (increasing CO_2) on Days 16 and 32. Continued uptake of CO_2 by the oceans may have progressively adverse effects on the immune function in mussels, with both ecological and commercial consequences. Whilst the current study does not identify the mechanism by which acidification causes suppression of the immune response, evidence from previous studies suggests that it is due to elevated calcium ion (Ca^{2+}) concentrations in the haemolymph.

Increased Ca^{2+} concentrations in association with increased haemolymph pCO_2 and decreased haemolymph pH occur in intertidal mussels during periods of shell closure (Lindinger et al. 1984). As mussels initially maintain a constant internal pH by decreasing their metabolic rates and/or dissolving their calcium carbonate shell (Gazeau et al. 2007) when exposed to elevated CO_2 levels, it is likely that increased Ca^{2+} concentrations in the haemolymph are caused by shell dissolution. Tissue acidosis compensation via calcium carbonate shell dissolution (Crenshaw & Neff 1969) and elevated Ca^{2+} concentrations in the haemolymph (Lindinger et al. 1984, Burnett 1997) have also been observed in other species of molluscs. Although shell dissolution was not directly investigated in the present study, mussel shells in the pH 6.7 treatment did become thinner and more brittle.

The concentration of Ca^{2+} within intracellular vesicles or the cytosol is thought to act as an important messenger at the molecular level (Massullo et al. 2006), transmitting signals for regulating haemocyte functions such as cell spreading and phagocytosis (Humphries & Yoshino 2003). If cellular disruptions were induced in mussels in the present study, this would explain the suppression of phagocytosis in response to decreasing levels of seawater pH. Increasing seawater acidity significantly reduces lysosome health as measured by the Neutral Red Retention assay (D. Lowe unpubl. data). As lysosomes play an important role in the defence system, by storing hydrolytic enzymes involved in intracellular degradation (Winston et al. 1996), a reduction in lysosome health would contribute to the disruption of cellular pathways and increase in membrane fragility due to acidified seawater.

Exposure to acidified seawater could also alter the reproductive condition of the mussels, which in turn could affect rates of phago-

cyctosis. Mussels expend energy for regulation in a variable and stressful environment, and growth and reproduction cannot be maximised. The energy invested in gametes and reproduction is accompanied by physiological costs that divert energy from reserves, possibly reducing adult survival rates, and stressful environmental conditions may cause delayed gamete release or gamete resorption (Stoeckmann & Garton 2001). The stress of exposure to acidified seawater may have caused the mussels to resorb their gametes as a survival or energy saving strategy. Resorption of gametes would require the phagocytic haemocytes to be transferred to the mantle, whilst proliferation of haemocytes would keep the circulating cell counts constant, as observed here. The maturity of haemocytes is thought to affect cell function; large eosinophilic cells are more phagocytic than immature small cells (Cheng 1981). Consequently, the number of immature haemocytes circulating in mussels at low pH may have been greater, with reduced phagocytosis levels on Days 16 and 32. A previous experiment conducted under similar conditions found no evidence that acidified seawater affected gamete maturation (D. Lowe unpubl. data), and suppression of phagocytosis as a result of changes in reproductive condition is unlikely in the present study.

Mesocosms do not reproduce *in situ* conditions such as tidal regime or diet. This can lead to increased levels of stress for the animals. In the current study, mesocosm induced stress was probably responsible for the increase in phagocytosis seen in all mussels on Day 16 and in the controls on Day 32. Phagocytosis levels in the pH treatment mussels declined on Day 32, compared to Day 16, indicating that exposure to acidified sea water may hinder the ability of stressed mussels to express an immune response.

Increased stress can result in an increase in the number of circulating haemocytes in bivalves (Renwrantz 1990, Mayrand et al. 2005, Malagoli et al. 2007). As eosinophilic cells are largely responsible for phagocytosis and release of reactive oxygen metabolites (Pipe et al. 1997), it is assumed that an increase in immune response is directly related to the number and type of circulating cells. Here, total cell counts remained consistent and the percentage of circulating eosinophils only increased from 40 to 50%. Therefore, the response in phagocytosis levels cannot be due solely to the slight increase in eosinophils, especially as there was no difference in eosinophil numbers between pH treatments. It is likely that the increase in levels of phagocytosis was partially due to changes in the activity and function of the haemocytes. In a 3 yr study, Cao et al. (2007) found no change in the average number of circulating haemocytes between summer and winter months, although immune response altered significantly. They suggested that the seasonal difference in immune response was due to the physiological state and health of the haemocytes. It is possible that in the present study, stress due to the holding conditions caused the haemocytes to become more active, whilst the increase in seawater acidity reduced haemocyte health and function. This could explain why immune response was altered, while haemocyte counts remained stable for the duration of the experiment.

The present study has demonstrated that seawater acidification can have a significant impact on the immune systems of mussels. Although this study did not demonstrate the exact mechanisms for this effect, one possible explanation is that dissolution of the mussel shells resulted in elevated levels of Ca^{2+} in the haemolymph, which in turn affected on cellular metabolism, function and signalling pathways. There is a need for molecular research regarding the disruption that ocean acidification may have on cellular systems involved in immune response. In addition, long term studies are required to assess possible adaptation or recuperation of the immune system during continuous acidification stress.

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